BIOCONVERSION OF BIODIESEL-DERIVED CRUDE GLYCEROL WASTE TO 1,3 PROPANEDIOL AND GELLAN USING ADAPTED BACTERIAL ISOLATES

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I hereby declare that this dissertation is my own work. It is being submitted for the degree, Master of Technology, to the Durban University of Technology, Durban, South Africa. It has not been submitted before for any degree or dissertation to any other institution.

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List of Abbreviations

1,3-PDO	1,3 propanediol
HPLC	High Performance Liquid Chromatography
Glc	Glucose
GlcA	Glucuronic acid
Ac	Acetate
Gly	Glycerate
Rha	Rhamnose
GWh	Gigawatt hour
UV	Ultra Violet light
NAD	Nicotinamide adenine dinucleotide
glyDH	glycerol dehydrogenase
DHA	Dihydroxyacetone
3-HPA	3-hydroxypropionaldehyde
1,3-PDODH	1,3-PDO dehydrogenase
GDHt	Glycerol dehydratase
PEP	Phosphoenolpyruvic acid
ATP `	Adenine Triphosphate
dhaT	1,3-PDO NAD oxidoreductase
yqhD	Aldehyde reductase
FOG	Fats, oils and greases
SEM	Scanning electron microscopy

ESEM	Environmental scanning electron microscopy
TEM	Transmission electron microscopy
LM	Light microscopy
PTT	Polytrimethylene terephthalate

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Abstract

The continual growth of the global biodiesel industry has resulted in a proportional increase in crude glycerol production. The by-production of glycerol waste during the manufacture of biodiesel has, with recent research, proven to hold use as a feedstock for the production of several commodity chemicals. The conversion of glycerol may be carried out by both chemical as well as biological means. The biological conversion of glycerol surpasses chemical conversion with respect to higher yield and selectivity, normal reaction conditions and the use of cheaper biological catalysts. Many microorganisms are known to convert glycerol to different value added products. This study involved the isolation of bacteria from soil and crude glycerol from a local biodiesel plant. Isolates were then used to convert crude glycerol supplemented with salts and a nitrogen source into commercially viable products. Isolates which successfully degraded glycerol were then identified via 16S PCR. A strain of Klebsiella pneumoniae, which is a known producer of 1,3-propanediol (1,3-PDO), was isolated from soil and two strains of Sphingomonas sp., which is a known gellan producer, was isolated from biodiesel waste. Gellan is an exopolysaccharide used in the food, cosmetic and pharmaceutical industries sold commercially as a product known as Gelrite or Gelzan while 1,3-PDO is an important component of fuels and polyesters (used widely in the petroleum industry) and is currently chemically produced. Using crude glycerol for producing 1,3-PDO is a good solution from an economic as well as ecological point of view. K. pneumoniae, Sphingomonas psueudosanguinis and Sphingomonas yabuuchiae were

subjected to a series of shake flask fermentations in order to determine optimal growth conditions. This microoganism was able to successfully produce significant amounts of 1,3-PDO and lactic acid using crude glycerol (80 g/l), without pre-treatment (37 and 6.8 g/l respectively). S psueudosanguinis and S. yabuuchiae were both able to produce two of the highest amounts of gellan gum than that reported by other studies using crude glycerol (80 g/l) as a sole carbon source in a minimal medium (50.9 and 52.6 g/l respectively).

Chapter 1: Literature Review

1.1   Introduction

Environmental concerns and the depletion of oil reserves have resulted in international attention to promote research on environmentally friendly and sustainable biofuels like bioethanol and biodiesel. Biodiesel made from renewable biological resources, has attracted much attention (Hu and Wood, 2010) as it is carbon neutral (Kiatkittipong *et al.*, 2009) indicating it generates less CO_2 than fossil fuels. As a result, biodiesel production is increasing; for example, the annual production capacity in the US and the EU were 1.2 and 5.7 million metric tons respectively, in 2007 (Hu and Wood, 2010). According to the monthly biodiesel production report for the year thus far approximately 66 million gallons of biodiesel was produced in January 2013. This was an increase from production of 57 million gallons in December 2012 (http://www.eia.gov/biofuels/biodiesel/production/).

Biodiesel is an alternative fuel chemically produced by transesterification of vegetable oil or animal fat with an alcohol (methanol or ethanol) in the presence of an alkali catalyst. Despite rapid development and commercialization of biodiesel, there are several key challenges that must be addressed efficiently. One such key problem is the production of glycerol as a co-product of biodiesel from transesterification of the vegetable oil. As a result the accumulation of tremendous quantities of glycerol into the market is expected in the near future (Johnson and Taconi, 2007). To give the

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magnitude of the imposed problem, it is pointed out that with the production of 10 kg of bio-diesel derived from transesterification of various oils; 1 kg of glycerol is generated (Johnson and Taconi, 2007). In 2007, Europe alone produced an over-capacity of more than 6×10^5 metric tons of glycerol (André *et al.*, 2010). Consequently, several research efforts being made in order to find ways for utilising glycerol in order to increase its value.

The three main approaches to resolving the problem of glycerol over-production are aqueous-phase reforming (APR), chemical conversion and bioconversion. There are a variety of methods employed for the chemical conversion of glycerol. Some of which allow for the production of other valuable products e.g. 1,3 propanediol (Dobson *et al.,* 2012). Other methods include breakdown of glycerol via epichlorohydrin (Miller *et al.,* 2009) and incineration of glycerol. However these methods of chemical conversion are not environmentally safe as well as costly since they require the use of high temperature and pressure, with concomitant production of harmful wastes; metal catalysts and low production of valuable products (chemical production of 1,3-PDO) (Dobson *et al.,* 2012). As a result, increased effort is being placed into the use of microorganisms to convert glycerol into valuable products (bioconversion). Such products include 1,3 propanediol, butanol, ethanol, succinate, dihydroxyacetone and hydrogen. In this study, the production of various products from fermentation of glycerol will be investigated.

There are several microorganisms that are capable of biologically producing 1,3 propanediol (1,3-PDO) from glycerol e.g. *Lactobacillus brevis*, *Lactobacillus buchnerii*, *Bacillus welchii*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Clostridium pasteurianum*, and *Clostridium butyricum* (Gonza´lez-Pajuelo *et al.,* 2005). 1,3 propanediol (1,3-PDO) is a versatile intermediate compound that has found importance in the synthesis of various polymers like polyesters and polyurethanes (Biebl *et al.,* 1999). This study will also focus on the production of 1,3-PDO from crude glycerol using *Klebsiella pneumoniae*.

The main aim of this study will be to ferment crude glycerol, using bacteria isolated from crude glycerol waste and soil to produce value-added products.

1.1.1 Objectives

1) Isolation and screening of potential bacterial strains from crude glycerol waste with glycerol as a sole carbon source.
2) Molecular Identification of bacterial isolates using 16S rDNA.
3) To optimise fermentation conditions for crude glycerol degradation using screened bacterial isolates.
4) To optimise crude glycerol fermentation using a strain of *Klebsiella pneumoniae*.
5) To detect, quantify and optimise the production of gellan by *Sphingomonas sp.*
6) To scale-up fermentation (5 L volume) for *Sphingomonas yabuucihiae*

1.2   Literature Review

1.2.1 Biodiesel

Overproduction of glycerol from the biodiesel industry has resulted in a drop in value of crude glycerol by as much as 10-fold (Yazdani and Gonzalez, 2008), subsequently encouraging the dumping of crude glycerol as waste by various industries. Consequently, much research is going into the conversion of this waste glycerol into more value-added products. Figure 1.1 outlines the transesterification reaction between triglycerides (usually waste vegetable oil or animal fat) and methanol to produce biodiesel and glycerol.

$$
\begin{array}{c}
CH_2\text{-O-COR} \\
| \\
CH\text{-O-COR} \\
| \\
CH_2\text{-O-COR}
\end{array}
\; + \; 3\,CH_3OH \; \longleftrightarrow \;
\begin{array}{c}
CH_2\text{-OH} \\
| \\
CH\text{-OH} \\
| \\
CH_2\text{-OH}
\end{array}
\; + \; 3\,R\text{-COOCH}_3
$$

Triglyceride	**Methanol**	**Glycerol**	**Methyl Ester (Biodiesel)**
1 mol	3 mol	1 mol	3 mol
10 litres	1 litre	1 litre	10 litres
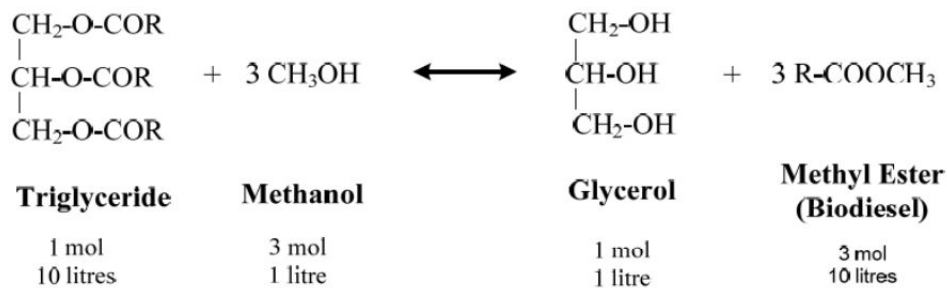
Figure 1.1: Schematic diagram of transesterification

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The vegetable oil used to make biodiesel has to be extracted from the seeds of the oil crop before it can undergo transesterfication to produce a mixture of biodiesel and glycerol. These products and unreacted reactants have to be separated and purified to obtain the final product of biodiesel. Figure 1.2 is an overview of the biodiesel manufacturing process. Despite the rapid development and commercialization of biodiesel, there are several key challenges that must be addressed efficiently (Andre *et al.*, 2010).
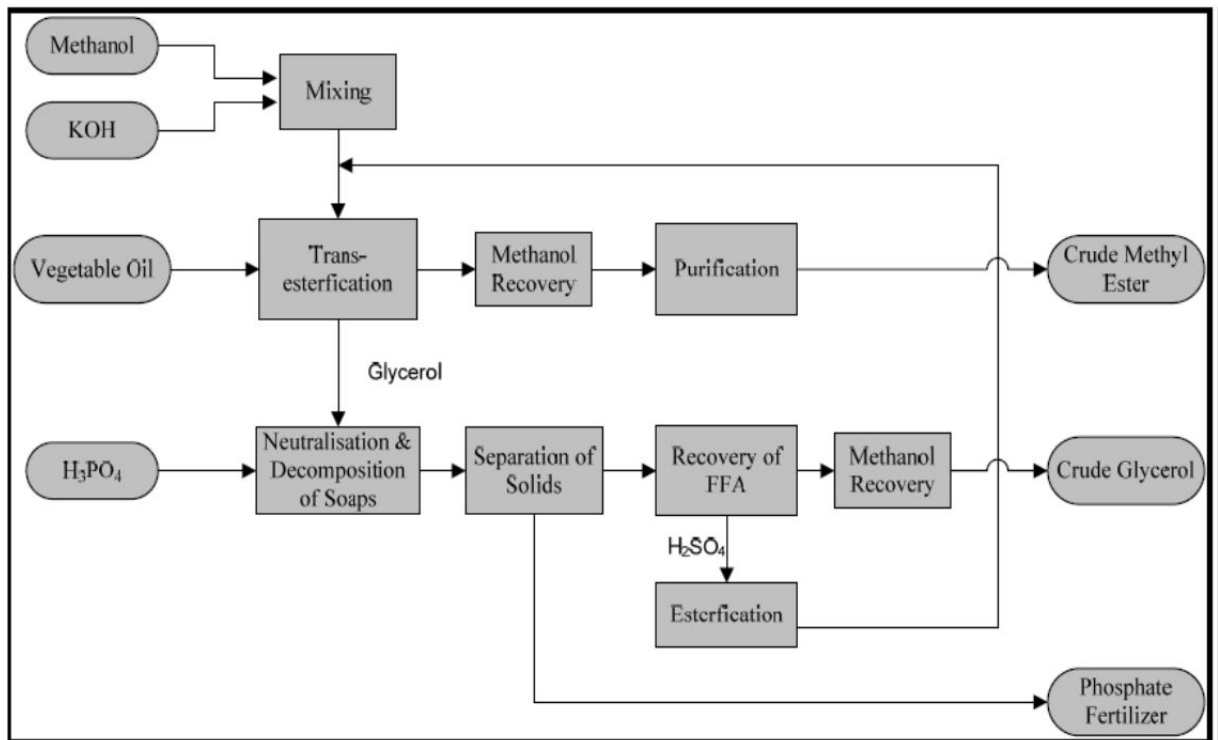
Figure 1.2: Schematics of the process of manufacturing biodiesel (Nolte, 2007)

Biodiesel currently accounts for only 10% of the world biofuel production, with ethanol making up the rest. It was estimated that between 2000 and 2005 biodiesel production has increased nearly fourfold with the top five producers in 2005 being Germany, France, USA, Italy and Austria (World Watch Institute, 2006). Table 1 summarize the five major biodiesel producers and their estimated annual production

Table 1.1: Top five biodiesel producing countries in 2005 with respective production yield (litres)

Biodiesel Producing Country	Production (litres)
Germany	1 920 000 000
France	511 000 000
USA	290 000 000
Italy	227 000 000
Austria	83 000 000

In South Africa, about 14% of the total primary energy supply is derived from imported crude oil. The constituency of the liquid fuel used in SA is 50 % imported crude oil, 30 % coal, 10 % domestic crude oil and 8 % natural gas (Wilson *et al.,* 2005). As a result South Africa has become one of the largest emitter of greenhouse gasses in Africa and one of the top twenty carbon-intensive countries in the world (Wilson *et al.,* 2005).

In 2005 South African Petroleum Industry Association reported an estimated 8.1 billion litre consumption of diesel in SA and this figure is set to increase by about 5 % per annum. The current price of diesel is more than R12.00 per litre (depending on sulfur content). As a result, the need for cheaper as well as cleaner fuel is in great need therefore, making large scale biodiesel production a promising prospect in South Africa. Biodiesel in South Africa has the following advantages (adapted from Nolte (2007) and their cited references):-

- decrease South Africa's dependence on fossil fuels and imported oil,

- promote renewable energy,

- decrease pollution, and

- assist South Africa in achieving the objectives of the White Paper on Renewable Energy, which states that by 2013, SA should be generating 10,000 GWh of energy from renewable sources.

- Ratification of the Kyoto Protocol by South Africa in 2002.

1.2.2 Glycerol

Glycerol is the simplest triol, and is a component of all natural fats and oils in the form of fatty acid esters and is an important intermediate in the metabolism of living organisms. It is obtained in yields of up to 90% by the splitting of triglycerides. It is the most important by-product of oleochemistry and its supply is increasing with accelerated oleochemical production. Glycerol is also the main waste component in biodiesel production, which is

becoming a seemingly popular biofuel with the current depletion of fossil fuels and increase in fuel prices (Dobson *et al.,* 2012).

Therefore the development of new applications for glycerol would be enthusiastically welcomed by the entire glycerol industry. A broad-based opportunity for the effective consumption of glycerol will arise from its use as a primary chemical building block. As the price of glycerol drops and its availability rises, glycerol ceases to become an "additive" for a fragmented list of small volume products and assumes a position as the starting point for the production of a smaller number of high-volume materials (Zheng *et al.*, 2007). Glycerol is reported to be a highly versatile product and has been under investigation since 1945. About 1583 different uses for glycerol have been documented from 1945 – 2008 (Adhikari *et al,.* 2009). Almost two third of the industrial uses of glycerol are in food and beverage (23%), personal care (24%), oral care (16%) and tobacco (12%) (Adhikari *et al,.* 2009).

Glycerol can also be utilised by aqueous-phase reforming, chemical synthesis and bioconversion (Dobson *et al.,* 2012). A number of microorganisms are capable of metabolising glycerol by fermentation, including yeasts, bacteria, and algae. An overview of the metabolic pathways used by these above-mentioned microorganisms during fermentation is outlined in Figure 3.
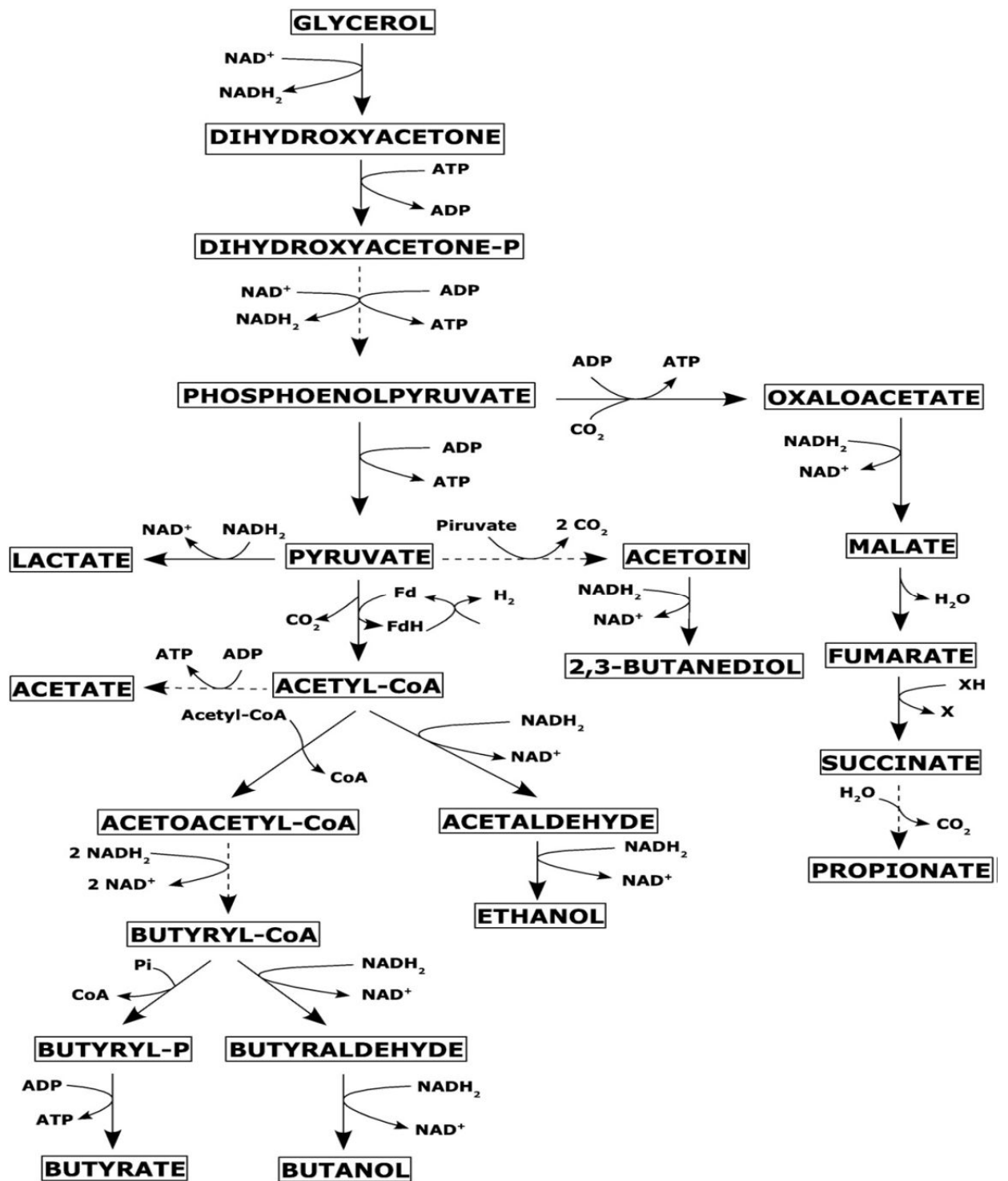
Figure 1.3: Overview of glycerol metabolism in different microorganisms (da Silva et al., 2009)

1.2.2.1 *Present Status of Glycerol Utilisation and Bioconversion*

Currently the chemical conversion of glycerol is widely used for the breakdown of this triol into either valuable chemicals or as a means of disposal (other than dumping of the effluent from industrial processes). Some of the experiments documented on glycerol utilisation include etherification of glycerol with either alcohols or alkenes to produce branched oxygen containing components, which could have suitable properties for use as a fuel or solvent (Adhikari *et al.,* 2009). In 2006, Karinen and Krause reported on the etherification of glycerol with isobutene. The experiment was conducted in a liquid phase with acidic ion exchange resin catalyst and the result was five ethers and, as a side reaction, isobutene reacted to C8–C16 hydrocarbons. The effect of the reaction conditions on the system was studied and conditions for optimal selectivity toward ethers were discovered with isobutene/glycerol molar ratio of 3 at 80 ˚C. These components may be used in the fuel industry as additives.

Another possible route is conversion of glycerol to hydrogen. Hydrogen is efficient, clean, and utilized for fuel cells as an energy source in portable electronics, power plants, and for the internal combustion engine. It has been reported that hydrogen can be produced by the pyrolysis of glycerol, (Hu and Wood, 2009) steam reforming (partial oxidation), gasification, autothermal reforming, aqueous-phase reforming (APR) and supercritical water reforming processes (Adhikari *et al.,* 2009). However, most of these processes involve expensive equipment, high temperatures and many other parameters which result in uneconomical production. Therefore production using

microorganisms is being investigated as a more desirable way of producing hydrogen from glycerol.

Conversion of glycerol to hydrogen by photo-fermentation with *Rhodopseudomonas palustris* has been investigated; however, the long fermentation process (200–300 h) and the difficulty in light penetration have proven to be undesirable factors in production. Anaerobic fermentation is likely to be more attractive as a means of producing hydrogen from glycerol as other useful by-products may also be produced (e.g. ethanol) (da Silva *et al.,* 2009). Hydrogen production from glycerol by fermentation has been studied with *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, and mixed cultures (Hu and Wood, 2009). The microorganisms capable of degrading glycerol into valuable products are further summarized in table 2. Another popular method for the bioconversion of glycerol is the use of genetically modified bacteria to increase their glycerol degradation potential. A recent study by, Hu and Wood (2009), demonstrated the conversion of glycerol to both hydrogen and ethanol, using a formate hydrogen lyase system from *Escherichia coli* which converts pyruvate to hydrogen. *E. coli* BW25113 frdC, lacking fumarate reductase, was exposed to a combination of adaptive evolution and chemical mutagenesis to produce improved strain HW2. This strain produced 20-fold more hydrogen in glycerol medium (0.68 ± 0.16mmol/L/h). HW2 also grew 5-fold faster (0.25 ± 0.01/h) than BW25113 frdC on glycerol. Ethanol production was also increased 5-fold in the evolved HW2 strain.

Table 1.2 : Summarised products formed from using glycerol as a carbon source with respective microorganisms adapted from da Silva et al., (2009) and Rahmat et al., (2010) (and their cited references).

Product	Microorganisms	Uses
1, 3 propanediol	*Klebsiella pnuemoniae* *Enterobacteriaceae* *Citrobacter freundii* *Lactobacillus buchnerii* *Bacillus welchii*	A monomer for poly-condensations producing plastics with special properties i.e. polyesters, polyethers, polyurethanes (a monomer for cyclic compounds) a polyglycol-type lubricant and also serve as a solvent.
Butanol	*Clostridium pasterianum*	Fuel additive and potential biofuel, chemical for solvent and textile process, paint thinner, hydraulic and brake fluid, perfume base.
Ethanol	*Escherichia coli* *Klebsiella panticola* *Enterobacter aerogenes*	Biofuel and fuel additive, fuel for direct ethanol cells (electricity), chemical feedstock for organic compounds and medical applications.
Succinic acid	*Anaerobiospirillum* *succinoproducens*	Used in food and pharmaceutical products, surfactants and detergents, green solvents, biodegradable plastics and ingredients to stimulate

		animal and plant growth, intermediate chemical and used in food and beverage industry
Dihydroxyacetone	*Gluconobacter oxidans*	Cosmetic industry, versatile building block for the organic synthesis of a variety of fine chemicals.
Propionic acid	*Propionibacterium acidipropionici* *Clostridium propionicum*	Antifungal agent in food and feed and as a basic chemical to produce cellulose-based plastics, herbicides, solvents and perfumes, arthritis drugs, flavors and thermoplastics.
Citric acid	*Aspergillus niger* *Yarrowia lipolytica*	Used in foods industry. Additive in detergents
Pigments:		
Prodigiosin	*Serratia marcescens*	Immuno-supressor and induce apoptosis of several cancer cell lines, such as hematopoietic cancer cells, colon cancer cells, B-lymphatic cancer cells and chronic lymphocytic leukemia cells.
Astaxantin	*Phaffia rhodozyma*	Feed for salmon, trout (freshwater) and crustaceans to give them a color that appeals to consumers.

1.2.3   1,3 Propanediol (1,3-PDO)

1,3 propanediol is a simple organic chemical. It can be used for various applications in industry. Due to high cost and limited availability, commercial use of this chemical has been restricted. It can be formulated into composites, adhesives, laminates, powder and UV-cured coatings, mouldings, novel aliphatic polyesters, co-polyesters, solvents, anti-freeze and other end uses (Pachauri and He, 2006). One of the recent successful applications of 1,3-PDO has been in the production of a new polyester, polytrimethylene terephthalate. The production of this compound has caused a great demand for 1,3-PDO due to its superior stretching, stretch recovery, better wash-fastness and biodegradable characteristics (Zhuge *et al.,* 2010 and Kurian 2005). It has also been reported that 1,3-PDO can be used as a liquid fuel as it has a heating value of 24.1 kJ/g, while methanol and ethanol have heating values of 22.1 and 29.01 kJ/g, respectively, which is higher than other liquid fuels (Flickinger, 1980; Yu *et al.,* 2011).

1.2.3.1   Current Production of 1,3 Propanediol

It is estimated that at least 1, 000, 000 tons of 1,3-PDO is produced annually. However, the means of production are known to be of a high cost and environmentally unfriendly. There are currently two processes for the chemical synthesis of 1,3-propanediol. Both of these processes produce toxic intermediates and require a reduction step under high hydrogen pressure

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(Sullivan, 1993). As a result, this has sparked interest in finding alternative methods of production of 1,3-PDO.

Currently there has been great interest in producing 1,3-PDO from microbial fermentation of glycerol. Microbial fermentation is an important technology for the conversion of renewable resources to chemicals of impotance. During the fermentation of glycerol it has been noted there is no generation of toxic by-products and due to the current increase in waste glycerol, it is also much more economically viable than using the chemical process for production.

1.2.3.2   *Microorganisms for Production of 1,3 Propanediol*

Several bacterial genus have been found to metabolise glycerol into 1,3-PDO. These include Klebsiella, Citrobacter and Clostridium which could convert glycerol to 1,3-PDO in significant quantities (Papanikolaou *et al.,* 2000). Dissimilation of glycerol in these organisms is strictly linked to their capacity to synthesize the highly reduced product 1,3 propanediol (1,3-PDO). It is known that a dismutation process involving two pathways is responsible for this phenomenon (Yazdani and Gonzalez, 2007). Through the oxidative pathway, glycerol is dehydrogenated by an NAD-linked glycerol dehydrogenase (glyDH) to dihydroxyacetone (DHA), which is then phosphorylated by PEP- and ATP-dependent DHA kinases (DHAK). Through the parallel reductive pathway, glycerol is dehydrated by the coenzyme B12-dependent glycerol dehydratase to form 3-hydroxypropionaldehyde (3-HPA). 3-HPA is then reduced to the

major fermentation product 1,3-PDO by the NADH-linked 1,3-PDO dehydrogenase (1,3-PDODH), thereby regenerating NAD+ (Yazdani and Gonzalez, 2007).
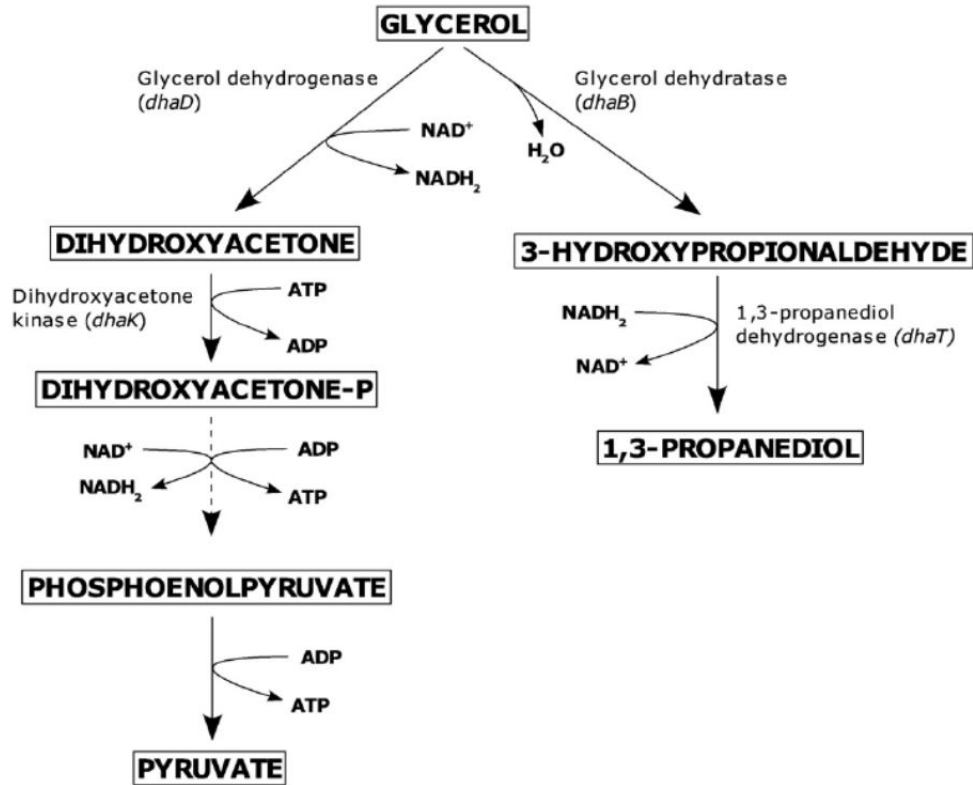
Figure 1.4: Basic pathway for the microbial fermentation of glycerol into 1,3 propanediol (da Silva *et al.,* 2007)

1.2.4 Gellan Gum

Gellan gum is the generic name for extracellular polysaccharide discovered by Kaneko and Kang in the laboratory of the Kelco Division of Merck and Co., California, USA in 1978. It was found to be produced aerobically by the non-pathogenic strain *Sphingomonas elodea* (Fialho *et al*., 2008), originally known as *Pseudomonas elodea,* and was isolated from the Elodea plant tissue. In 1994, it was discovered that another strain of *Sphingomonas* viz. S*phingomonas paucimobilis* could also produce gellan*,* and was subsequently classified in the a-4 subclass of the *Proteobacteria*. Since then there have been several other gellan- producing Sphingomonas species that have been discovered. Gellan is a linear anionic exopolysaccharide that has an average molecular mass of 500 kDa with double-helical structure when placed in solution (Fialho *et al.,* 2008). It composed of glucose, glucuronic acid and rhamnose linked in a molar ratio of 2:1:1 in a linear tetra saccharide repeat (Figure 1.5) (Vanderhoff *et al*., 2010).
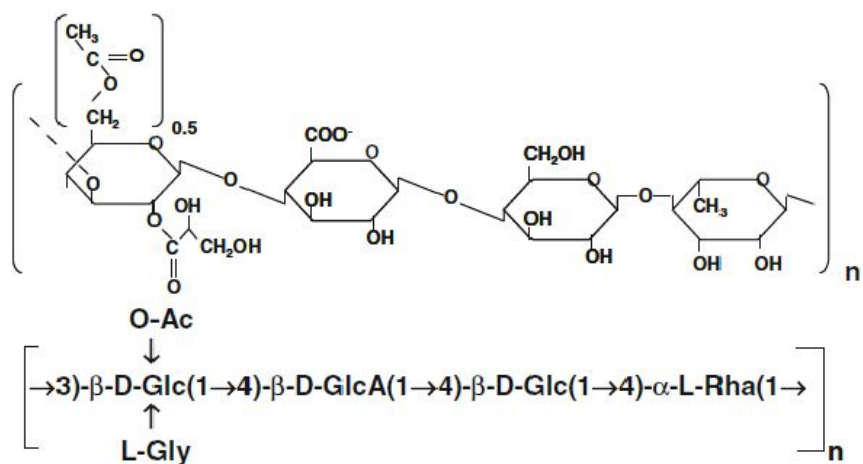
Figure 1.5: Molecular structure of a gellan repeat unit, Glc glucose, GlcA glucuronic acid, Rha L-rhamnose, Ac acetate, Gly glycerate (Fialho et al., 2008)

1.2.4.1   Applications of gellan gum

Gellan is able to withstand four autoclaving cycles, able to tolerate high salt concentrations and has proven to be tolerant to a variety of pH changes. Successful toxicity trials were completed and gellan gum received approval for use in food in Japan in 1988. The US FDA approved gellan gum for use as a food additive in 1992 (Bajaj et al., 2007). Due to these characteristics, gellan has great commercial applications in the pharmaceutical, food, biotechnology and cosmetic industry. Gellan also has potential for use as a low-cost substitute in soil erosion control products like Soil Guard™ (Vanderhoff *et al.*, 2010). Gellan is commercially available under the following names Gelzan® (agar substitute from Sigma-Aldrich), Gelrite® (agar substitute as well as for environmental applications such as biodegradation of gasoline), Kelcogel® F and Kelcogel® LT100 (both used as gelling agents in food and personal care).

In the biotechnology industry gellan gum is used as an agar substitute for microbiological application and is known as Gelrite (Lobas *et al.,* 1992). It is particularly useful for the culture of thermophilic microorganisms, as the gels are thermostable and can withstand prolonged incubations at high temperatures. In the biomedical field gellan is used in nasal, ocular, gastric and colonic drug delivery applications (Fialho *et al.,* 2008).

In the food industry, gellan gum is used as an additive which may function as a stabilizer, thickening agent, structuring and versatile gelling agent in a wide variety of foods (Anderson *et al.,* 1988). Food products containing gellan gum include bakery fillings, confectionaries, dairy products, dessert gels, frostings, icings and glazes, jams and jellies, low-fat spreads, microwavable foods, puddings, sauces, structured foods, and toppings (Duxbury, 1993).

The following Table outlines the various gellan-based products that have been patented in various industries adapted from Fialho *et al.,* 2008.

Table 1.3 : Gellan Products and Patents (Fialho *et al.,* 2008)

Patent	Country and Patent No.
Gellan gum and beverage process for making a gelled beverage	US5597604
Gelatin-free gummy confectionary using carrageenan and gellan	US6586032
Calcium stable high acyl gellan gum for enhanced colloidal stability in beverages	CN101001538
Food containing native gellan gum	JP2005253473
Gellan gum tablet film coating	US2004033261
Liquid aqueous ophthalmic composition with gellan gum	NZ217662
Spray able wound care with gellan gum	NZ323126
Controlled release compositions comprising gellan gum	WO9922768
PHB-free gellan gum broth	US5300429
Sphingomonas elodea mutant for non-acetylated gellan gum	US2003100078
Production of modified gellan gum	JP11341955
Cosmetic composition with gellan gum and carrageenan	GB2384705
Toothpaste with improved stability	KR20050023598
Paper coating composition comprising gellan gum/starch blend	US6290814
Purification and use of gellan in electrophoresis gel	US204168920
Media/methods for prompting maturation of conifer somatic embryos	US20050003415

1.2.4.2   Current Production of Gellan

Gellan gum is produced by a group of 'sphingan'-producing bacteria from the genus Sphingomonas (Pollock 1993). *S. paucimobilis* has the ability to convert six carbon mono- and disaccharides into gellan. A number of carbon and nitrogen sources have been shown to support gellan production (Vanderhoff *et al.,* 2010). Figure 1.6 illustrates the pathway of gellan production using glucose which is the current carbon source since there are no reports on glycerol metabolism.

In order to reduce gellan production costs other carbon sources and nitrogen sources have been investigated as alternatives to glucose and yeast extract. Industrial wastes cheese whey and molasses, contain high sugar concentrations and have shown significant potential for use as raw materials for low-cost fermentative production of gellan (Fialho *et al.,* 1999; Banik *et al.,* 2006). The results were considered successful as the study contributed to reducing these common industrial wastes. However, the amount of gellan produced was not at its optimal. It was also shown that mutant strains of *S. paucimobilis* ATCC 31461 can utilize glucose, corn syrup and soybean pomace (Wang *et al.,* 2006).

Nitrogen sources are also an important parameter in gellan production. The most commonly used are corn steep liquor, yeast extract or tryptone (Bajaj *et al.,* 2007). According to Vanderhoff *et al.,* the total cost of Manna medium is around $66.55 per 1000 L while the cost of gellan was estimated at $4.75. In

their study they showed that *S. paucimobilis* was able to utilize a low-value co- product from dry-mill corn ethanol production called condensed corn solubles (CCS) as a nitrogen source with 30 g/l of glucose only to yield significant amounts of gellan at almost half the price of total conventional production.
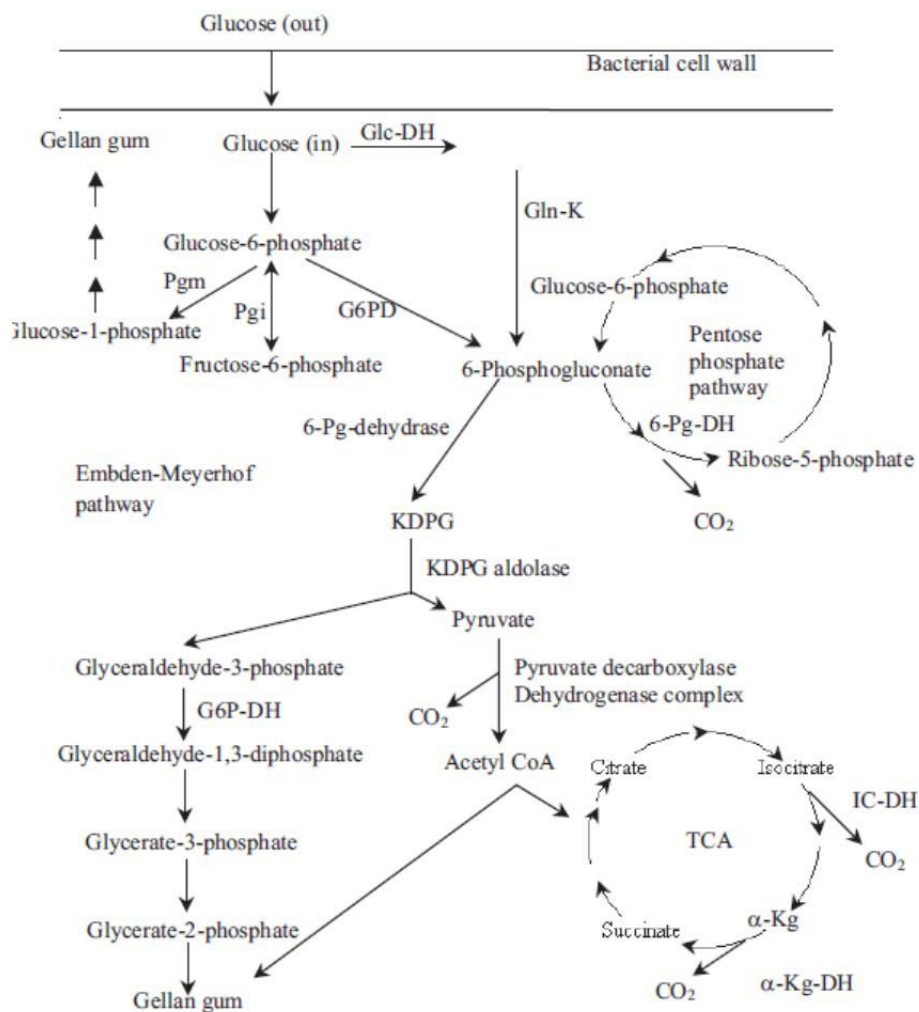
Figure 1.6: Metabolic pathway of glucose degradation in *Sphingomonas paucimobilis* (Martins and Sá-Correia, 1991)

Factors affecting gellan production include temperature and aeration. *Sphingomonas* are strict aerobes therefore agitation speed and the amount of dissolved oxygen within the medium plays a great role in production.

Chapter 2: Isolation and screening of bacterial strains from biodiesel waste and optimisation of crude glycerol degradation

2.1   Introduction

Due to the rapid increase in biodiesel production, the production of glycerol is reaching significantly high levels and thus leading to a decrease in value. Estimates indicate that biodiesel could represent as much as 20–22% of all on-road diesel used in Brazil, Europe, China and India by the year 2020 (Kumar and Sharma, 2011). This influx in biodiesel production would lead to large quantities of waste glycerol placing this once-valuable triol, which finds applications in various cosmetic, food and biotechnological industries, in the category of an industrial waste. Dobson *et al.* (2012) reported that to date the effective use of glycerol waste from the biodiesel process remains a low key priority. This could possibly be the result of the lack of an incentive to companies producing biodiesel to further purify this glycerol for resale, as the current value is around US $0.34/lb. This has subsequently led to the increase in research into new cheap and environmentally safe methods of converting this waste into by-products of commercial value thereby increasing the value of glycerol.

There are several methods being investigated for both the chemical and biological transformation of glycerol (Nimje *et al.,* 2011). In the chemical conversion of glycerol there are many environmental impacts and additional costs to consider. One such method is the epichlorohydrin method of conversion wherein, the glycerol is converted to propylene glycol and

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thereafter separation allows for the conversion of 50 % of the glycerol. Glycerol and hydrogen are reacted in the presence of a metal catalyst in a closed reaction vessel. The temperature of the reaction is kept at 180° C - 220° C and at a pressure between about 800 - 1500 psi (Miller *et al.,* 2008). But this, and other methods, of chemical conversion are environmentally unfriendly as the usual factors associated with these methods include high temperature and pressures (as in the case of incineration), addition of poisonous organic solvents (1,3-PDO production) and the further production of waste chemicals and harmful by-products (Dobson *et al.,* 2012).

At present the biological conversion of crude glycerol, either into by-products that are of commercial value or are less harmful to the environment, is the most attractive process. This conversion was described as being able to circumvent the disadvantages of chemical catalysis (Nimje *et al.,* 2011). Due to toxins that may exist within the crude glycerol, arising from the transesterification process (Manosak *et al.,* 2011), chemical catalysis of crude glycerol may also be a dangerous process. However, this setback can be overcome by subjecting the crude glycerol to an initial purification process so that it may be separated from the other chemical compounds existing in the waste. This would result in additional costs, which are not viable as the current value of glycerol is already low. Consequently biological conversion would not only be a more cost-effective method but it would also be safer. The process whereby microorganisms are used to degrade wastes in the environment is referred to as bioremediation. This process may be carried out by one of two approaches: The first is termed mineralization, whereby the

microorganisms are used to oxidate the organic contaminants completely. The second is termed biotransformation, whereby the microorganisms are used to convert or biotransform these organic contaminants into less harmful metabolites or, as in the case of this study, by-products that may be of industrial value (Adhikari *et al,.* 2009, Yazdani and Gonzalez, 2008).

The by-products of the biological conversion of glycerol are generally useful chemicals such as succinate, ethanol (Hu and Wood, 2010), butanol, 1,3-PDO, hydrogen and formic acid, amongst others (Yazdani and Gonzalez, 2008), allowing this to be a more preferred method of glycerol utilization. Several microorganisms have been shown to successfully convert crude glycerol into some of these products (Table 1.2).

Recent studies have proven that the most efficient way of isolating bacteria capable of converting wastes, that maybe toxic to the environment, is to screen for these particular microorganisms within the waste sample itself or from environmental samples such as nutrient-rich soils (Fredrickson *et al.,* 1995; Govender and Pillay, 2008).

This chapter focuses on isolating and identifying bacterial species using enrichment techniques to screen for potential glycerol-degraders already existing in the crude glycerol as well as to investigate the maximum concentration of crude glycerol that these bacteria are able to tolerate.

2.2   Materials and Methods

2.2.1   Screening and isolation of glycerol-degrading microorganisms

2.2.1.1   Soil and crude glycerol waste

Environmental samples used for screening of the presence og glycerol-degrading bacteria were soil and crude glycerol waste. Crude glycerol waste from a biodiesel process was obtained from GC Biofuels, Durban for screening of glycerol-utilising microorganisms existing in the waste. The samples collected were from the top of the storage vat and from the bottom of the vat while, the soil used for screening was taken near a pond at the Botanical Gardens in Durban.

2.2.1.2   Enrichment technique

A 9-day enrichment technique, adapted from Govender and Pillay (2008), was used with minor modifications. The growth medium used was M9 medium (pH 7) which comprised of g/l: 1, NH_4Cl; 3, KH_2PO_4; 5, NaCl; 6, Na_2HPO_4 (autoclaved at 121°C; 15 min). $MgSO_4$ (1mM) and $CaCl_2$ (0.1 mM) were filter-sterilized separately and then added to the medium (Nimje *et al.,* 2011). All chemicals were purchased from Sigma-Aldrich and Merck.

Enrichment experiments were done in 250 ml Erlenmeyer flasks with 100 ml working volumes using M9 broth medium with glycerol concentrations (analytical grade, Merck) of 10, 15, 20 and 25 g/l. Each flask was inoculated

with 1% of crude glycerol (GC Biofuels) as inoculum and incubated at 30 ºC with a shaking speed of 200 rpm for 3 d. A further 1% of inocula from each of these flasks was thereafter transferred to a similar series of flasks and incubated under identical conditions. This procedure was repeated after 3 d before serial dilutions were made from each flask (1:10) and 100 µl of each were then plated out onto M9 medium containing, 12 g/l technical agar and 1 % analytical grade glycerol as the carbon source.

Plates were incubated at 30, 34 and 37°C for 48-72 h to isolate bacteria. Colony morphologies were thereafter inspected on all plates. Single colonies were then isolated onto fresh M9 plates and re-incubated at 30, 34 and 37°C to determine the temperature which allowed for the most efficient growth of each isolate.

For the screening of bacteria from soil, a similar technique was used except the 250 ml Erlenmeyer flasks containing glycerol (analytical grade, Merck) concentrations of 10, 15, 20 and 25 g/l were inoculated with 1 g of soil and identical fermentation parameters were used.

2.2.2 Preliminary optimisation of glycerol degradation

2.2.2.1 Pre-Inoculum preparation

A 10 % starter inoculum was used for each fermentation, unless specified, and were prepared in 250 ml Erlenmeyer flasks containing a 100 ml M9 medium with 30 g/l analytical grade glycerol (Merck, 99,5 % purity) as the sole

carbon source. Flasks were incubated at 34°C with shaking at 200 rpm on an Infors platform shaker for 48 h.

2.2.2.2   Fermentation conditions

All fermentations were carried out in 250 ml Erlenmeyer flasks with 100 ml working volumes. M9 medium was used with varying concentrations of glycerol (analytical grade, Merck) (50 – 120 g/l) or crude glycerol (60 – 120 g/l). Flasks were incubated at 34 °C and 200 rpm for 7 d. In order to improve glycerol degradation, fermentation conditions were optimised by investigating the effects of co-fermentation and the addition of yeast extract to M9 medium. For the co-fermentation study, 2 g/l glucose (Sigma-Aldrich) was added as a co-substrate to M9 medium and crude glycerol at 34°C and 200 rpm. For the addition of yeast extract, 2, 4 and 6 g/l of yeast extract was used to investigate its effect on glycerol degradation.

2.2.3 Investigating the effect of bacterial consortia on glycerol degradation

Individually isolated bacterial colonies from M9 plates were grown as described in section 2.2.2.1 and thereafter combined (10% total inoculum) in pairs (bacterial isolates K8+K9, K4+K8, K9+K10, K10+K8, K4+K10 and K9+K4), combinations of three (bacterial isolates K4+K9+K10, K4+K9+K8, K4+K9+K10, K8+K9+K10) as well as combined altogether (bacterial isolates K4+K8+K9+K10).

Each combination was thereafter grown as described in section 2.2.2.2 using 50 g/l analytical grade glycerol as the main carbon source.

2.2.4 Analytical methods

2.2.4.1 High performance liquid chromatography

Glycerol degradation was detected and quantified using a high performance liquid chromatography (Merck-Hitachi Lachrom) equipped with a Hi-Plex H Fast Acid Column (100 x 7.7 mm, Polymer Labs) which is known to detect glycerol, sugars, certain organic acids and alcohols with a refractive index detector. Culture broth was centrifuged at 5000 rpm for 10 min at 4°C (Eppendorf Centrifuge 5415R). The supernatant was then filtered through a 0.22 μm Millipore filter into HPLC vials. The mobile phase used was 5 mM H_2SO_4 with a flow rate of 0.7 ml/min and a temperature of 65 °C. Analytical grade glycerol (Merck) was used as a standard.

2.3   Results and discussion

2.3.1 Screening and isolation of bacteria from crude glycerol waste.

For this study, bacterial strains were isolated directly from crude glycerol waste which was collected from a biodiesel manufacturer (GC Biofuels). The reasoning behind this was based on the ability of pre-existing microorganisms to already have an established tolerance for crude glycerol and any other toxins that may be in the waste. Due to this waste being kept in a storage vat outside the plant, it was also proposed that these bacteria would have adapted to degrading glycerol under environmental conditions. This would be advantageous should bioremediation be an option in crude glycerol degradation. Studies have shown that this method of screening for bacteria is a promising approach to isolating microorganisms capable of degrading environmental contaminants (Govender and Pillay, 2008).

After the screening process (outlined in section 2.2.1), similar colony morphologies were observed on all M9 plates, with the most number of colonies being observed at all four glycerol concentrations (10, 15, 20 and 25 g/l) at 34 °C. These bacteria were then purified on M9 plates and incubated at 30, 34 and 37 °C in order to distinguish which temperature allowed for the most efficient growth. The best growth was observed at 34°C (48 h). At the end of the screening process, a total of 11 isolates were obtained from the crude glycerol and one isolate from soil.

These isolates were then used in shake flask fermentations with both crude and analytical grade glycerol to determine the glycerol-degrading potential. Results are summarised in Table 2.1

Table 2.1: Degradation of analytical grade glycerol (50 g/l) with 12 bacterial isolates at 34°C and 200 rpm for 72 h

	Residual Glycerol (g/l)			
Strains	6 h	24 h	48 h	72 h
K1	50	47	43	41
K2	50	37.9	391	37.7
K3	48	46	44.2	43
K4	48.9	45	36.1	27.5
K5	49.1	49	48.3	47.9
K6	47.3	43.1	36.8	34.7
K7	50	45.2	43.4	42
K8	47.6	42.9	33.4	28.9
K9	50	45.8	40.2	29.3
K10	49.6	45.7	31	26.3
K11	50	43.2	41	39.1
S1	48	40.6	33.5	28.2
Control	50	50	50	50

Lowest glycerol degradation (10 – 20%) was evident by bacterial isolates K1, K2, K5 and K6, whilst isolates K3, K7 and K11 showed only 20 – 30% degradation after 72 h using 50 g/l analytical grade glycerol at 34°C and 200 rpm (Table 2.1). Isolates S1, K4, K8, K9 and K10 yielded good glycerol degradation values (>40%) (Table 2.1) and were consequently subjected to

more intensive fermentation studies to assess their degradation potential (Fig 2.1).

2.3.2 Assessment of the effect of bacterial consortia on glycerol degradation

The four bacterial isolates from biodiesel waste were then used in an extended study to determine the synergistic effect of these isolates from the same environment (biodiesel waste) on glycerol degradation. Previous studies have reported that using bacterial consortia is a viable method for degradation since microorganisms do not occur individually in the environment. Rossi *et al.* (2012) reported that bacterial consortia isolated from sludge at a biodiesel plant (Passo Fundo, Brazil), were able to degrade 70 g/l of both analytical grade and crude glycerol within 32 h. For the purposes of testing the effect of naturally-occurring consortia within biodiesel waste, S1 was excluded as it was isolated from soil and not biodiesel waste.

The consortia investigation involved the degradation of analytical grade glycerol at a 50 g/l concentration after 7 d at a temperature of 34°C and 200 rpm. The isolates were also used in an individual degradation study whereby each one was placed into the minimal media separately (Fig 2.1 a) in order to compare their degradation to consortia experiments (Fig 2.1 b and c).

K8 and K10 were able to degrade 50 g/l glycerol at 86 and 90% respectively, (Fig 2.1 a). K4 and K9 were able to degrade 67 and 54% respectively, (Fig 2.1 a). Maximum degradation of 93% was achieved with K8+K10. The remaining pairs *viz.*, K4+K8, K8+K9, K9+K10, K4+K10 and K9+K4, exhibited

degradation levels ranging from 52 to 66% (Fig 2.1 b). However, these mixed cultures did not yield much higher levels of glycerol degradation in comparison to experiments using individual bacterial cultures. As a result, the pairing of isolates was not investigated further.

From the combination of three isolates (Fig 2.1 c), K8+K9+K10 showed the best degradation at 72% whereas K4+K8+K10 showed 70% degradation (Fig 2.1 c). The combination of all isolates with viable degrading potential (Fig 2.1 c) showed a 12% less glycerol degradation level. Additional mixed culture combinations *viz*. K4+K9+K8, K4+K9+K10, K4+K8 +K10 showed 62, 60 and 70% glycerol degradation levels, respectively. This was not considered an exceptional finding in comparison to the single isolates assessed, which was similar to that achieved with the paired isolates described above. Hence further investigations with these mixed cultures were also terminated. In this study the use of a mixed consortium did not enhance glycerol degradation substantially as in the case of the study previously discussed by Rossi *et al.* (2012).

Based on the above, three bacterial isolates were then chosen from the biodiesel waste along with the isolate from soil for further studies viz., K4, K8, K10 and S1. K9 was eliminated due to its inability to significantly degrade 50 g/l glycerol (<60%) significantly.
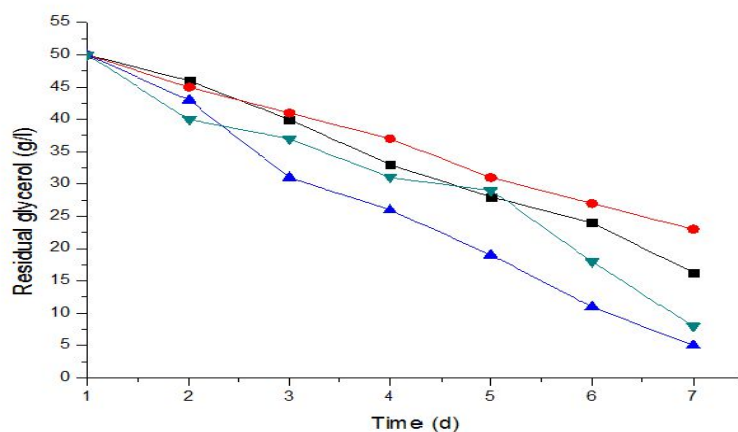
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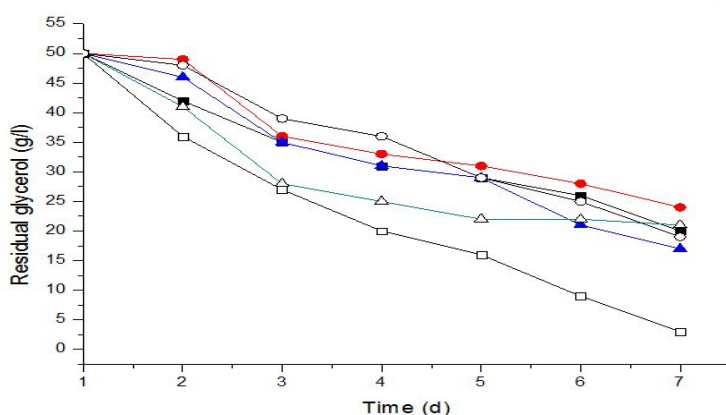
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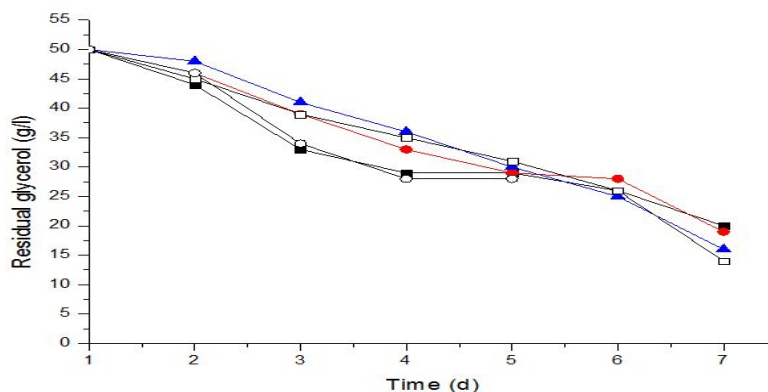
(c)

Figure 2.1: Assessment of 50 g/l analytical grade glycerol degradation for (a) individual isolates K4 (■), K8 (▲), K9 (●), K10 (▼),(b) combined isolates K8+K9 (■), K4+K8 (●), K9+K10 (▲), K8+K10 (□), K4+K10 (△), K4+K9 (○), (c) tripled isolates K4+K9+K10 (■), K4+K8+K9 (●), K4+K8+K10 (▲), K8+K9+K10 (□) and all isolates combined (○) at shaking flask conditions 34°C and 200 rpm for 7 d.

2.3.3 Preliminary optimisation of glycerol degradation

Having isolated and established the ability of the four isolates to degrade analytical grade glycerol it was necessary to assess their capabilities at higher glycerol concentrations as well as to optimise the M9 medium by adding glucose and yeast extract as potential growth enhancers (outlined below).

Analytical grade glycerol concentration was increased to 60 g/l and fermentation conditions were at 34°C and 200 rpm over 7 d (Fig 2.2 a). A concurrent experiment was also conducted using 60 g/l of crude glycerol at identical fermentation conditions (Fig 2.2 b). K4 was only able to degrade 33% of analytical grade (Fig 2.2 a) glycerol and 42% of crude glycerol (Fig 2.2 b). K8 was able to degrade 73% analytical grade glycerol (Fig 2.2 a) and 91% crude glycerol (Fig 2.2 b). For isolate K10, glycerol degradation was at 78% analytical grade glycerol (Fig 2.2 a) and 94% crude glycerol (Fig 2.2 b). Degradation of crude glycerol by isolate S1 yielded similar degradation results to isolates K8 and K10. Approximately 78% of analytical grade glycerol was degraded and 90% of crude glycerol after the 7 d analysis.

All four strains were able to degrade crude glycerol more efficiently than analytical grade glycerol. This could be due to there being a reduced concentration of actual glycerol (50%) in the crude sample as well as the metabolism of these bacterial isolates (K4, K8, K10) being better adapted to the content of crude glycerol from which they were isolated or the dynamic environment such as that of soil (S1).

The content and variety of impurities found in crude glycerol is different due to the use of alcohols, salts, heavy metals and even soaps used for the initial biodiesel reaction by various industries or process. The by-product glycerol is also dependent on the parent feedstock used for the biodiesel reaction. Biodiesel may be processed from rapeseed, soybean, waste cooking oil or animal fat, thus making all of these important factors in determining crude glycerol composition (Dobson *et al.,* 2012). The isolation of bacteria from soil, rich in organic matter, which have the ability to degrade glycerol based wastes, has been widely studied with great success. Čipinytė *et al.* (2009) isolated approximately 124 microorganisms from soil that had the ability to degrade glycerol-containing FOG's (fats, oils and greases).
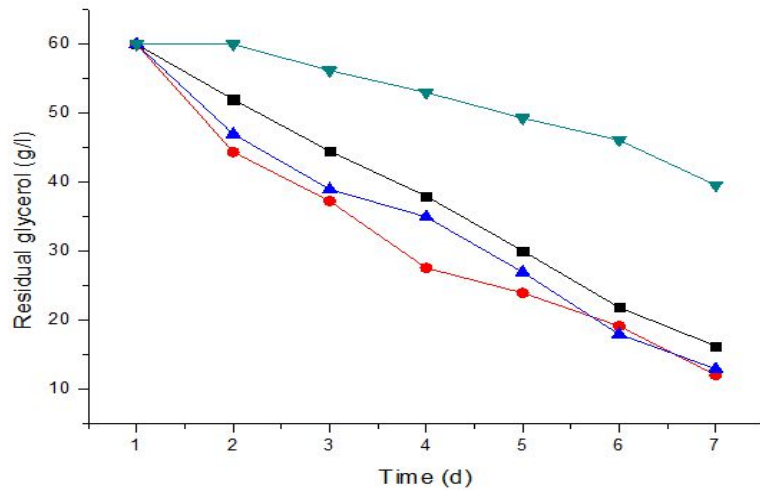
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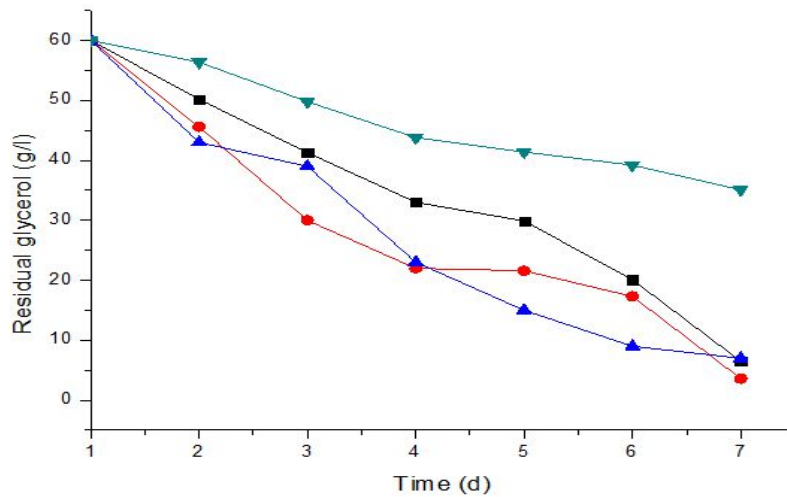
(b)

Figure 2.2: Assessment of glycerol degradation by isolates K4 (▼), K8 (■), K10 (●) and S1 (▲) using (a) analytical grade glycerol (60 g/L) and (b) crude glycerol after shake flask incubation at 34°C and 200 rpm over 7 d.

In the next study, bacterial isolates K8, K10 and S1 were used in fermentation with 80 g/l crude glycerol at 34°C and 200 rpm. Isolate K8 degraded 93% crude glycerol (Fig 2.3 a), K10 degraded 97% crude glycerol (Fig 2.3 b) and S1 was able to degrade 91% crude glycerol during fermentation (Fig 2.3 c).

From these results, a co-fermentation study was conducted in order to increase the rate of glycerol degradation. Previous studies have shown that the addition of a low concentration of glucose as a co-substrate induces the glycerol-3-phosphate pathway in bacterial metabolism, thereby increasing the rate of glycerol degradation (Celińska, E, 2010). Glucose is also a sugar that is known to increase the energy of the cell in bacterial metabolism as glucose catabolism increases NADH and ATP levels. Jalasutram and Jetty (2011) reported that glucose-glycerol co-fermentation at a molar ratio of 0.4 mol glucose per mol glycerol improved fermentation during the production of 1,3-propanediol, whereby glucose was used for the generation of energy and glycerol was used for the production of 1,3-propanediol. Studies involving genetic modification of microorganisms such as *E. coli,* have shown that deleting the glucose phosphotransferase significantly improves glycerol degradation from 64% to 78% which indicates that glucose does affect the cell's uptake of glycerol (Tian *et al.,* 2012).

For the purposes of this study, each isolate (K8, K10 and S1) was used in M9 medium with 2 g/l of glucose and 80 g/l glycerol over 7 d at 34°C and 200 rpm.

From the co-fermentation experiment conducted, isolate S1 showed an increase in the glycerol degradation rate after 2 d, however, this did not improve overall degradation as 88% of glycerol was degraded after 7 d as compared to 91% degraded without glucose (Fig 2.3 c). Isolate K8 degraded 85% of crude glycerol when glucose was used as a co-substrate which

indicated that the addition of glycerol had no effect on the fermentation when compared to that without glucose (Fig 2.3 a). The glycerol degradation rate of K10 initially followed a similar trend of degradation for both regular fermentation and co-fermentation during the first 3 d (Fig. 2.3 b) however; only 89% of crude glycerol was degraded after 7 d during co-fermentation which indicated that the addition of co-substrate glucose had reduced glycerol degradation by 8%.

All three isolates depicted better degradation without the addition of glucose as a co-substrate. From an economic perspective this has an immense long-term benefit. Currently the most commonly used source of carbon in the bioprocessing industry is glucose (Dobson *et al.,* 2012). However, there has been a rise in the price of this feedstock in recent years from between US $0.21-0.23/lb to around US $0.40/lb in 2010 (Dobson *et al.,* 2012). Another setback which is of importance is that glucose is popularly used as an important additive in many foods thus placing it in the ongoing 'food versus fuel' debate (Yazdani and Gonzalez, 2008; Dobson *et al.,* 2012). This contributes to boosting the use of glycerol as a feedstock in industry as well as finding microorganisms capable of solely degrading glycerol.
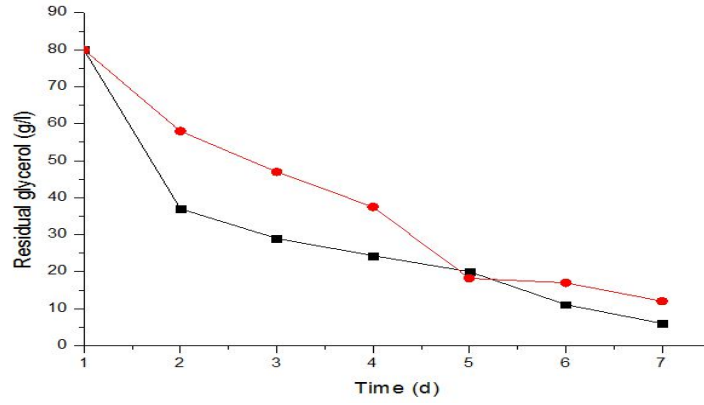
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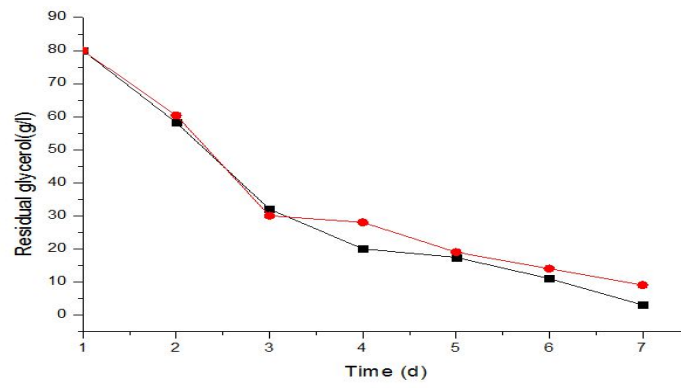
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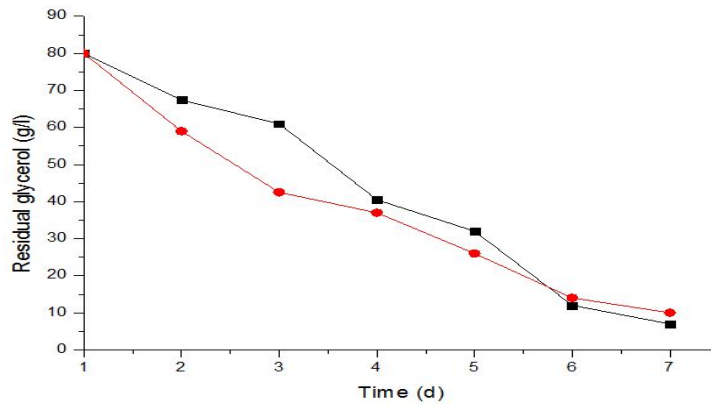
(c)

Figure 2.3: Assessment of glycerol degradation by isolate K8 (a), K10 (b) and S1 (c) using co-fermentation in 80 g/l crude glycerol with 2 g/l glucose added (●) and 80 g/l crude glycerol without the addition of glucose (■) after shake flask incubation at 34 °C and 200 rpm for 7 d

In order to investigate the influence of an organic nitrogen source on the rate of glycerol degradation, 2g/l of yeast extract was added to the fermentation medium.

Isolates K8 (Fig 2.4 a), K10 (Fig 2.4 b) and S1 (Fig 2.4 c) had approximately 100% degradation by the 6th day of fermentation with the addition of 2 g/l yeast extract in the M9 medium. Thereafter, further studies investigated the effect of increasing the amount of yeast extract added to the fermentation to 4 g/l and 6 g/l for each isolate (Fig 2.4). The addition of 4 g/l yeast extract resulted in complete degradation by the 7th day for isolates K8 and K10 and by the 6th day for isolate S1. The addition of 6 g/l yeast extract confirmed 92, 93 and 89% degradation levels by the 7th day for isolates K8, K10 and S1 respectively (Fig 2.4).

The addition of 4 g/l of yeast extract was observed as having a more significant impact on glycerol degradation rates than the addition of 6 g/l yeast extract; however, 2 g/l was best due to fermentation time being reduced from 7 d to 6 d.

The use of complex organic nitrogen sources, such as yeast extract, are known to enhance fermentation as compared to inorganic nitrogen sources like NH_4NO_3 and $(NH_4)_2SO_4$, as they act as both energy and nitrogen sources for the cell, however high concentrations may result in inhibition of product formation or decrease degradation rates due to feedback inhibition in the glycerol metabolism pathway (Jalasutrum and Jetty, 2011).

M9 medium was thereafter modified by adding 2 g/l yeast extract for further degradation experiments with a reduced incubation time of 5 d.
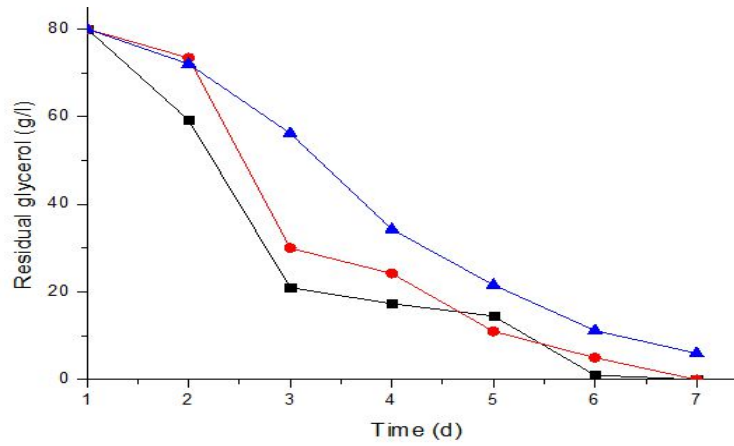
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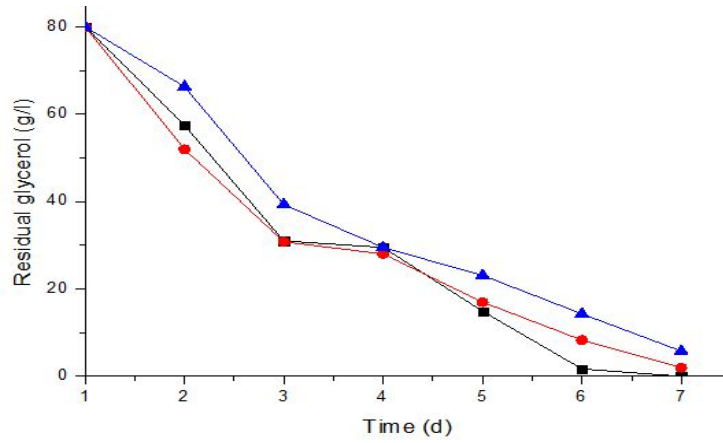
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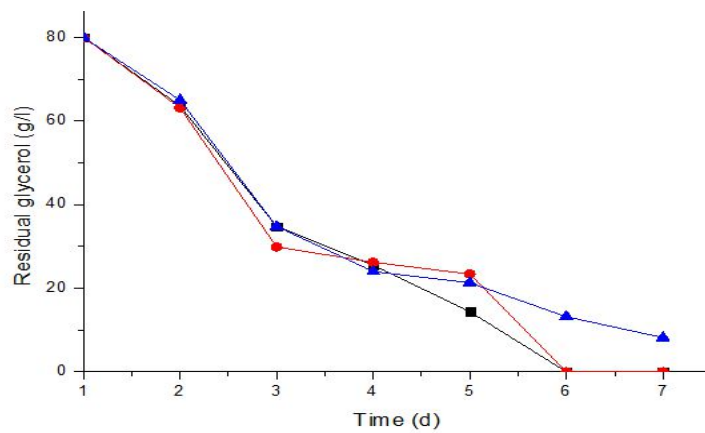
(c)

Figure 2.4: Evaluation of crude glycerol degradation (80 g/l) by isolates K8 (a), K10 (b) and S1 (c) using 2 (■), 4 (●) and 6 g/l (▲) yeast extract after shake flask conditions at 34 °C and 200 rpm for 7 d

All 3 isolates were then subjected to increased crude glycerol concentrations of 100 and 120 g/l in modified M9 media to investigate their ability to tolerate high levels of crude glycerol (Fig 2.5).

Isolate K8 was able to degrade 54% of crude glycerol at 100 g/l and 58% of crude glycerol at 120 g/l concentrations (Fig 2.5 a). K10 degraded 58% at 100 g/l and 51% at 120 g/l (Fig 2.5 b), while S1 degraded 59% at 100 g/l and 41% at 120 g/l crude glycerol concentration (Fig 2.5 c). The inability to completely degrade crude glycerol waste at these increased concentrations could be due to suicidal inactivation of glycerol dehydratase (GDHt) at high glycerol concentrations (Toraya, 2003).
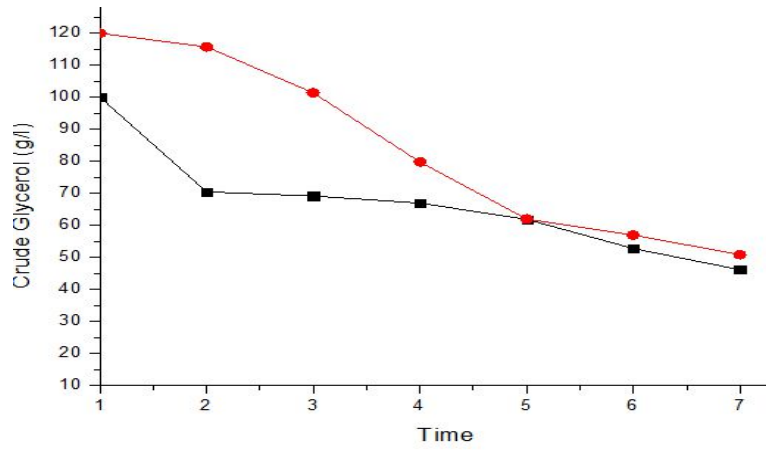
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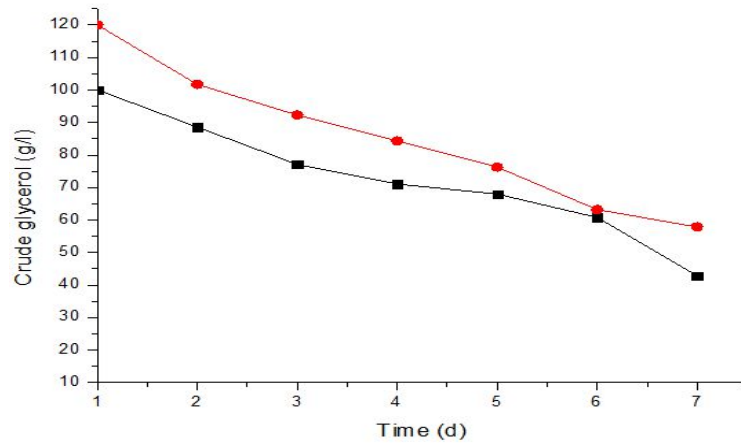
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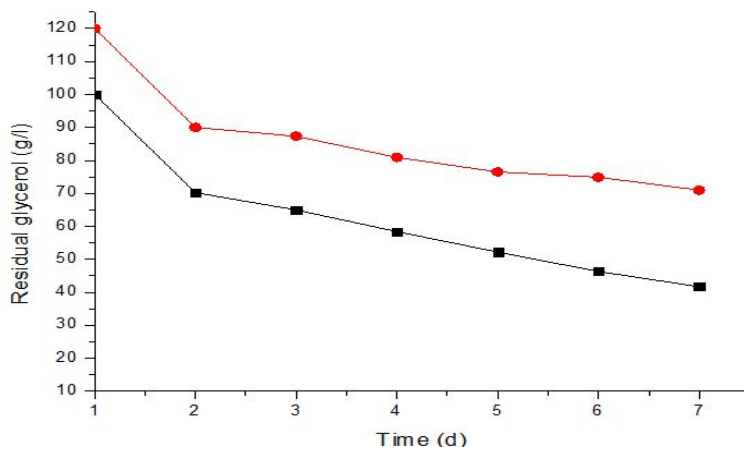
(c)

Figure 2.5: Evaluation of crude glycerol degradation by bacterial isolates K8 (a), K10 (b) and S1 (c) using 100 (■) and 120 g/l (●) crude glycerol at 34°C and 200 rpm for 7 d

This phase of work focussed on finding bacteria that may be used to degrade significantly high concentrations of crude glycerol. Despite a low number of potential isolates, two bacteria species were procured from a crude glycerol sample and one from nutrient-rich soil. All three isolates were able to degrade a maximum of 80 g/l of crude glycerol within 5 to 6 d. This is considerably high in relation to studies done on other microorganisms able to degrade glycerol. A case in perspective is the work of Yazdani and Gonzalez (2008). It was reported that genetically modified strains of *E.coli* (SY03 and SY04) were able to take up approximately 10-12 g/l of analytical grade glycerol. These strains were both modified by mutations that inactivated fumarate reductase (DfrdA) and phosphateacetyl transferase (Dpta) respectively, to favour the production of ethanol and hydrogen and ethanol and formate respectively. In another study, Andre *et al* (2010) reported that *Aspergillus niger* and *Lentinula edodes* were able to metabolise approximately 100% of 30 g/l crude glycerol and 60% of 60 g/l crude glycerol over a period of 300 h.

Chapter 3: Bioconversion of glycerol to 1,3-propanediol and gellan using screened bacterial isolates

3.1   Introduction

Due to the current over-production of glycerol from biodiesel manufacture, hydrolysis of fats in soap and from the production of petro-chemical feedstocks (Wang *et al.,* 2001), the price of glycerol in its crude form has dropped in value, allowing it to now be cheaper than glucose. As a result, glycerol is being extensively studied as a viable alternative carbon source in microbial fermentations rather than using glucose (Dobson *et al.,* 2012). This chapter will focus on the biological conversion of crude glycerol, from the transesterification reaction in biodiesel, to 1,3-PDO and gellan using a strain of *Klebsiella pneumoniae* (S1) isolated from soil and two strains of *Sphingomonas sp.* isolated from biodiesel waste.

1,3 Propanediol

1,3 propanediol (diglycol, trimethylene glycol, propylene glycol), was first discovered in 1881 by August Freund (Ringel *et al.,* 2012). 1,3-PDO is a chemical compound efficiently used as a polyglycol-type lubricant, and can improve the properties of various adhesives, laminates, cosmetics and solvent systems (Chatzifragkou *et al.,* 2011). Recently, 1,3-propanediol (1,3-PDO) has gained significant interest due to its use as a building block in polymerization, especially in the manufacture of polytrimethylene terephthalate (PTT). PTT is a new type of polyester that requires 1,3-PDO as

a monomer (Ji *et al.,* 2010). PTT is currently sold as Sorona ® (DuPont) or Corterra ® (Shell) and has application in carpet and textile manufacture (Wilkens *et al.,* 2012).

Currently, 1,3-PDO is synthesised chemically via two major routes. The first is via acrolein to 1,3-PDO through 3-hydroxypropionaldehyde (hydration process by Degussa/DuPont) while the second is ethylene oxide to 1,3-PDO via 3-hydroxypropionaldehyde (hydroformulation process by Shell). However, these processes involve high pressures, expensive catalysts and the production of toxic by-products. As a result, alternative pathways like the biological conversion of glycerol to 1,3-PDO has attracted much attention in recent years (Chatzifragkou *et al.,* 2011; Sattayasamitsathit *et al.,* 2011; Wilkens *et al.,* 2012). Several microorganisms are capable of producing 1,3-PDO from glycerol under both aerobic and anaerobic conditions. Some of these microorganisms include *Klebsiella oxytoca, Klebsiella pneumoniae, Enterobacter agglomerans, Citrobacter freundii, Clostridium butyricum* and some *Lactobacilus* sp*.* (Rossi *et al.,* 2012; Sattayasamitsathit *et al.,* 2011; da Silva *et al.,* 2009). The microbial fermentation of glycerol to 1,3-PDO has become an attractive method due to being low-cost, environmentally-friendly, requiring low energy and has been proven in recent studies as having a higher production yield when compared to chemical synthesis (Wilkens *et al.,* 2012; da Silva *et al.,* 2009; Jun *et al.,* 2009).

Gellan

Exopolysaccharides produced from microorganisms are water soluble polymers known to have a wide range of applications in the food, pharmaceutical, cosmetic and biotechnology industries (Nampoothiri *et al.,* 2003; Bajaj *et al.,* 2007). Due to their stable and pliable properties, microbial polysaccharides are becoming industrially attractive as compared to gums produced from plants or algae. Gellan is a gum generally produced extracellularly by *Sphingomonas paucimobilis* ATCC 31461 (Kang *et al.,* 1982; West 2003). Sphingan-producing bacteria were classified originally into diverse genera such as *Alcaligenes*, *Azotobacter*, *Pseudomonas*, *Xanthobacter* and *Xanthomonas*; although a re-examination of their phenotypic characteristics indicated that they are all closely related to each other and to *Sphingomonas* species (Pollock 1993; Nampoothiri *et al.,* 2003; Denner *et al.,* 2003).

Gellan gum is found in a variety of applications such as a thickening agent in food, an agar substitute with stability at high temperatures in biotechnology (Fialho *et al.,* 2008), drug delivery in pharmaceuticals (Sultana *et al.,* 2006; Fialho *et al.,* 2008) and as an additive in the cosmetic industry. There are three types viz. native gellan, deacetylated gellan, and clarified gellan. Native gellan is produced into the fermentation medium and has a backbone of repeating units of β-1,3-D-glucose, β-1,4-D-glucuronic acid, β-1,3-D-glucose, α-1,4-L-rhamnose, and two acyl groups, acetate and glycerate, bound to a glucose residue adjacent to glucuronic acid. Deacetylated gellan involves

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removal of acetyl groups in native gellan gum producing a deacetylated nature. Clarified gellan gum is deacetylated gellan that has been heated and filtered to remove any cell protein residue (Bajaj et al., 2007).

Alternative methods for detecting gellan currently being explored involve the interaction between soluble dyes and the polysaccharide. Some examples of dyes being used in polysaccharide detection are aniline blue, brilliant blue, tryptan blue, congo red binding to pachyman and laminaran as well as toluidine blue O and methylene blue staining xanthan (West *et al.,* 2000). Another method for detecting gellan proposed by West *et al.* (2000) was using a dye reagent of 50 µM toluidine blue O dissolved in 50 mM Tris-HCl (pH 7.3). Gellan or gellan-containing supernatant was then added to the dye reagent (1:39). This was incubated at 25 °C for 20 min; the absorbance of the mixture was measured at 530 nm. The gellan concentration in the supernatant was derived using an experimentally-calculated absorption coefficient.

Published reports showed the positive role of *Sphingomonas sp.* strains in bioremediation of toxic wastes in the environment. Tao *et al.* (2006) reported on the successful biotransformation of phenanthrene by *Sphingomonas sp.* GY2B. Zhao *et al.* (2007) also reported on the degradation of phenanthrene using *Sphingomonas sp.* ZP1. Fredrickson *et al.* (1995) showed that bacteria isolated from Southeast Coastal Plain subsurface sediments had 16S ribosomal similarity to *Sphingomonas capsulate* and was able to degrade toluene, naphthalene, and other aromatic compounds.

Most studies have shown gellan to be produced from *Sphingomonas paucimobilis* using mainly glucose as a carbon source. In this chapter, gellan production will be investigated from two *Sphingomonas* strains isolated from crude glycerol waste viz. *Sphingomonas pseudosanguinis* and *Sphingomonas yabuuchiae.*

3.2 Materials and Methods

3.2.1 Identification of bacterial isolates

3.2.1.1 DNA Isolation

DNA was isolated from the bacterial isolates using a cell lysis method. Single bacterial colonies (3-4) were added to 100 µl of double distilled water and resuspended using a vortex (IKA Vortex Genius 3). This was centrifuged for 5 min at 10 000 rpm (Eppendorf Centrifuge 5415R) to pellet cells. The supernatant was then discarded and 100 µl of lysis buffer was added to the pellet. This was boiled for 15 min at 85 ºC and centrifuged for 10 min at 13 000 rpm. The supernatant was used for the PCR reaction.

3.2.1.2 16S PCR

The PCR reaction conditions were adapted from Marchesi *et al.,* (1998) and carried out in an automated thermal cycler (Bioer XP-Cycler). The primers used for the PCR were 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) 1492R (5′-ACGGCTACCTTGTTACGACTT-3′). The PCR mixture contained 1 µl DNA (isolated from lysis method), 5 µl dNTP, 5 µl 27F primer, 5 µl 1492R primer, 3 µl MgCl$_2$, 5 µl Taq Buffer (Fermantas), 5 µl Taq enzyme and 21 µl deionised water. The 16 S PCR product was then sequenced by Inqaba Biotech. PCR products were sequenced using ABI 3130XL genetic analyser (Applied Biosystems). Resultant genomic sequences and graphs were emailed used for sequence alignment using DNA analysis software, DNAMAN (Lynon

Biosoft) and edited using Chromas. Sequence comparison was done by the basic local alignment search tool - BLAST.

3.2.1.3 *Phylogenetic tree construction*

The method for the construction of a phylogenetic tree was adapted from Kumar *et al.* (2004) with minor modifications. Mega 5.05 software was used for phylogenetic analysis after multiple alignments of sequence data using CLUSTAL_X. Distances were calculated using Kimura's two-parameter model (Kimura 1980) and clustering was performed using neighbour-joining method. Bootstrap analysis was used to evaluate tree topology by means of 1000 re-samplings. The similarity between *K. pneumoniae* and other 1,3-PDO producers was thus established, as well as the similarity between *S. pseudosanguinis* and *S. yabuuchiae* and other *Sphingomonas sp.*

3.2.2 *Microscopy of gellan*

Gellan being an exopolysaccharide, will most probably be visible as a layer attached to the cell wall of these isolates. Since no literature was available on this phenomenon, we focused on applying different microscopic techniques to best visualise gellan. Sample preparation and viewing for scanning electron microscopy and transmission electron microscopy were as per UKZN instruction manual at the EM unit in Pietermaritzburg.

3.2.2.1 Light microscopy of S. pseudosanguinis and S. yabuuchiae

Staining of bacterial capsule for negative imaging

Sample broth of each isolate (20 µl) was placed on a slide, together with nigrosine stain dissolved in ethanol. This was allowed to mix and was subsequently smeared across the slide. A 30 min period was allowed for the stain to fix cells onto the slide which was then viewed on an Olympus (Provis) AX 70 light microscope with a Nikon DSR camera.

3.2.2.2 Scanning electron microscopy

Sample broth of each isolate (20 µl) was placed onto a filter membrane (Nucleopore Track E-Tech Membrane, 47 mm, 4 µm) and allowed to air-dry. Thereafter the membranes were transferred into plastic vials and covered with 3% glutaraldehyde in order to fix cells. The glutaraldehyde was then removed and the discs were washed with 0.05 M Cacodylate buffer twice for 30 min each. The discs were then dehydrated using 30, 50, 70, 80, 90 and 100% ethanol for 10 min each. The 100% ethanol was applied three times for 10 min durations before transferring to individual critical point dryer baskets soaked in 100% ethanol. These baskets were then placed into the critical point dryer (Hitachi HCP-2) and allowed to dry for 1 h to remove all ethanol from the dehydration step. Thereafter the discs were fixed onto stubs and

sputter coated with gold (Argon) using an Eiko IB 3 Ion coater before being viewed on a Hitachi S-570 SEM.

3.2.2.3   Environmental scanning electron microscopy of S. pseudosanguinis and S. yabuuchiae

Sample preparation using the air-drying method

Sample broth of each isolate (20 µl) was placed onto a filter membrane (Nucleopore Track E-Tech Membrane, 47 mm, 4 µm) and allowed to air-dry. Thereafter the discs were fixed onto stubs and sputter coated with gold (Argon) using an Eiko IB 3 Ion coater. These were then viewed on a Hitachi S-570 SEM.

3.2.2.4   Transmission electron microscopy of Sphingomonas strains

Staining method for negative imaging

Sample broth of each isolate (20 µl) was transferred onto a piece of parafilm. A copper grid coated with Formvar was then inverted onto the droplet. This was allowed four to five min to bind and then wicked dry with Whatman filter paper. A drop of uranyl acetate (2%) was then placed onto a piece of parafilm and the grid was subsequently inverted onto it to stain. After 30 s the grid was wicked dry with filter paper and thereafter viewed on a JEOL JEM-1400 transmission layer electron microscope with Orius SC 600A camera.

3.2.3 Strain maintenance and fermentation conditions

3.2.3.1   Strain maintenance

Isolates S1, K8 and K10 were maintained on M9 plates with 1% analytical grade glycerol (99.5%, Merck) at 4°C. M9 medium plates contained in g/l: 1, NH_4Cl; 3, KH_2PO_4; 5, NaCl; 6, Na_2HPO_4; 12, technical agar (autoclaved at 121°C; 15 min). Thereafter 1mM $MgSO_4$ and 0.1mM $CaCl_2$ was added (filter-sterilized with a 0.22 µm Millipore filter) (Nimje *et al.,* 2011).

3.2.3.2   Shake-flask experiments and fermentation conditions

Shake-flask fermentations were carried out as described in section 2.2.2.2 using modified M9 medium at varying concentrations of crude glycerol (70-90 g/l). Modified M9 contained in g/l: 2, yeast extract; 1, NH_4Cl; 3, KH_2PO_4; 5, NaCl; 6, Na_2HPO_4. The pH of the medium was adjusted with 1M NaOH for high pH or 1M HCl for low pH experiments.

A 10% inoculum was used for all fermentation experiments. Inoculum was prepared using modified M9 media with 50 g/l crude glycerol in an Infors platform shaker grown for 48 - 72 h at 34 °C and 200 rpm. All inocula were gram-stained before each fermentation to confirm that the culture was free of contamination. Samples (5 ml) were removed every 24 h for 7 d for the analysis of crude glycerol degradation, biomass and product formation.

3.2.3.3   Scale-up Fermentation (5 L)

For scale-up, a 5 L fermentor with a 3 L working volume was used (Infors, Minifors). Inocula were grown in a 500 ml Erlenmeyer flask with a 300 ml working volume using a modified M9 medium with 50 g/l crude glycerol and incubated at 34°C with shaking at 200 rpm over 48 h. from shake flask experiments, media conditions for pH was adjusted to 8 as this was the optimal pH.

Modified M9 medium was made up to a volume of 2.7 L in the fermentor and autoclaved at 121°C for 20 min and thereafter cooled till room temperature. A 10% inoculum was added before fermentation (300 ml) to make up a volume of 3 L. Conditions were as follows: crude glycerol concentration 80 g/l, temperature 34 °C, agitation speed of 600 rpm, pH 8 and dissolved oxygen was set at 0.5 vvm. Samples (25 ml) were removed every 24 h intervals for 7 d.

3.2.4 Analytical methods

3.2.4.1   Dry cell weight of K. pneumoniae

Cell biomass was measured using a method adapted from Ji *et al.* (2009) with minor modifications. Cell culture broth (1 ml) was centrifuged at 10 000 rpm for 15 min at 4°C (Eppendorf Centrifuge 5415R). The pellet was then resuspended with 10 ml saline and re-centrifuged. Cells were then dried at 80°C till constant weight.

3.2.4.2   *Quantification of deacetylated gellan and dry cell weight for S.*

pseudosanguinis and S. yabuuchiae

The method for gellan quantification and dry cell weight was adapted from Nampoothiri *et al.* (2003) with minor modifications. The fermentation broth (3.5 ml) was immersed in a boiling water bath for 15 min, cooled and the pH was increased to 10.0 using 1.0 M NaOH. The broth was then kept at 80 °C for 10 min, after which the pH was adjusted to 7.0 using 1.0 M HCl. The medium was then centrifuged at 10 000 rpm for 25 min at 4 °C to separate the cell mass. This cell mass was then dried till constant weight at 80 °C in an oven for dry cell weight determination. Cell free supernatant (3 ml) was then added to three volumes of ice cold isopropyl alcohol (-70°C) to precipitate the deacetylated gellan. The alcohol supernatant mixture was kept overnight at 4°C to enhance precipitation. The produced precipitate was then recovered by centrifuging at 10 000 rpm for 25 min at 4 °C. The precipitated deacetylated gellan was then dried to a constant weight in a hot air oven (80 °C, 24 h).

3.2.4.3   *Evaluation of glycerol degradation*

HPLC analysis was conducted as described in section 2.2.4.1 to determine glycerol degradation and for the detection of organic acids. Standards used were 1,3 propanediol (Sigma-Aldrich), D-lactic acid (Merck) and glycerol (Merck).

3.3 Results and discussion

3.3.1 Klebsiella pneumoniae

3.3.1.1 Identification of bacterial isolate S1

Molecular identification of S1, isolated from soil was carried out using 16S rDNA techniques. Gram-stain results done in the laboratory showed S1 as a gram-positive short rod. Strain S1 was identified as *Klebsiella pneumoniae* subsp ozaenae strain ATCC11296 (99% similarity) from the 16S sequence results and BLAST analysis (Table 3.1). A phylogenetic tree was then constructed to analyse this strain's similarity to other glycerol degrading strains (Fig 3.1).

Several reports have supported the screening of glycerol-degrading bacteria, 1,3-PDO producers in particular, from soil. *K. pneumoniae* is currently a well-known producer of 1,3-PDO from glycerol, making it an extensively researched bacterium. Jalasutram and Jetty (2011) isolated a strain of *K. pneumoni*ae from soil that was able to metabolise 100 g/l glycerol and produce 38.7 g/l 1,3-PDO. In a another report, 76.2 g/l of 1,3-PDO was produced from crude glycerol as a carbon source with an initial 28 g/l concentration in a 1 L volume using a strain of *Clostridium butyricum* isolated from soil (Wilkens *et al.,* 2012). Ringel *et al.* (2012) also reported up to 77 g/l 1,3-PDO production from a strain of *Clostridium butyricum* found in soil.
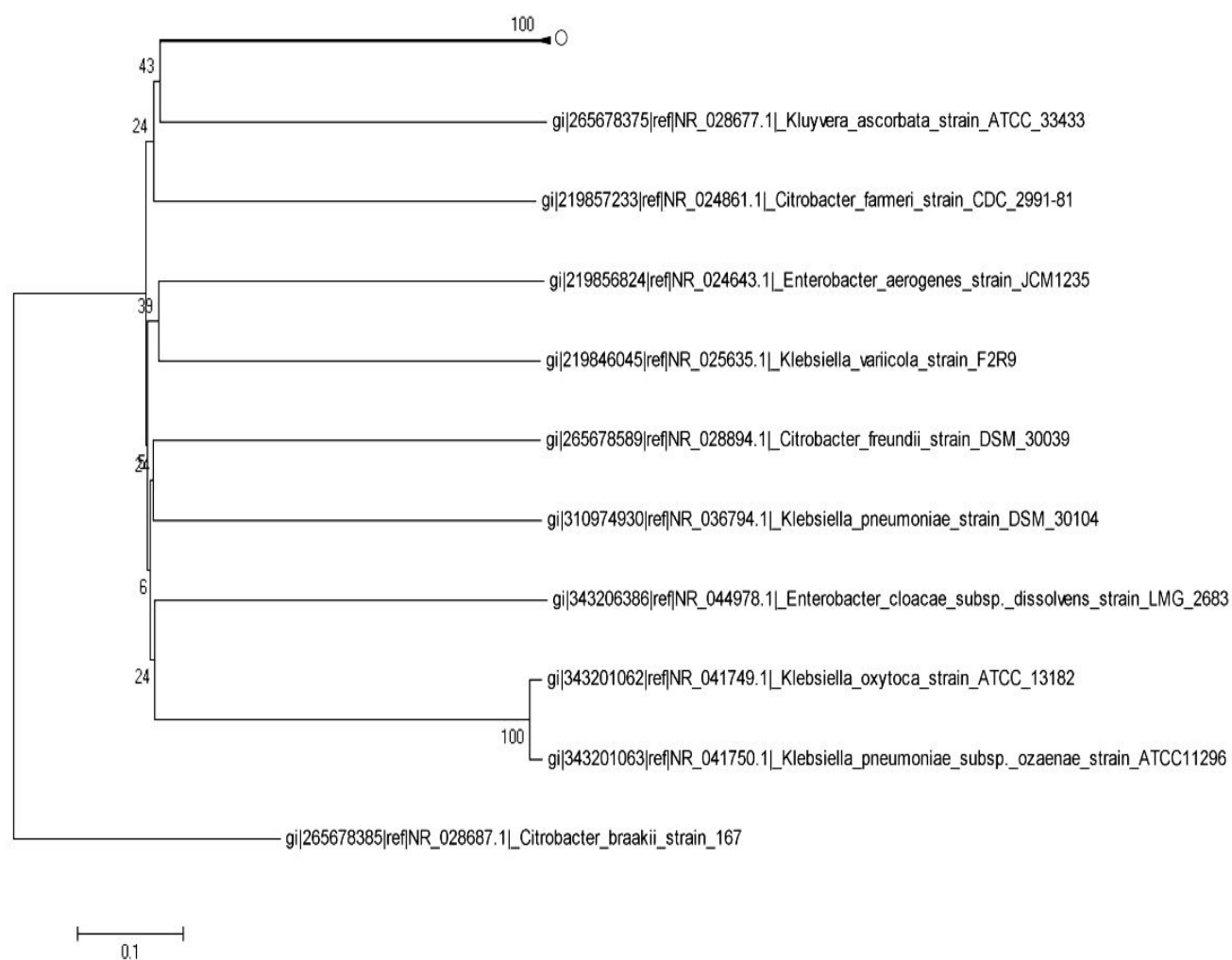
Figure 3.1: Phylogenetic tree representing the similarity of *Klebsiella pneumoniae* subsp ozaenae strain ATCC11296 with other strains of Klebsiella and other 1,3-PDO producers

Table 3.1: Blast search results for isolate *K. pnuemoniae* (S1)

Accession	Description	Max score	Total score	Query coverage	E value
NR_041750.1	*Klebsiella pneumoniae* subsp. *ozaenae* strain ATCC11296 16S ribosomal RNA, partial sequence	1615	1615	99%	0.0

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3.3.1.2   Shake flask experiments to evaluate the influence of pH on
Klebsiella pneumoniae

This study was conducted using three different pH values (6, 7 and 8) at three crude glycerol concentrations (70, 80 and 90 g/l); (Fig 3.2). Samples were taken daily and analysed using HPLC (section 3.2.3). HPLC analysis of glycerol degradation at pH 6 showed best degradation at 70 g/l crude glycerol (100%), while at 80 g/l and 90 g/l crude glycerol degradation was 99.3 % and 77.7%, respectively after 7 d (Fig 3.2 a). Data shown in Fig 3.2 b & c revealed that for pH 7 and 8 respectively, complete degradation (100%) occurred after 5 d at a glycerol concentration of 70 and 80 g/l. However, at the higher glycerol levels (90 g/l) approximately 90 % degradation was observed for pH 7 after 7 d and complete degradation was achieved after 6 d for pH 8.

In comparison to pH 7 and pH 8, pH 6 results showed a slower rate of degradation overall. *K. pneumoniae* was only able do completely degrade 70 g/l of crude glycerol over 7 d whereas at pH 7 and pH 8 for 70 g/l crude glycerol, both 70 and 80 g/l glycerol concentrations were degraded after 5 d. The rationale behind a lower pH (pH 6) demonstrating a slower rate of degradation could be due to the enzymes involved in glycerol degradation being lower in activity due to the impurities in the crude glycerol.

There are many impurities present in crude glycerol such as methanol, fatty acids, soap, heavy metals or salts, depending on the catalysts and feedstock used during the transesterification of biodiesel (Gonzalez-Pajuelo *et al.,* 2004;

Chatzifragkou *et al.,* 2010; Wilkens *et al.,* 2012). A higher concentration of toxic substances within the glycerol may have an effect on cell division and can reduce cell viability due to sodium and heavy metal ions (Gonzalez-Pajuelo *et al.,* 2004). These impurities could be the reason for slower uptake of crude glycerol by *K. pneumoniae* at a concentration of 80 and 90 g/l. A study by Matsumura *et al.* (2008) tested *C. diolis* DSM 15410 on crude glycerol from sunflower oil transesterification. The result was inhibition of growth due to a high concentration of unsaturated fatty acids being present in the glycerol.

Cell dry weights were then measured to establish a correlation between cell growth and glycerol degradation at the pH's investigated (6, 7 and 8). At pH 6 (Fig 3.3 a), 70, 80 and 90 g/l crude glycerol reached maximum cell dry weight at 7 d (27.8, 32.6 g/l and 35.4 g/l respectively), while at pH 7 (Fig 3.3 b), maximum cell dry weights are reached after 5 d for 70 and 80 g/l (30.5 and 27.8 g/l). At 90 g/l, maximum weight is 38.9 g/l at 7 d. At 90 g/l concentrations a maximum weight was attained after 6 d (23.7 g/l) (Fig 3.4 c). For 70 and 80 g/l, maximum weight was reached after 5 d (39.8 g/l and 35.9 g/l). Dry cell weight notably increased with time which indicated good cell growth at the three pH values assayed. High dry cell weights were noted for *K. pnemoniae*, it was assumed that this could be due to the untreated crude glycerol not being completely washed off the cells, thus increasing the weight of the cells after drying.

It was observed at all three pH values, the rate of glycerol degradation increased with increasing initial pH values. This phenomenon was also noted in a report where *K. pneumoniae* SU6 was able to completely degrade crude glycerol in 14-16 h at pH 7.5 rather than at pH 6.5 (Sattayasamitsathit *et al.,* 2011). Barbirato *et al.* (1997) also reported that *E. agglomerans* had a more efficient rate of glycerol degradation at pH 8, resulting in complete degradation after 35 h. From pH readings taken before and after glycerol degradation there was a noticeable decline in final pH readings during the uptake of glycerol over the 7 d period. This drop in pH may be attributed to production of organic acids such as lactate during fermentation. For pH 6, 70 g/l crude glycerol, after 7 d pH declined to 5.55, pH 7 at a 70 g/l crude glycerol concentration declined to 5.96 and pH 8, 70 g/l crude glycerol reduced to 7.11. For 80 g/l crude glycerol pH 6 reduced to 5.3, pH 7, 80 g/l crude glycerol to 6.11 and pH 8 at 80 g/l crude glycerol concentration declined to 6.91. At 90 g/l crude glycerol, pH 6 reduced to 5.12, pH 7 to 6.07 and pH 8 to 6.69. This decline in pH could also be due to the impurities in the biodiesel waste becoming more prominent in the fermentation as the major component (glycerol) decreases (Ji *et al.,* 2008; Sattayasamitsathit *et al.,* 2011; Wilkens *et al.,* 2012).
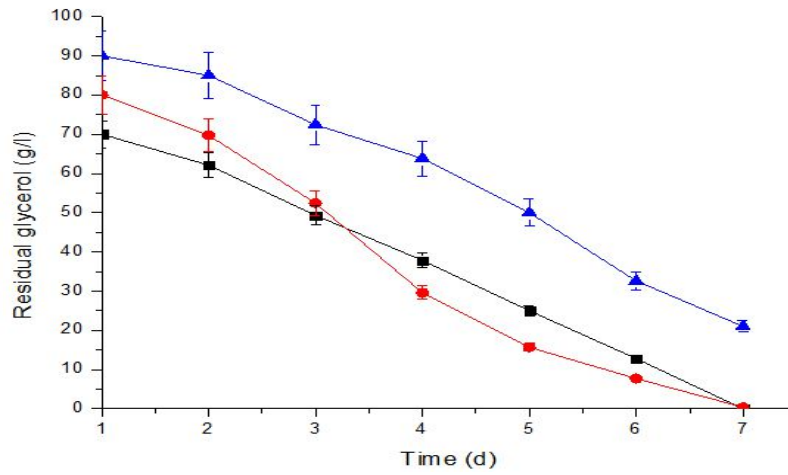
(a)
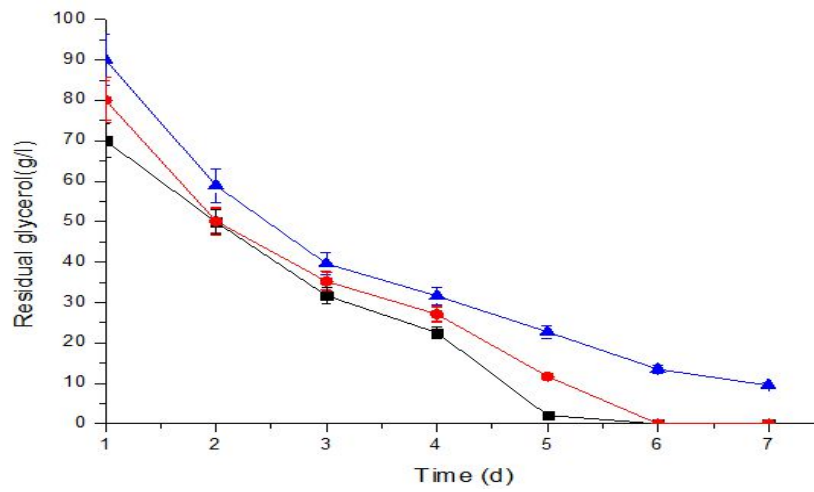
(b)
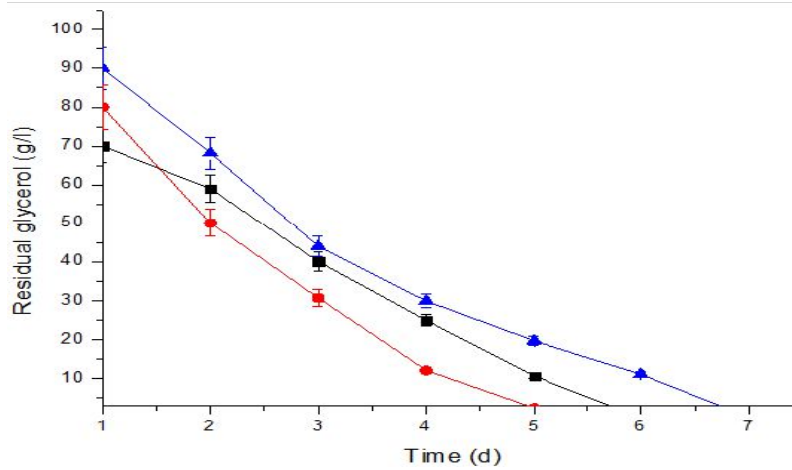
(c)

Figure 3.2: Crude glycerol degradation by *K. pneumoniae* during shake flask cultivations at 34°C and 200 rpm over 7 d at (a) pH 6; (b) pH 7 and (c) pH 8 using glycerol concentrations of: (■) 70 g/l), (●)80 g/l and (▲) 90 g/l.
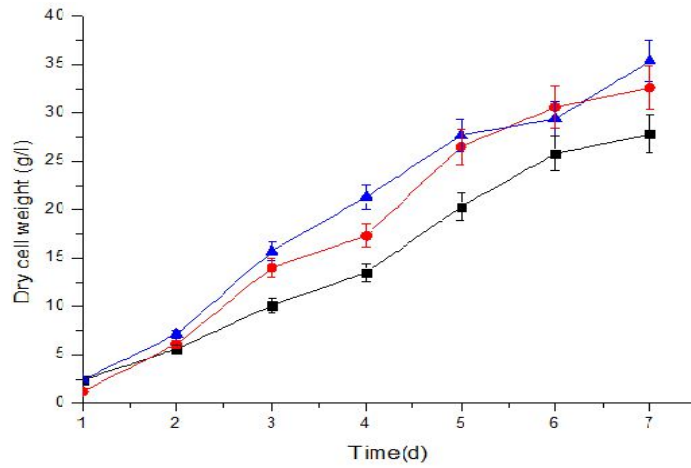
(a)
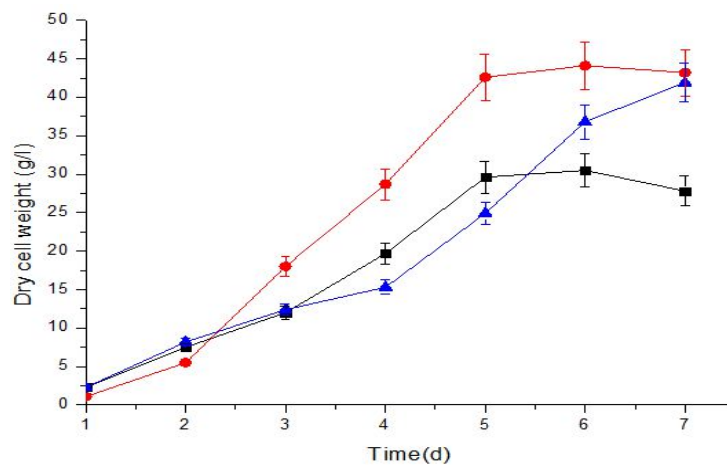
(b)

(c)

Figure 3.3 Assessment of dry cell weight of *K. pneumoniae* during shake flask cultivations at 34°C and 200 rpm over 7 d at (a) pH 6; (b) pH 7 and (c) pH 8 using glycerol concentrations of: (■) 70 g/l), (●) 80 g/l and (▲) 90 g/l

3.3.1.3 Bioconversion of crude glycerol into 1,3-PDO and lactic acid

1,3-propanediol and lactic acid concentrations were also quantified for the 3 pH values at 70, 80 and 90 g/l crude glycerol concentrations. At pH 6, 1,3-PDO and lactic acid was not produced. This could be attributed to a lower pH having an effect on the coenzyme glycerol dehydratase (GDHt). This enzyme along with related diol dehydratases (da Silva *et al.,* 2009) is responsible for catalysing the conversion of glycerol to 3-hydroxypropionaldehyde (3-HPA), which is subsequently reduced by NADH to 1,3-PDO via 1,3-PDO-NAD oxidoreductase (*dhaT*) (Liu *et al.,* 2007). Such knowledge of glycerol metabolism in *K. pneumoniae* is vital in genetically modifying this bacterium for optimum 1,3-PDO production. A study by Zhuge *et al.* (2010) reported higher 1,3–PDO production from a strain of *K. pneumoniae* which had expression of the genes for enzyme *dhaT* and *yqhD.* Both these enzymes are involved in the conversion of 3-HPA to 1,3-PDO. Yields of 1,3-PDO were reported at 18.3 g/l as compared to its wild-type which was found to produce 17.1 g/l. These values however, are not as high as the values yielded from the *K. pneumoniae* in this study at pH 7 and 8.

At pH 7 (Fig 3.4 a) the best 1,3-PDO production was quantified at 80 g/l (19.1 g/l after 4 d). At pH 8 (Fig 3.4 b), 80 g/l was once again the best concentration with 37 g/l of 1,3-PDO produced, while 90 g/l produced 30.4 g/l of 1,3-PDO. There was also production of lactic acid at pH7 and pH8 (Fig 3.5 a, b)

In comparison with other studies, the amount of 1,3-PDO at pH 7 and pH 8 was relatively high. Ma *et al.* (2009) reported 8.73 g/l of 1,3-PDO produced from 20 g/l from a batch culture using *K. pneumoniae* XJPD-Li. Zhang *et al,* (2007) reported 12.2 g/l 1,3-PDO using a strain of *K. pnuemoniae* isolated from soil and was able to produce significant amounts of 1,3-PDO with only analytical grade glycerol (20 g/l). Batch cultivations of *K. pneumoniae* BLh-1 was able to produce 6.2 g/l 1,3-PDO from 25-30 g/l crude glycerol (Rossi *et al.,* 2012). A study done by Hao *et al.* (2008) reported *K. pneumoniae* and *C. freundii* strains isolated from soil produced a maximum of 11 g/l 1,3-PDO from 30 g/l analytical grade glycerol in aerobic shake-flask cultivations.

From the results of this study*, K. pneumoniae* demonstrated optimum degradation at pH 8 with 80 g/l crude glycerol concentration as well as best 1,3-PDO production at these conditions over 5 d. Other studies show the use of low concentrations of glycerol in either crude or pure form in the production of 1,3-PDO (20-50 g/l) as compared to this study. Sattayasamitsathit *et al.* (2011) reported that the consumption for 40 g/l glycerol was 100% when compared to 60, 80 and 100 g/l where rates were 56.7, 32.5 and 18 % respectively, for *K. pneumoniae* SU6. One of the reasons for this is the suicidal inactivation exhibited by the glycerol dehydratase enzyme at higher glycerol concentrations. Sattayasamitsathit *et al.* (2011) also noted that 1,3-PDO concentrations increased even though degradation of glycerol decreased, up to 80 g/l. This was noted at pH 7 and 8 in this study where 1,3-PDO was lower at 90 g/l than 80 g/l (Fig 3.4 a, b). Barbirato *et al.* (1997) reported that at high glycerol concentrations the accumulation of

intermediates (3-hydroxypropioaldehyde) during 1,3-PDO production resulted in inhibition of growth for *E. agglomerans* before glycerol fermentation. This bacteria follows the same pathway of 1,3-PDO production as *K. pneumonie* and *C. freundii.*
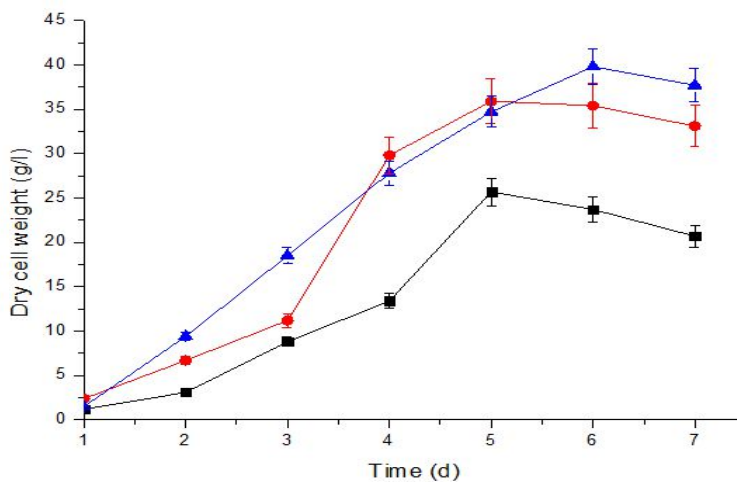

(a)

(b)

Figure 3.4: 1,3-PDO (g/l) production by *K. pneumoniae* during shake flask cultivations at 34°C and 200 rpm over 7 d at (a) pH 7 and (b) pH 8 using glycerol concentrations of: (■) 70 g/l), (●)80 g/l and (▲) 90 g/l.

At pH 7 (Fig 3.5 a) with 70 g/l crude glycerol, the most lactic acid produced was 4.3 g/l after 3 d. The maximum lactic acid produced was at pH 7 was at 80 g/l were lactic acid was produced at 5.9 g/l after 4 d. At 90 g/l crude glycerol, 5.1 g/l lactic acid was produced after 4 d. At pH 8 (Fig 3.5 b) there was best lactic acid production at 80 g/l crude glycerol (6.8 g/l) while 70 and 90 g/l produced a maximum of 4.8 and 5.7 g/l respectively, at 4 d.

In this study, lactic acid production occurred at pH 7 and 8. The highest amount of lactic acid (6.8 g/l) was produced at pH 8 at a crude glycerol concentration was at 80 g/l. The rationale behind higher pH producing more lactic acid can be attributed to a more alkaline environment, thus allowing for better activity of enzymes in crude glycerol. Ji *et al.* (2009) reported higher lactic acid production (11.2 g/l) at an increased pH (pH 7.3) and a higher production of 2,3 butanediol at pH 5 to pH 6. The inference was that a more alkaline broth allows for lactic acid to be the dominant product as undissociated organic acids were favoured.
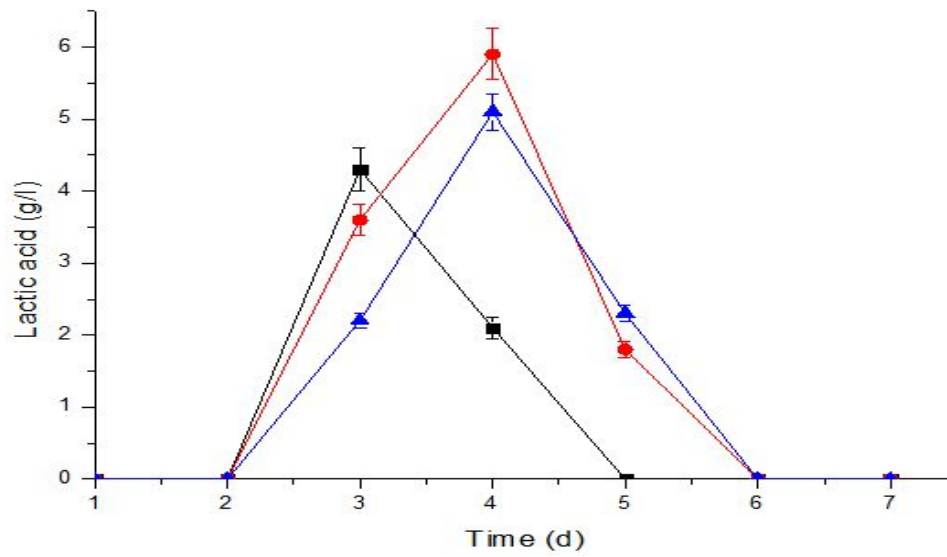
(a)
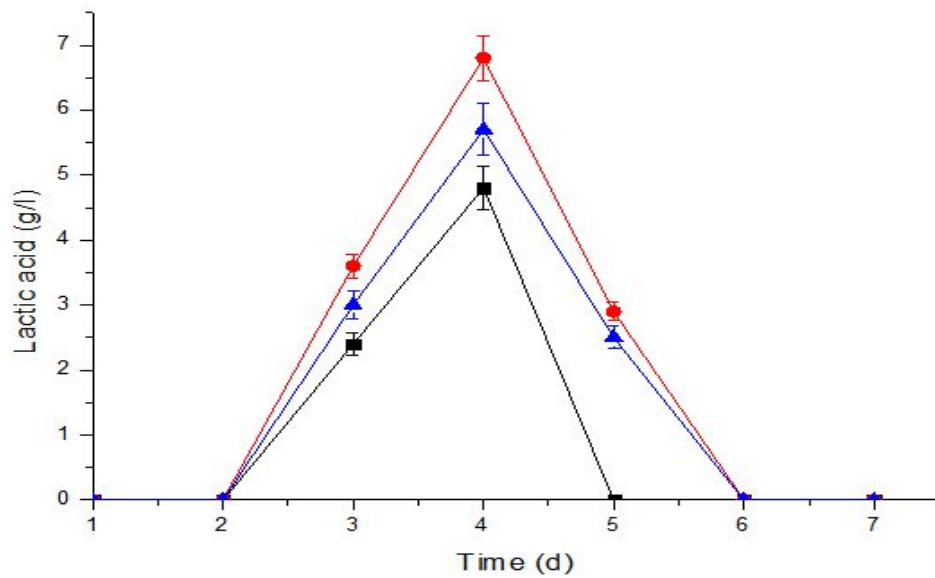
(b)

Figure 3.5: Lactic acid (g/l) production by *K. pneumoniae* during shake flask cultivations at 34°C and 200 rpm over 7 d at (a) pH 7 and (b) pH 8 using glycerol concentrations of: (■) 70 g/l), (●)80 g/l and (▲) 90 g/l.

The next experiment involved the scaling up of fermentation to a 5 L fermenter with a 3 L working volume. Data shown in Fig 3.6 depicts 54 % degradation of crude glycerol after 7 d. A maximum of 18.5 g/l of 1,3-PDO was produced at 4 d with 4.9 g/l lactic acid after 6 d (Fig 3.6). The lack of glycerol degradation could be due to micro-aerobic growth conditions of 0.1 vvm. The production of 18.5 g/l of 1,3-PDO was high in comparison to other studies done (Jun *et al.,* 2010; Wilkens *et al.,* 2012). Studies reported on large fermentations volume are generally done at lower concentrations of glycerol. Wilkens *et al.* (2011) used 28 g/l as an initial concentration in a 1 L volume fermentor and 20 g/l was the initial concentration at a 200 L volume. Jun *et al.* (2010) reported an initial glycerol concentration of 40 g/l in a 3 L fermenter with a 1.5 L working volume.

A chromatogram of HPLC results yielded from the detection and quantification of glycerol, 1,3-PDO and lactic acid during fermentation is shown in Fig 3.7.
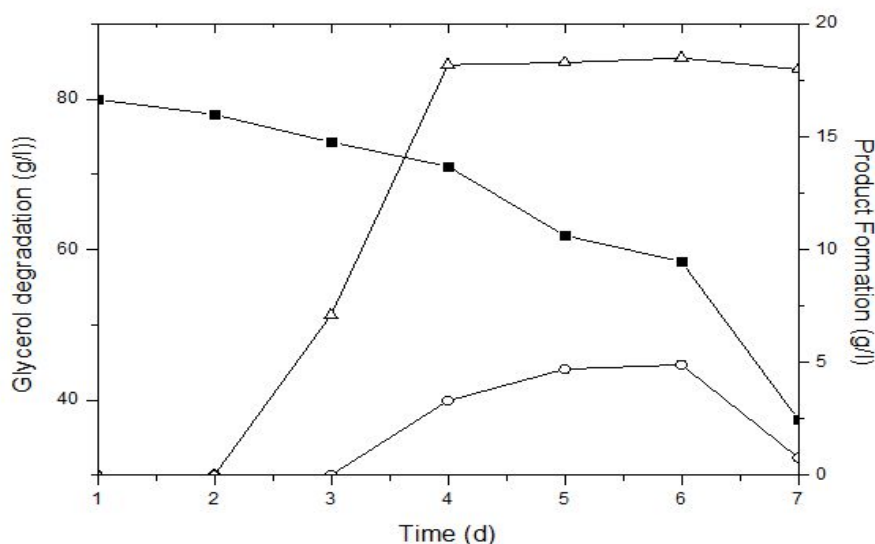
Figure 3.6: Crude glycerol degradation (■) (80 g/l) and production of 1,3-PDO (Δ) and lactic acid (O) by *K. pneumoniae* during cultivation in a 5L fermentor at 34°C and 600 rpm over 7 d
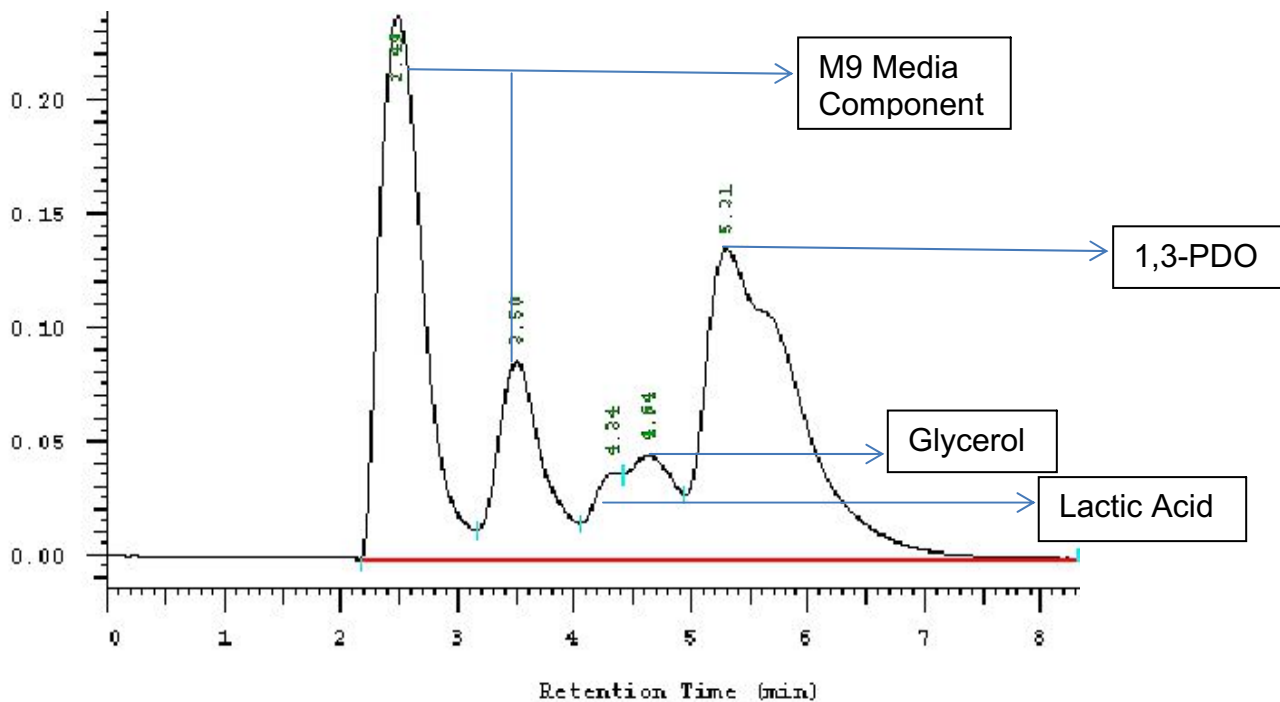
Figure 3.7: HPLC chromatogram for glycerol, 1,3-PDO and lactic acid detection using _K. pneumoniae_

The ability of _K. pneumoniae_, isolated from soil, to completely degrade crude glycerol to a maximum concentration of 90 g/l as well as produce significantly high amounts of 1,3-PDO under shake flask conditions was a significant finding in this study. This isolate showed most efficient degradation of glycerol and best 1,3-PDO production at pH8 and crude glycerol concentration 80 g/l. From the comparisons reported in this study it is evident that this microorganism has significant potential to biologically convert crude glycerol to 1,3-PDO. The ability of this isolate to metabolise high concentrations of crude glycerol also gives it an added advantage when compared to other 1,3-PDO producers which are mostly able to convert analytical grade or purified forms of glycerol to 1,3-PDO.

3.3.2 S. pseudosanguinis (K8) and S. yabuuchiae (K10)

3.3.2.1   Identification of Strain K8 and K10

The results from the 16 S rDNA sequences identified the bacterial isolates, isolated from untreated biodiesel waste, K8 and K10 as *S. pseudosanguinis* and *S. yabuuchiae* respectively, using BLAST (Table 3.2). Isolating *Sphingomonas sp.* from industrial wastes has been previously reported (Govender and Pillay 2011; *Tao et al.,* 2007). Govender and Pillay (2011) isolated *S. paucimobilis* UT26 from DCA-containing industrial waste. Tao *et al.,* (2007) isolated *Sphingomonas sp.* GY2B was isolated from soils contaminated with polycyclic aromatic hydrocarbons (PAHs).

A phylogenetic tree was then constructed to analyse this strain's similarity to other *Sphingomonas sp.* (Fig 3.8). The phylogenetic tree for *S. pseudosanguinis* and *S. yabuuchiae* demonstrates their similarity to each other as well as other strains of *Sphingomonas sp*. A direct relationship was noted between both these strains and a strain of *S. paucimobilis* which is most commonly used in gellan production (Fig 3.8).

Table 3.2: Blast search results for *S. psuedosanguinis* (K8) and *S. yabuuchiae (*K10)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NR_028634.1	*Sphingomonas pseudosanguinis* strain : G1-2 16S ribosomal RNA, partial sequence	2401	2401	99%	0.0	99%
NR_028634.1	*Sphingomonas yabuuchiae* strain A1-18 16S ribosomal RNA, partial sequence	2298	2298	100%	0.0	99%

3.3.2.2   Evaluating the effect of pH on glycerol degradation by S. pseudosanguinis and S yabuuchiae at shake flask level

In this study, isolates *S. pseudosanguinis* and *S. yabuuchiae* was exposed to crude glycerol at three different concentrations (70, 80 and 90 g/l) and three different pH's (6, 7 and 8).

For *S. pseudosanguinius*, complete degradation was observed after 6 d at pH 6 using both 70 g/l and 80 g/l crude glycerol concentrations(Fig 3.9 a) while at 90 g/l there was 83 % degradation after 7 d (Fig 3.9 a). After 7 d pH values declined to pH 5.3 – 5.5 for crude glycerol concentrations 70, 80 and 90 g/l.

For *S. yabuuchiae*, pH 6 results yielded 90% crude glycerol degraded for 70 and 80 g/l crude glycerol concentrations while at 90 g/l crude glycerol, 86 % degradation occurred after 7 d (Fig 3.10 a). The final pH values after 7 d for all experiments at pH 6 were around pH 5.

A similar trend of glycerol degradation was observed for 70 and 80 g/l crude glycerol at pH 7 and pH 8 for *S. pseudosanguinis* (Fig 3.9 b & c) and *S. yabuuchiae* (Fig 3.10 b & c). After 5 d, all glycerol was degraded at the 70 and 80 g/l crude glycerol concentrations for both strains, while 90 g/l degraded after 6 d. The overall pH of each medium decreased after 7 d from pH 7 to between pH 6 and pH 6.3. At pH 8 the overall pH value for both strains declined to approximately pH 7.

Most efficient degradation was found to be after 5 d for pH 7 and 8 at 70 and 80 g/l crude glycerol concentrations. The ability of *Sphingomonas sp.* to degrade industrial wastes has been reported in many studies. *Sphingomonas sp.* ZP1, isolated from industrial waste samples was able to degrade naphthalene, phenanthrene, toluene, methanol and ethanol, salicylic acid and Tween 80. Moreover, it could remove nearly all the phenanthrene at a concentration of 0.025% in 8 d (Zhao *et al.,* 2007). SS3 isolated from industrial waste contaminated soil in Germany utilizes diphenyl ether and its 4-fluoro, 4-chloro, and (to a lesser extent) 4-bromo derivatives as a sole source of carbon and energy (Schmidt *et al.,* 1992). It was noted that at 90 g/l crude glycerol concentration at pH 7 and 8, the glycerol took a longer time to degrade (7 d), while at pH 6 only 86 % for *S. yabuuchiae* could be degraded

and 83 % for *S. pseudosanguinis*. The rationale behind this could be due to non-reacted components in the crude glycerol accumulating in the broth and thus inhibiting or lowering the enzyme activities resulting in longer degradation time (Freitas *et al.,* 2011).
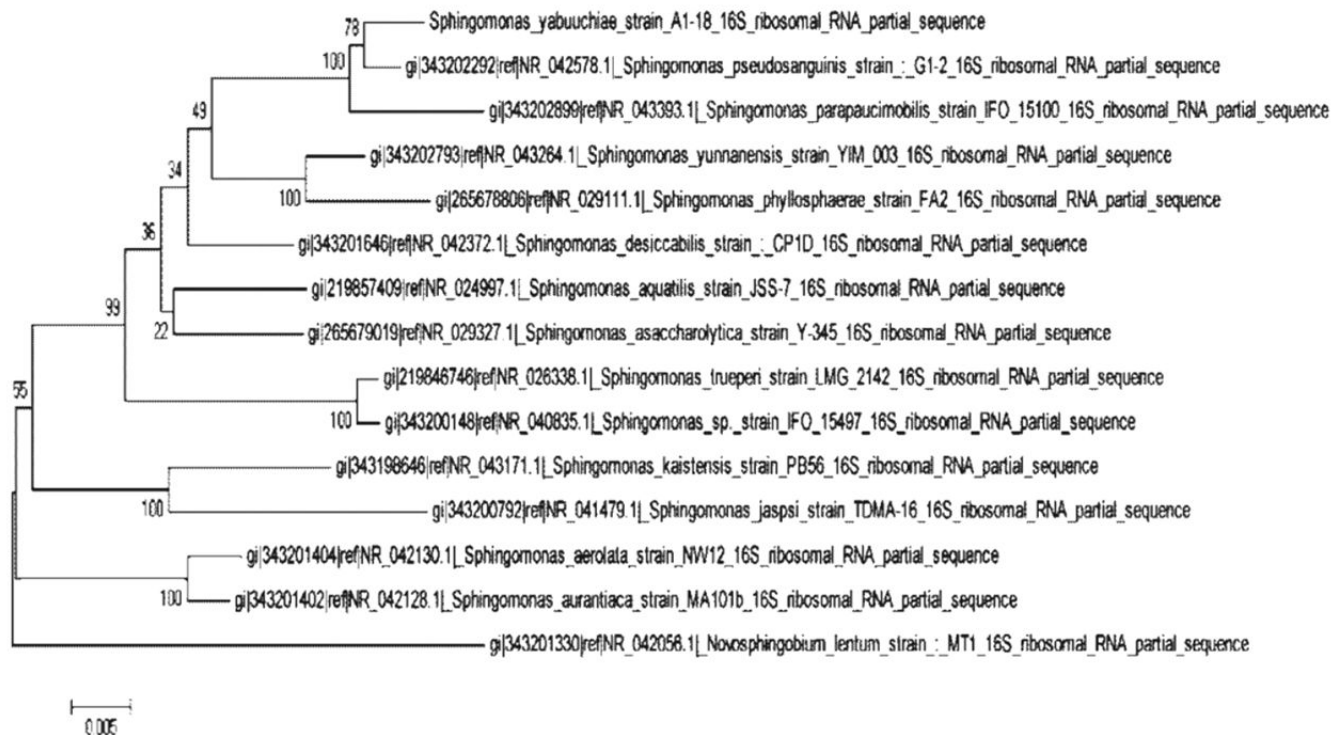
Figure 3.8: Phylogenetic tree representing the similarity of *S. pseudosanguinis* and *S. yabuuchiae*
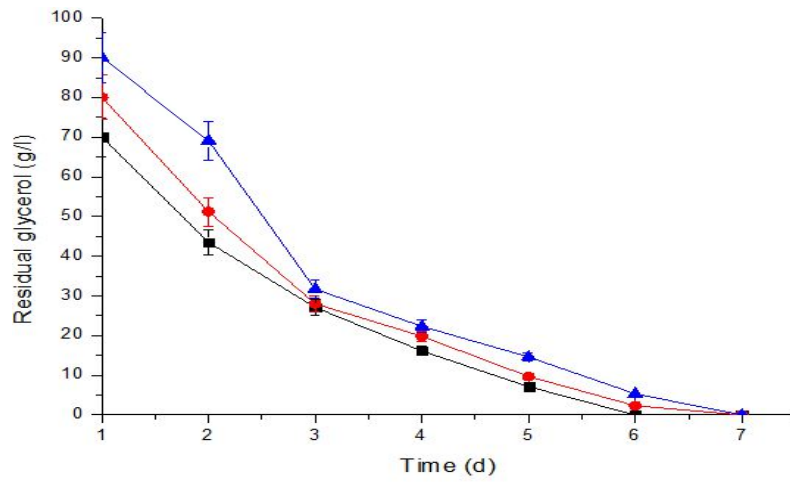
(a)
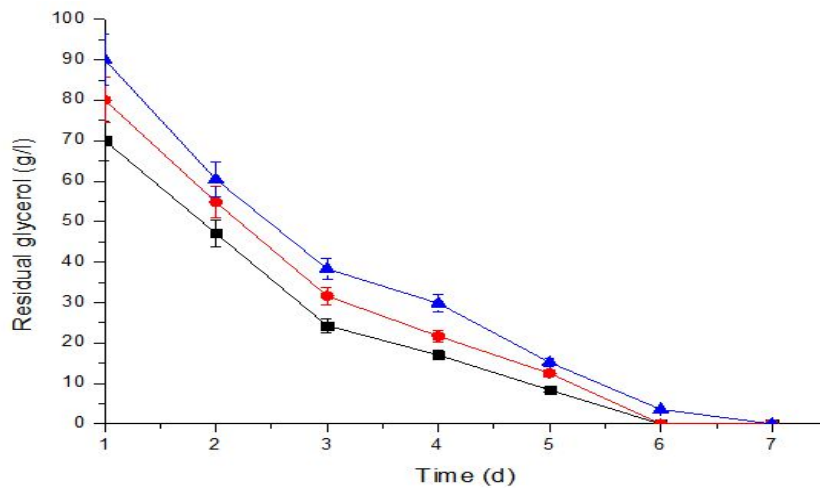
(b)
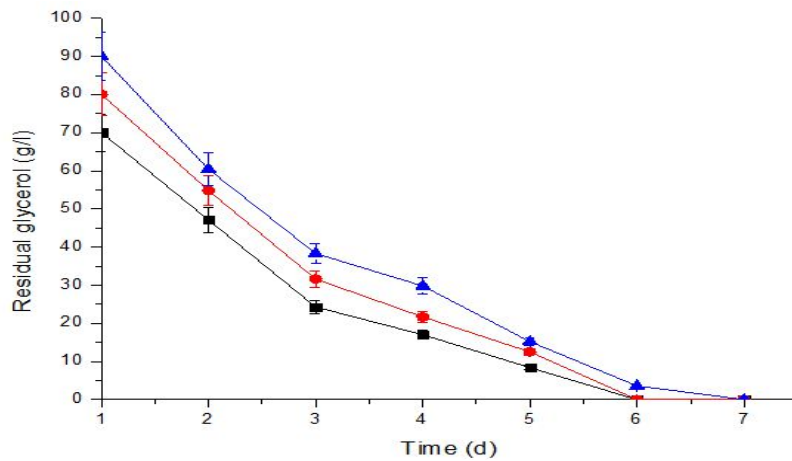
(c)

Figure 3.9: Crude glycerol degradation by *S. pseudosanguinis* during shake flask cultivations at 34°C and 200 rpm over 7 d at (a) pH 6, (b) pH 7 and (c) pH 8 using glycerol concentrations of: 70 (■), 80 (●) and 90 (▲) g/l

Figure 3.10: Crude glycerol degradation by *S. yabuuchiae* during shake flask cultivations at 34°C and 200 rpm over 7 d at (a) pH 6, (b) pH 7 and (c) pH 8 using glycerol concentrations of: 70 (■), 80 (●) and 90 (▲) g/l

3.3.2.3   Evaluating the effect of pH on gellan production by S. pseudosanguinis and S. yabuuchiae

Gellan production at different glycerol concentrations (70 to 90 g/l) and at the three pH values (6 to 8) over 7 d of fermentation is shown in Fig 3.11 and Fig 3.12 for *S. pseudosanguinis* and *S. yabuuchiae* respectively.

The production of gellan was assessed using the method described in section 4.2.4.1. This procedure has great similarity to many other exopolysaccharide quantifications. Most procedures require the heating of the broth to boiling point before cell removal in order to kill the bacterial cells and deactivate enzyme processes. The heating step also allows for broth viscosity to be reduced (Bajaj *et al.,* 2007; Freitas *et al;* 2011).

For *S. pseudosanguinis*, at pH 6 (Fig 3.11 a) maximum gellan production was recorded at 80 g/l crude glycerol after 6 d (48.8 g/l) followed by 70 g/l crude glycerol which resulted in 42.2 g/l gellan produced after 6 d and 90 g/l crude glycerol which yielded 46.7 g/l gellan after 7 d. In comparison to pH 6, gellan production was higher at pH 7. At pH 7 (70 g/l crude glycerol) (Fig 3.11 b) *S. pseudosanguinis* produced a maximum of 44.7 g/l gellan after 5 d, 80 g/l crude glycerol produced 50.9 g/l after 6 d and 90 g/l crude glycerol produced 49.8 g/l gellan after 6 d. At pH 8, (Fig 3.11 c) highest gellan production was achieved with 80 g/l crude glycerol (49.9 g/l after 5 d) whilst similar values were attained with 70 g/l and 90 g/l crude glycerol after 5 and 6 d respectively.

S. yabuuchiae produced 46.3 g/l gellan after 5 d at pH 6 70 g/l crude glycerol, 80 g/l crude glycerol produced 36.3 g/l after 7 d while 90 g/l crude glycerol yielded 41.9 g/l gellan after 6 d (Fig 3.12 a). At pH 7 70 g/l crude glycerol, 37 g/l gellan was produced after 7 d, at 80 g/l crude glycerol concentration 52.7 g/l was produced after 6 d and 90 g/l crude glycerol produced 48.7 g/l gellan after 6 d (Fig 3.12 b). In the pH 8 experiments, (Fig 3.12 c) 70 g/l crude glycerol produced 35.3 g/l gellan after 6 d, 80 g/l crude glycerol produced 50.8 g/l gellan after 6 d and 90 g/l crude glycerol produced 48.7 g/l gellan after 6 d. From this study, it was found that *S. pseusosanguinius* was able to produce a maximum of 50.9 g/l gellan at pH 7 and 80 g/l crude glycerol while *S. yabuuchiae* produced the best gellan yield of 52.6 g/l at identical conditions.
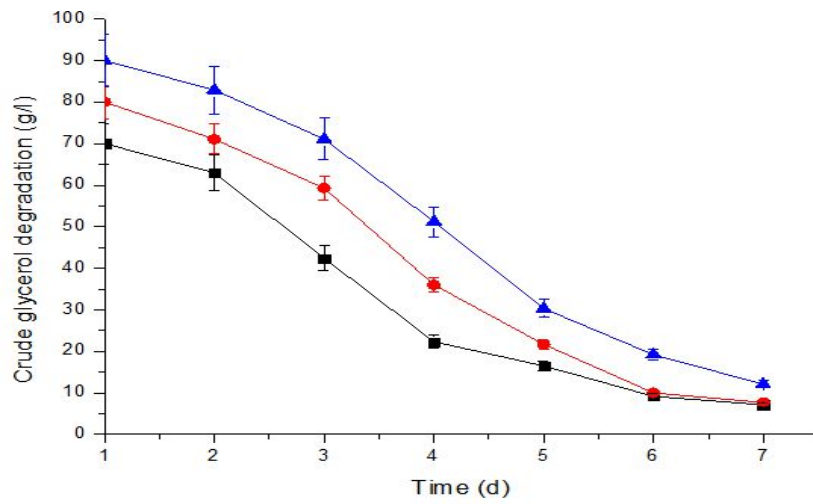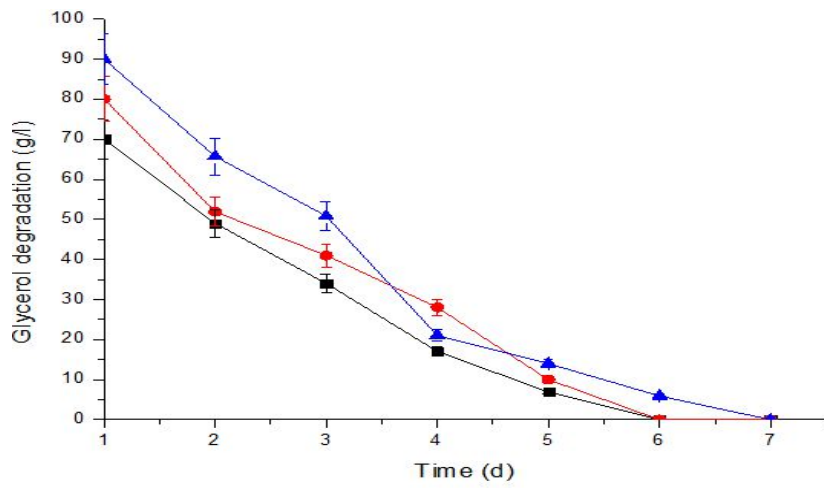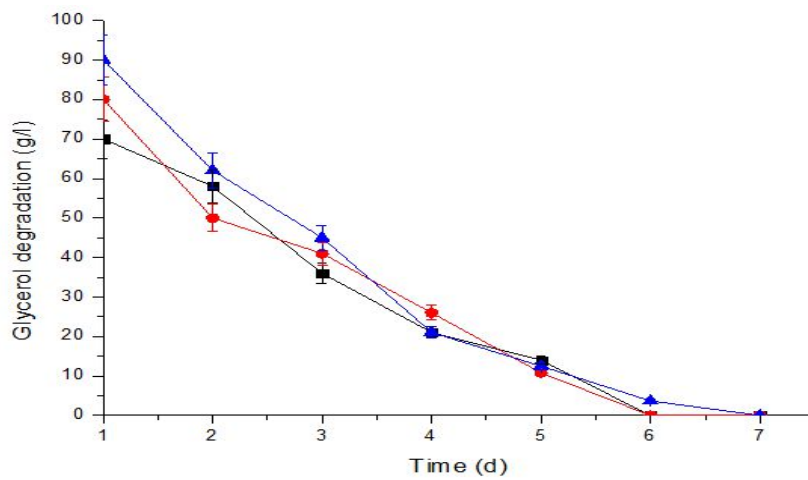
(a)
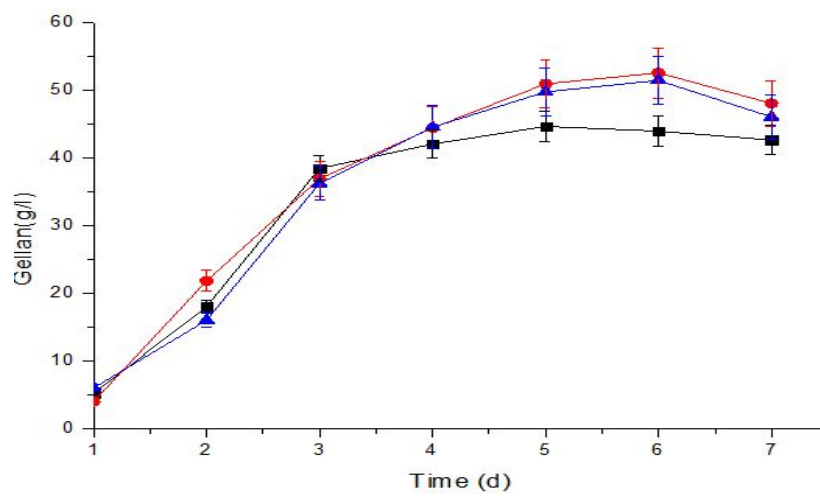
(b)
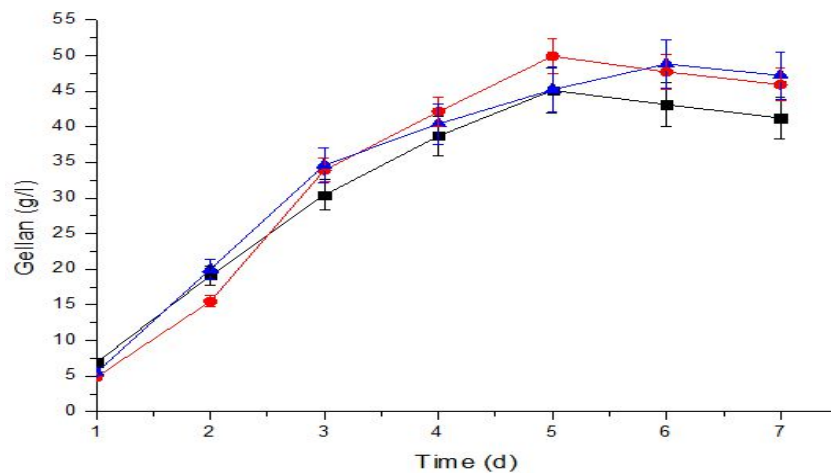
(c)

Figure 3.11: Gellan production by *S. pseudosanguinis* during shake flask cultivations at 34°C and 200 rpm over 7 d at (a) pH 6, (b) pH 7 and (c) pH 8 over 7 days at pH 6 (a), pH 7 (b) and pH 8 (c) using glycerol concentrations of: 70 (■), 80 (●) and 90 (▲) g/l
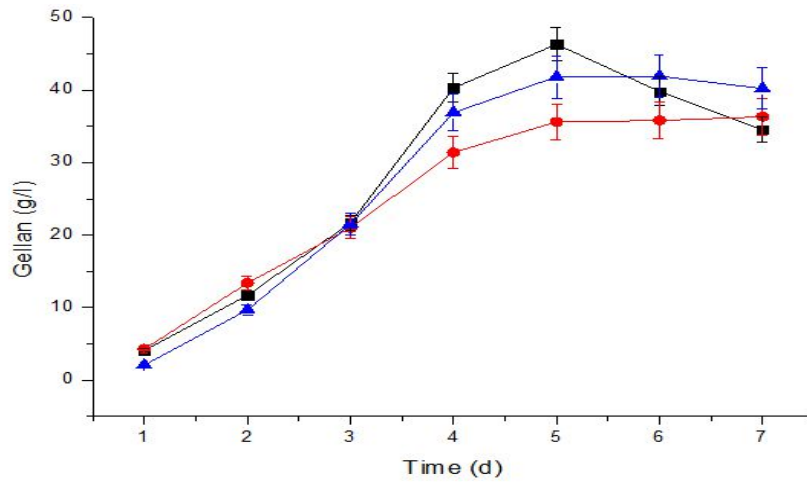
(a)
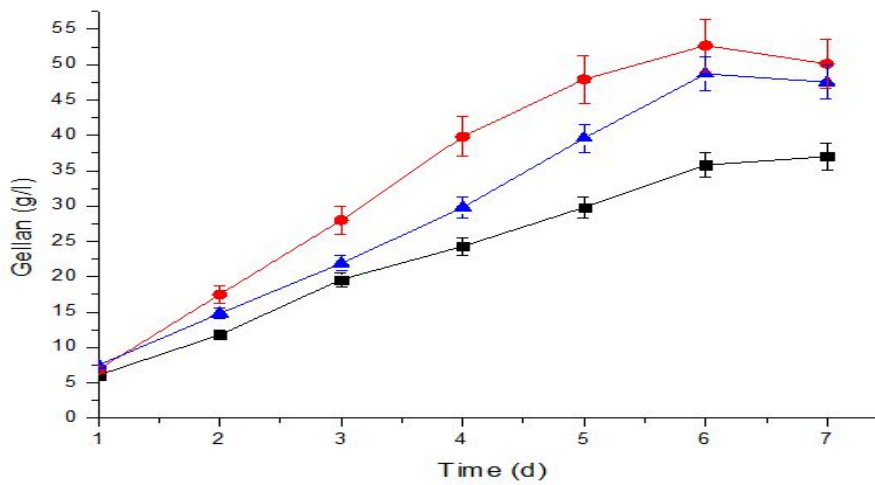
(b)
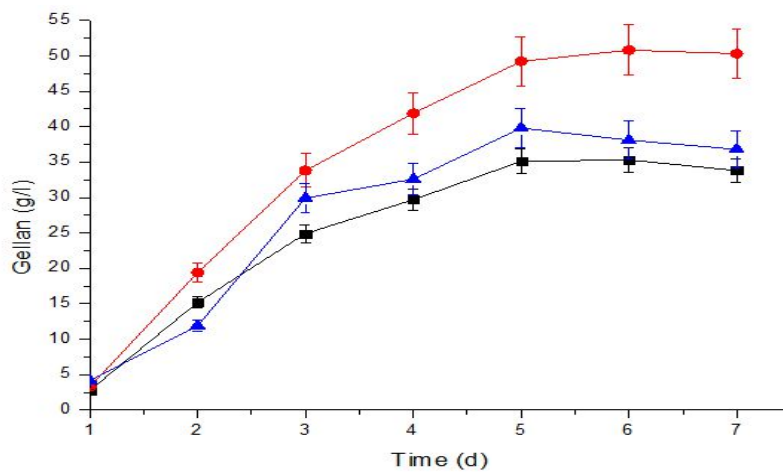
(c)

Figure 3.12: Gellan production by *S. yabuuchiae* during shake flask cultivations at 34°C and 200 rpm over 7 d at (a) pH 6, (b) pH 7 and (c) pH 8 using glycerol concentrations of: 70 (■), 80 (●) and 90 (▲) g/l
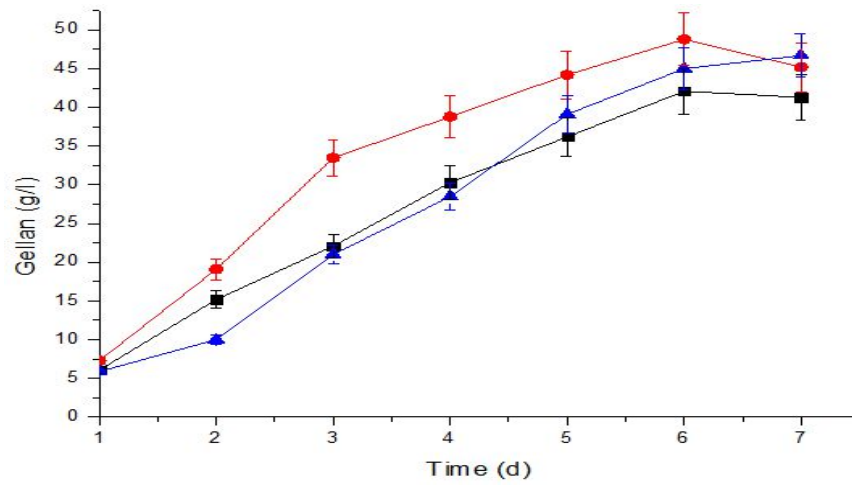
In order to compare the results obtained from this study of using crude glycerol to produce gellan by *S. pseudosanguinis* and *S. yabuuchiae* with other published reports, a table was constructed to show the various carbon sources currently used and the corresponding amounts of gellan yielded (Table 3.3).

The amount of gellan produced in this study was found to be comparatively high and it is evident that the use of crude glycerol as a carbon source is not only cost effective, due to it being an industrial waste, but extremely efficient when compared with other studies which utilised different types of sugars as carbon sources (Table 3.3). In gellan production, the most commonly used carbon sources are glucose, lactose or sucrose. To the best of our knowledge, no other published report has demonstrated the use of glycerol to produce gellan.

Table 3.3: An overview of various carbon sources utilised to produce maximum yields of gellan from *Sphingomonas sp.*

Carbon Source	Gellan Yield (g/l)	Reference
Glucose	15	Huang *et al.,* 2010
Sucrose	23.2	Banik and Santhiagu 2006
Glucose with condensed corn solubles	12.5	Vanderhoff *et al.,* 2010
Glucose	15	Nampoothiri *et al.,* 2003
Starch	23.5	Nampoothiri *et al.,* 2003
Sucrose	18	Nampoothiri *et al.,* 2003
Lactose	20	Nampoothiri *et al.,* 2003
Maltose	15	Nampoothiri *et al.,* 2003
Glucose	9	Fialho *et al.,* 1999
Lactose	15	Fialho *et al.,* 1999
Cheese Whey	7.9	Fialho *et al.,* 1999
Glucose	14.75	Wang *et al.,* 2006
Glucose	20.3	Huang *et al.,* 2011
Glucose	35.7	Bajaj *et al.,* 2007
Molasses	13.8	Bajaj *et al.,* 2007
Glucose	15.9	Aal and Attallah 2007
Crude Glycerol	50.9	Present study
Crude Glycerol	52.6	Present study

The use of industrial wastes as viable carbon sources in exopolysaccharide production has been receiving much attention recently. Some of the most attractive feed stocks have been cheese whey in the production of gellan from *S. paucimobilis* (Fialho *et al.,* 1999), sugar cane molasses in the production of cellulose by *Acetobacter xylinum* ATCC 10245 (Premjet *et al.,* 2007), glycerol byproduct to produce an exopolysaccharide from *Pseudomonas oleovorans* (Freitas *et al.,* 2009) and GalactoPol and FucoPol from biodiesel waste crude glycerol (Alves *et al.,* 2010; Freitas *et al.,* 2011).

From Table 3.3, it is noted that the production of gellan reported is lower than that produced by both *S. pseudosanguinis* and *S. yabuuchiae* using crude glycerol. The highest amount of gellan reported was 35.7 g/l from glucose (Bajaj *et al.,* 2007). The lowest amount of gellan yielded in this study was 35.3 g/l at pH 8, 70 g/l crude glycerol while the highest was 52.6 g/l using *S. yabuuchiae* at identical conditions. *S. pseudosanguinis* produced the lowest amount of gellan at pH 6, 70 g/l crude glycerol (42.1 g/l) while the highest amount (50.9 g/l) was produced at pH 7 80 g/l crude glycerol.

Data related to dry cell weight determination of *S. pseudosanguinis* at different pH is presented in Fig 3.13. At 70 g/l crude glycerol, dry cell weight peaked after 4-5 d (7.8 g/l) (Fig 3.13 a). At 80 g/l and 90 g/l crude glycerol dry cell weight peaked at day 6 (7.9 and 9.3 g/l respectively). At 70 and 80 g/l crude glycerol concentrations dry cell weight peaked at day 7 (8.8 and 9.1 g/l respectively) (Fig 3.13 b). At 70 and 90 g/l crude glycerol concentrations dry

cell weight peaked at day 5 (8.2 and 7.5 g/l respectively) while, 80 g/l crude glycerol peaked at day 6 (8.7 g/l) (Fig 3.13 c).

Dry cell weights were measured over 7 d at pH 6, 7 and 8 with results shown in Fig 3.14 for *S. yabuuchiae*. At 70 g/l crude glycerol, dry cell weight peaked at day 5 (6.7 g/l). Crude glycerol at 80 g/l and 90 g/l concentrations peaked at day 6 (6.4 and 6.9 g/l respectively) (Fig 3.14 a). In Fig 3.14 b, 70 and 90 g/l crude glycerol concentrations, dry cell weight peaked at day 5 (5.9 and 6.9 g/l respectively). The dry cell weight at 80 g/l crude glycerol concentration peaked after 6 d at 6.9 g/l. Results depicted in Fig 3.14 c shows that 70, 80 and 90 g/l crude glycerol peaked at day 6 (6.5, 7.1 and 6.4 g/l, respectively).
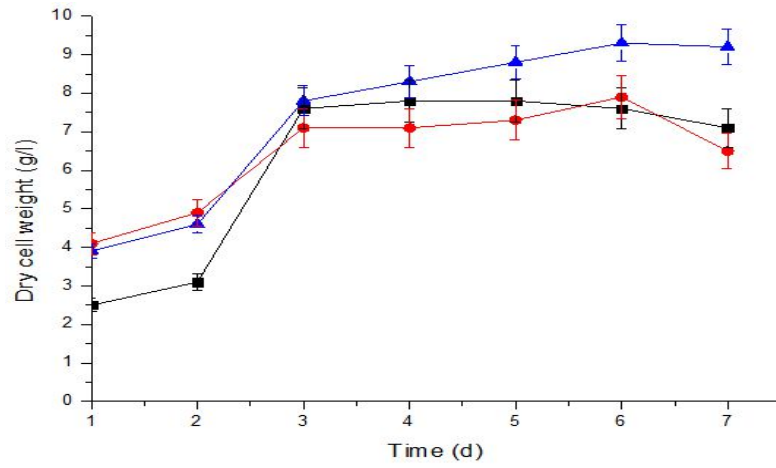
(a)
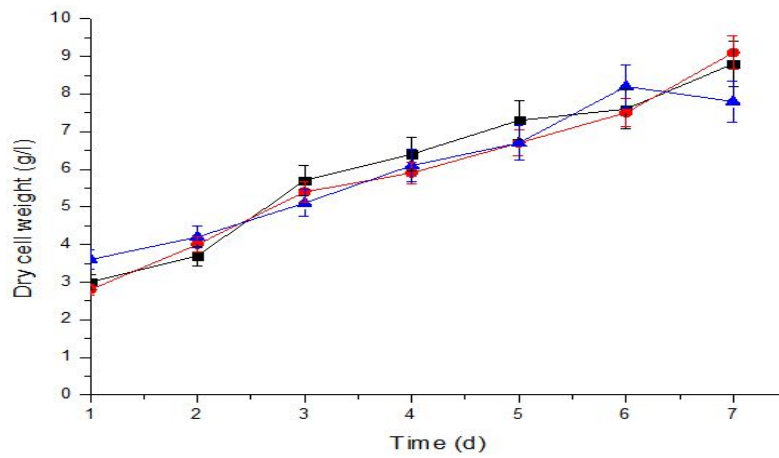
(b)
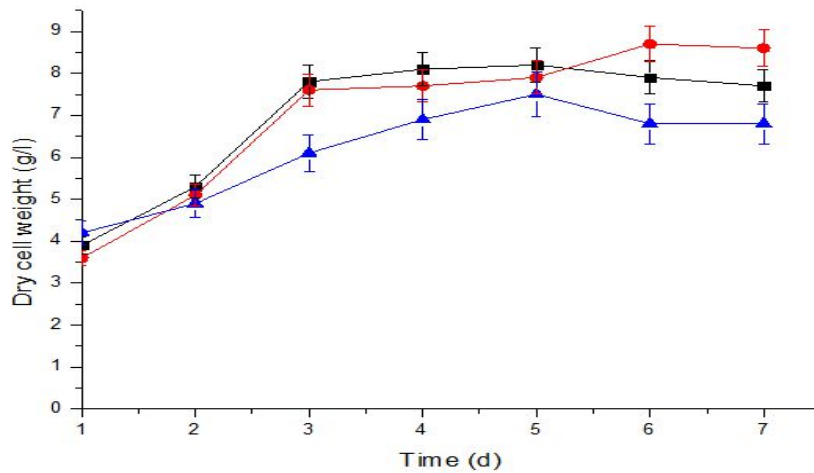
(c)

Figure 3.13 : Assessment of dry cell weight by *S. pseudosanguinis* during shake flask cultivations at 34°C and 200 rpm over 7 d at (a) pH 6, (b) pH 7 and (c) pH 8 using glycerol concentrations of: 70 (■), 80 (●) and 90 (▲) g/l
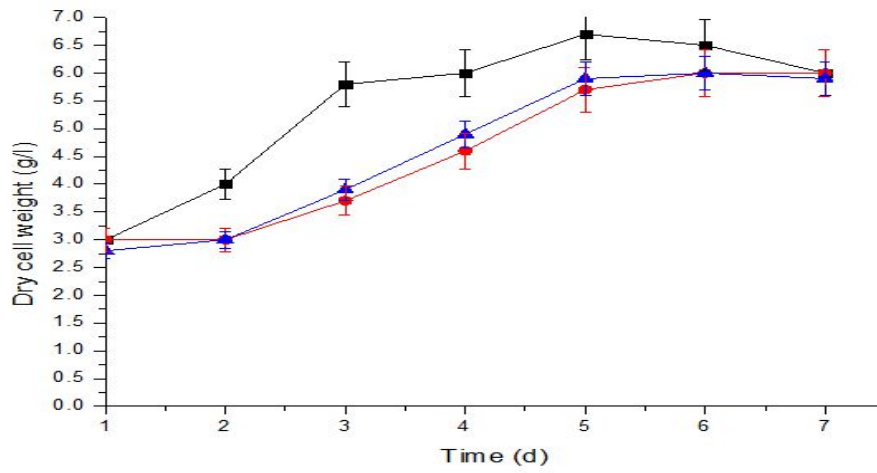
(a)
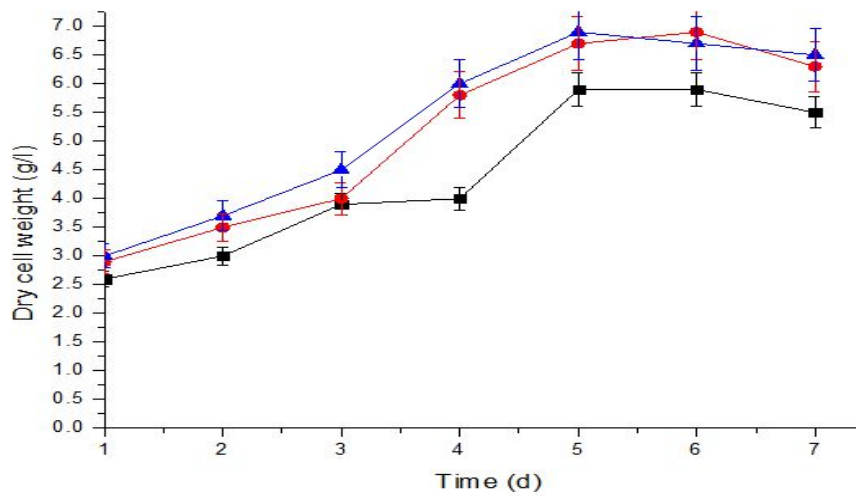
(b)
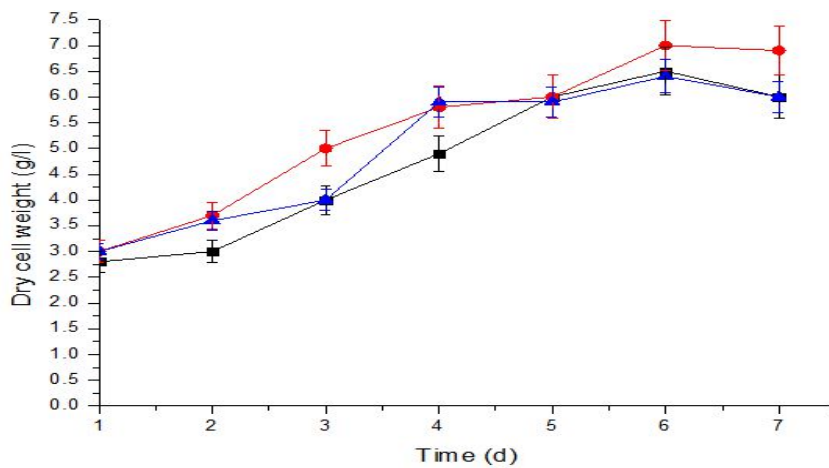
(c)

Figure 3.14: Assessment of dry cell weight by *S. yabuuchiae* during shake flask cultivations at 34°C and 200 rpm over 7 d at (a) pH 6, (b) pH 7 and (c) pH 8 using glycerol concentrations of: 70 (■), 80 (●) and 90 (▲) g/l

3.3.2.4 Scale up fermentation of S. yabuuchiae

The optimal concentration of crude glycerol for the production of gellan was found to be 80 g/l at pH 7 (52.6 g/l) using *S yabuuchiae* during shake-flask cultivations. Therefore, these media conditions were chosen for the scale-up (5 L) fermentation study in order to investigate its ability to produce gellan at a larger volume.

From the 5 L scale-up results (Fig 3.15) only 51 % of the 80 g/l crude glycerol was consumed after 7 d with 25.7 g/l of gellan production. From previous studies it has been shown that at higher fermentation volumes, the carbon source does not deplete in the same time period as shake flask experiments (Wang *et al.,* 2006 and Freitas *et al.,* 2010). Wang *et al.,* (2006) reported 49.17 % degradation in a 30 L batch fermenter with 14.75 g/l of gellan. The amount of gellan produced is also a fairly high amount of exopolysaccharide production when compared to a study done by Freitas *et al.,* (2010). It was reported that *Pseudomonas oleovorans* was able to produce 11.82 g/l of exopolysaccharide after 7 d in a 5 L fermenter with a 3 L working volume whereas *S. yabuuchiae* produced 25.7 g/l after 5 d.
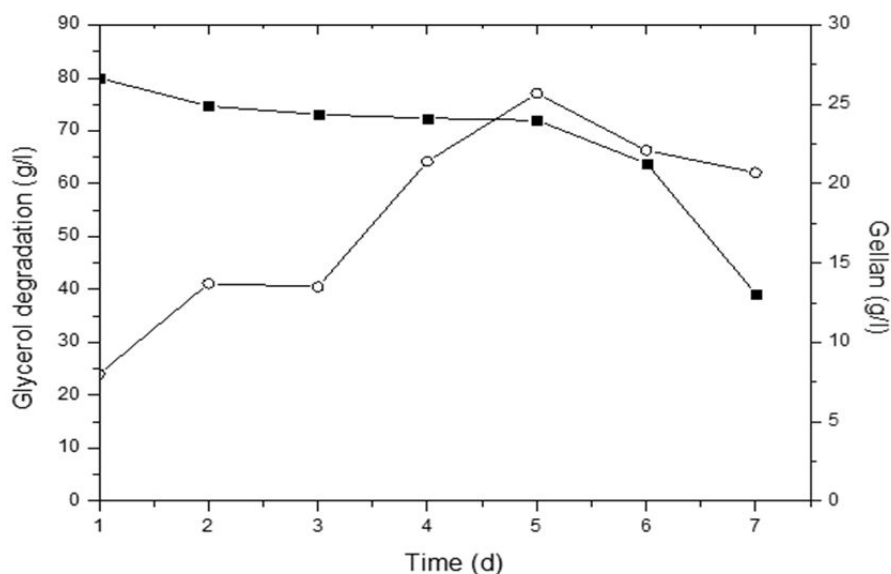
Figure 3.15: Evaluation of crude glycerol degradation (80 g/l) (■) and production of gellan (g/l) (o) at 34°C and agitation speed 200 rpm *S. yabuuchiae*

3.3.2.5   Assessment of gellan using microscopy

The next study done was microscopy of *S. yabuuchiae*. Different microscopy techniques were employed to visualise *S. yabuuchiae*. Samples from the pH 7 80 g/l crude glycerol broth for *S. yabuuchiae* over 7 d was used in LM, SEM, TEM and ESEM to further analyse cell morphology and the exopolysaccharide produced (gellan).

Light microscopy with negative staining was used to visualise the bacterial capsule at day 1 (least amount of gellan in broth) and day 6 (most amount of gellan in broth) respectively (Fig 3.16 a & b). Nigrosine stain was used as described in section 3.2.1.1. Nigrosine is commonly used in light microscopy

to negative stain the bacterial capsule. From the images, it is noted that the bacterial capsule darkened over a 6 d period. This is due to the production of gellan which is excreted from the cell as an exopolysaccharide. This was further noted in transmission electron microscopy were the cell appeared more translucent on day 1 as compared to day 6. After 6 d the cell is seen to be completely covered by the exopolysaccharide which is excreted through the capsule (Fig 3.17 a & b).

In the scanning electron microscopy images of *S. yabuuchiae*, different preparation methods were employed in order to view gellan as an exopolysaccharide secreted by the cell more clearly. In the first set of images, regular preparation of bacterial and fungal samples was used (section 3.2.1.2). This involves an extensive washing and dehydrating of the sample in order to remove the fermentation broth from the cells (Fig 3.18 a & b). The individual cells are viewed more clearly on day 1 (Fig 3.18 a) as compared to day 6 were long chains of cells are seen closely bound together in chains (Fig 3.18 b). The next sample was prepared in a method described in section 3.2.1.3 whereby sample broth was untreated and left to air-dry before viewing as an environmental sample. The images show the broth becoming more viscous in nature from day 1 to day 6 (Fig 3.19 a & b) which is indicative of gellan production. The final sample preparation was similar to the method described in section 3.2.1.4 except samples were viewed without air-drying (fresh sample). Bacterial cells are more visible on day 1 were gellan concentration is very low (Fig 3.20 a). On day 3 cells are less visible and appear to coalesce together (Fig 3.20 b). By day 6 (Fig 3.20 c) the

fermentation broth is too viscous to be viewed on the ESEM as it is completely covered in the biofilm produced by the cells. This image contradicts Fig 3.18 b were cells are completely visible after the gellan-containing broth is washed off the cells. This is due to the gelatinous nature of the broth not allowing electrons to pass through so the bacterial cells may be visible on day 6 which further substantiates the presence of gellan.

(a)

(b)
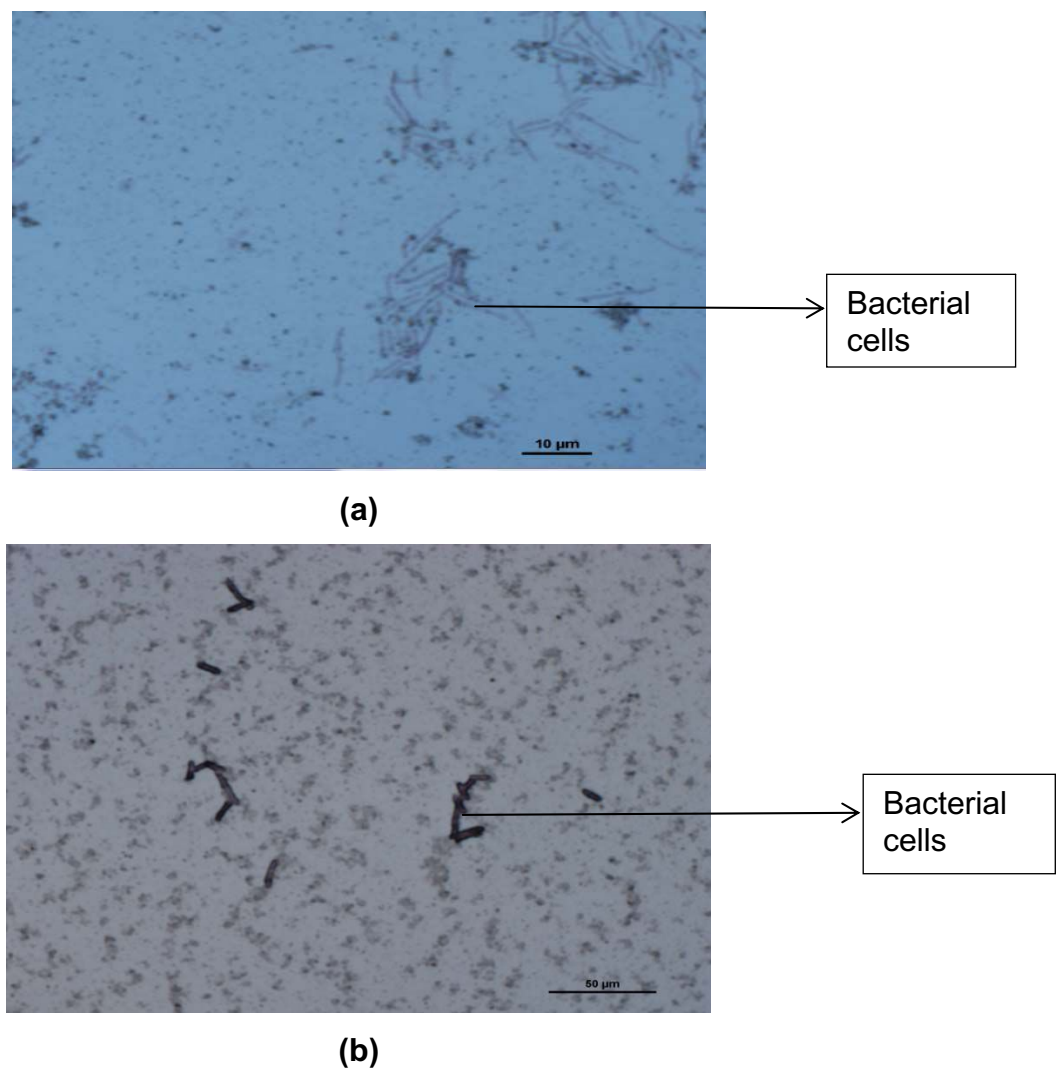
Figure 3.16: Light microscopy of *S. yabuuchiae* at day 1 (a) and day 6 (b) at a scale of 10 and 50 µm respectively
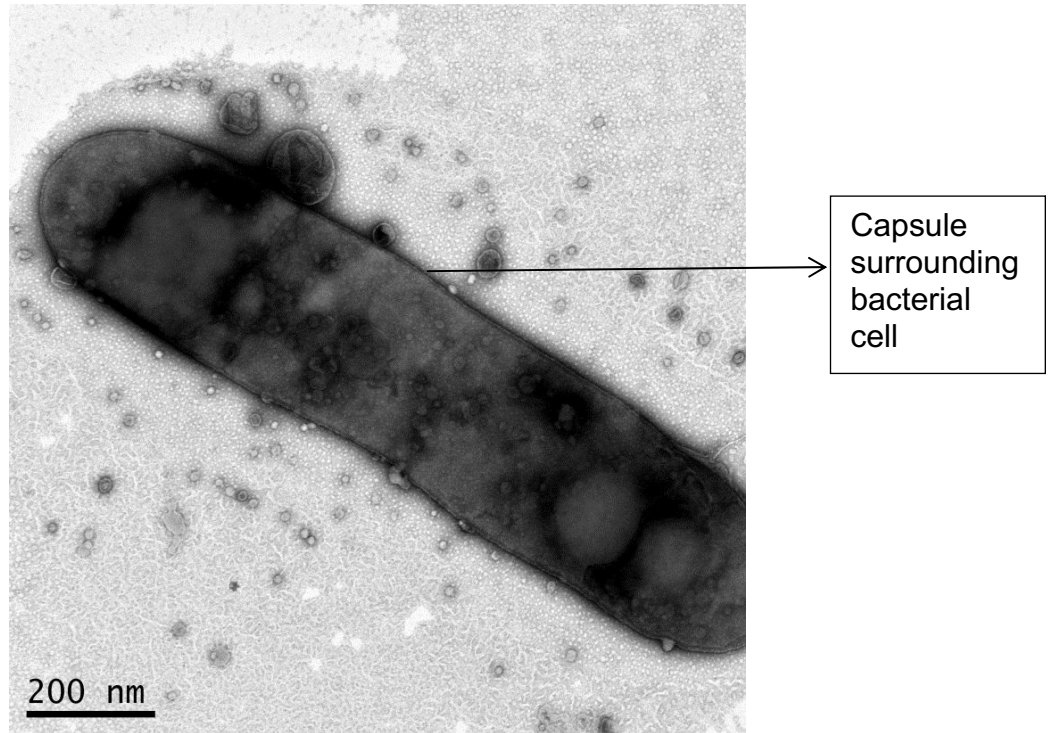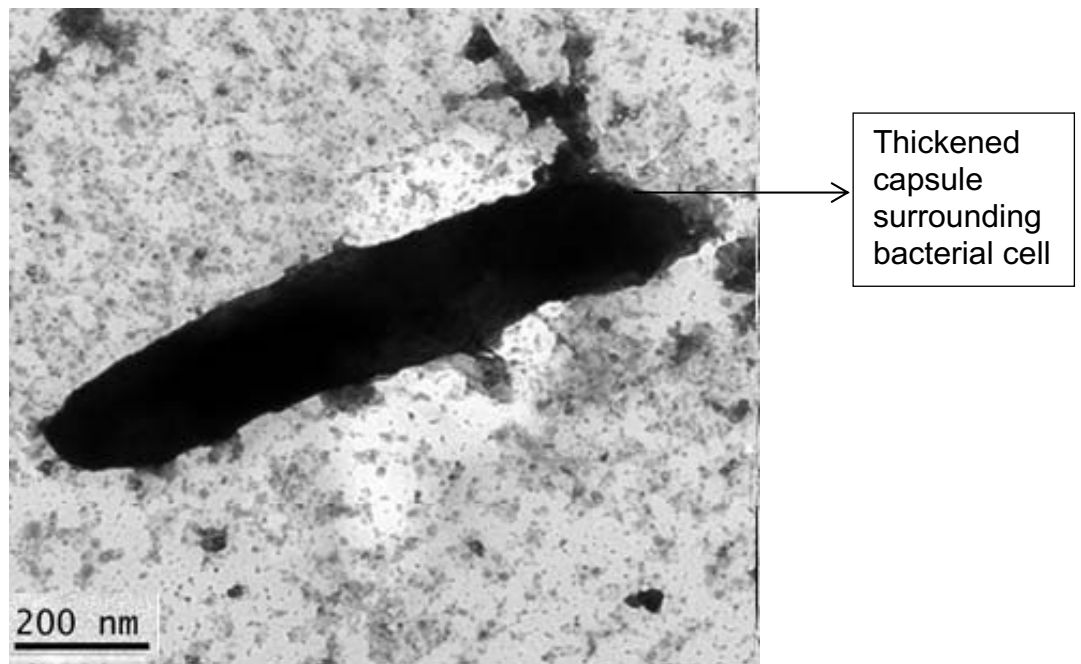
Capsule surrounding bacterial cell

(a)

Thickened capsule surrounding bacterial cell

(b)

Figure 3.17: Transemission electron microscopy of *S. yabuuchiae* at day 1 (a) and day 6 (b) at a scale of 200 nm

(a)

(b)

Figure 3.18: Scanning electron microscopy of *S. yabuuchiae* at day 1 (a) and day 6 (b) at a scale of 1 and 10 µm respectively
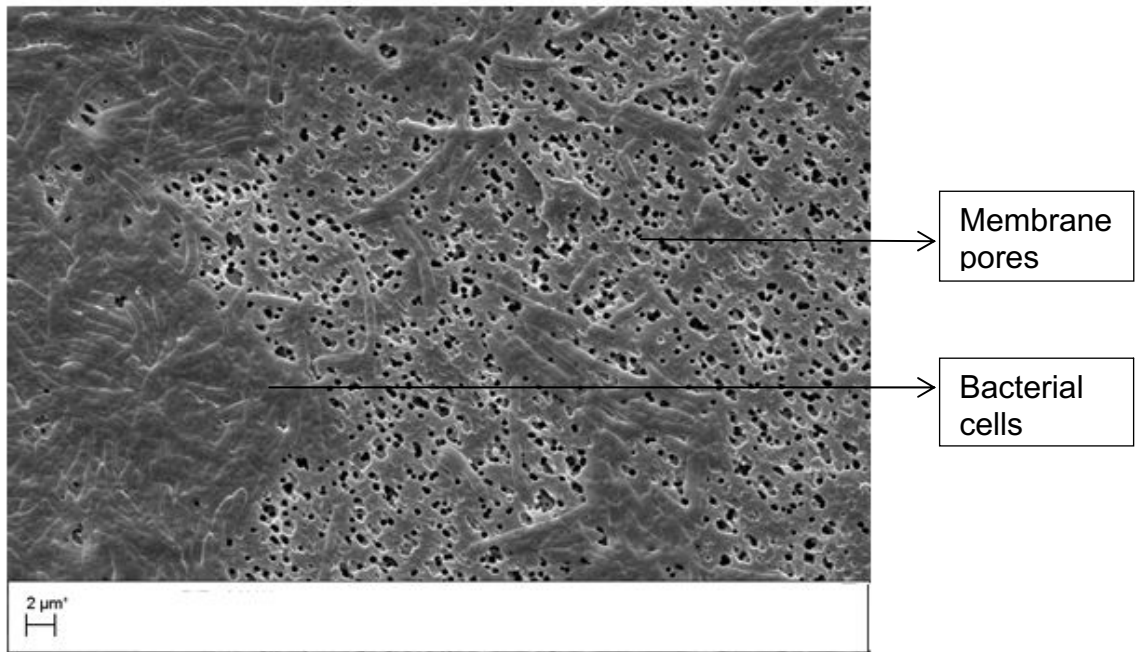
(a)
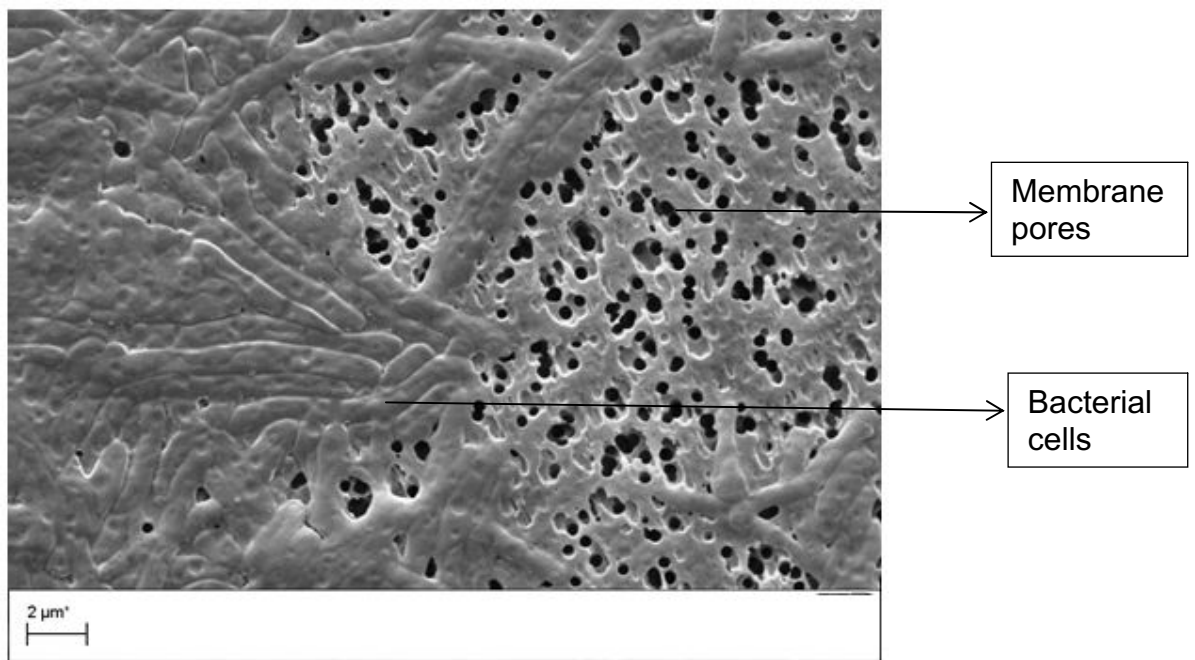
(b)

Figure 3.19: Environmental scanning electron microscopy of *S. yabuuchiae* using the air-drying method at day 1 (a) and day 6 (b) at a scale of 2 μm

Bacterial cells

(a)

Bacterial cells

(b)

Viscous broth covering cells
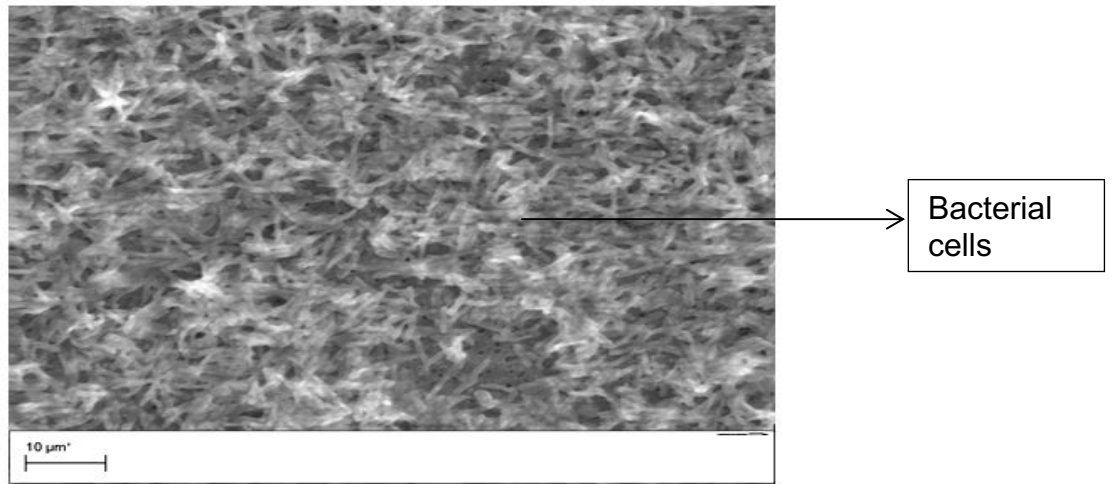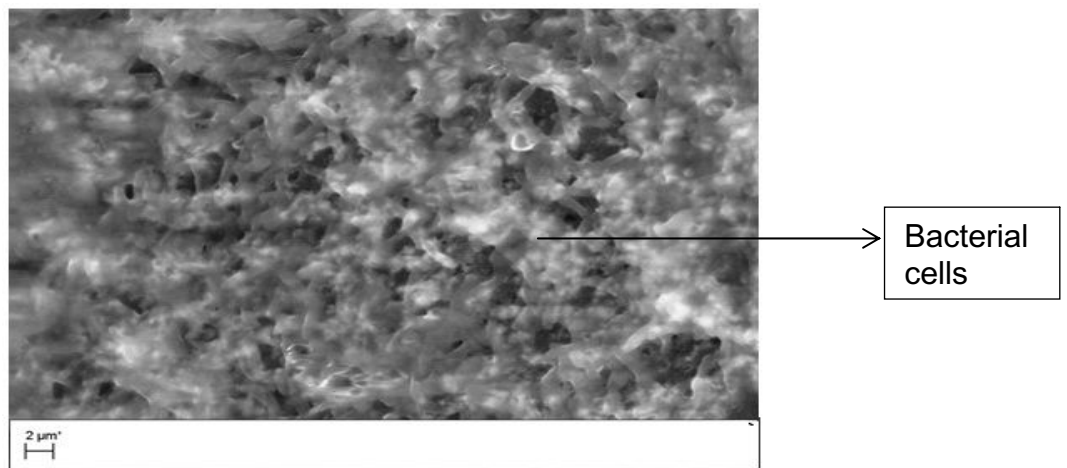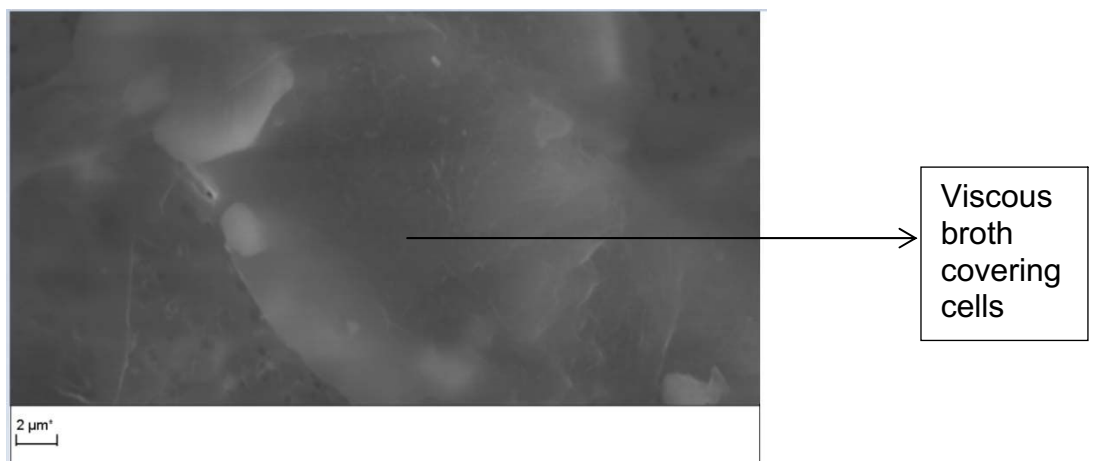
(c)

Figure 3.20: Environmental scanning electron microscopy of *S. yabuuchiae* using fresh sample at day 1 (a), day 3 (b) and day 6 (c) at a scale of 10 and 2 µm respectively

Freitas *et al.* (2011) reported that the gellan market in the USA is currently a $15, 000, 000 industry with a price of $55-66/kg. In previous studies, gellan production is optimally produced at 15-30 g/l using mainly glucose as a carbon source using *S. paucimobilis* (Table 3.3). From this study both *S. pseudosanguinis* and *S. yabuuchiae* were able to produce higher yields of gellan utilising a low cost carbon source, thus making this method of gellan production industrially attractive.

In this chapter crude glycerol was a successful carbon source used in fermentation using two different bacterial genus'. From the experiments conducted we may draw the following conclusions:-

i. *K. pneumoniae* was able to successfully produce significant amounts of 1,3-PDO and lactic acid using crude glycerol, without pre-treatment (37 and 6.8 g/l respectively).

ii. *S. yabuuchiae* and *S psueudosanguinis* are both able to produce high amounts of gellan gum than that reported by other studies using crude glycerol as a sole carbon source in a minimal medium (50.9 and 52.6 g/l).

iii. SEM, TEM and LM may be employed as alternative methods of viewing gellan production. This may be improved by slicing transverse sections through the cell to better visualise the thickening of the capsule during exopolysaccharide production.

iv. Both experiments are industrially viable as large scale production (5 L) was fairly successful. However, with further optimisation it is clearly possible that higher volume fermentations may be achieved.

Chapter 4: General Discussion

The current global biofuels market is rapidly expanding due to the need for more environmentally safe alternatives to petroleum-based fuels. Many countries have also implemented regulations in order to ensure the production and implementation of biofuels. In the US the target volume is 36 billion gallons annual production by 2022 under the under the Renewable Fuel Standard, as called for in the Energy Independence and Security Act of 2007 (Ashby *et al.,* 2011). In South Africa, a biofuel policy was approved by government in 2007 for the country to accomplish a 2% biofuel production of the national liquid energy supply level by 2013. This is in compliance with Millenium Development Goals (MDGs) by economically developing rural communities and creating approximately 25 000 jobs (Sparks *et al.,* 2010).

Many countries are also actively participating in manufacturing and selling biofuels such as biogas, bioethanol and biodiesel either as independent petroleum alternatives or in a mix of biofuel with petroleum-based fuel. However, the technology used in the manufacturing of biofuel is less economically attractive than the existing technology that is used for petroleum based equivalents. Therefore the use of inexpensive non-food materials and the innovation of finding commercially viable uses for by-products, like glycerol, are fundamental in boosting the biofuel industry.

Crude glycerol is of low value due to the presence of impurities. Typically crude glycerol derived from a base-catalysed transesterification reaction contains approximately 50 to 60% of glycerol, 12 to 16% of alkalis, 15 to 18%

of methyl esters, 8 to 12% of methanol, and 3 to 8% of water. There also various elements present such as K (0 to 217 ppm), P (12 to 37 ppm), S (14 to 128 ppm), Na (1.06 to 1.40%), C (24 to 37%), N (0.04 to 0.12%), and protein (0.05 to 0.44%) (Santibáñez *et al.*, 2011). In order to convert crude glycerol into a grade suitable for commercial use, it has to undergo filtration, the use of chemical catalysts, and fractional vacuum distillation. This refining process is costly and unattractive to industry thus encouraging the dumping of crude glycerol (Thompson and He, 2005). As a result the fermentation of crude glycerol into value-added products is being extensively investigated. However, attaining microorganisms that are tolerant to the above mentioned impurities is a principal hindrance. In this study, screening for readily-existing bacteria in the crude glycerol was attempted in order to find strains capable of degrading this by-product.

Previous studies have shown great success using crude glycerol to isolate microorganisms capable of degrading glycerol. Choi *et al.* (2011) isolated a novel *Kluyvera cryocrescens* S26 strain from crude glycerol which was capable of converting biodiesel-derived crude glycerol, supplemented with yeast extract to as much as 27 g/l of ethanol. Dąbrowski *et al.* (2012) isolated twenty nine bacteria after a microbial screening process using crude glycerol. Several of these isolates were identified as *Clostridium* species viz., *C. lituseburense* (one strain), and *C. sartagoforme* (one strain) which were able to produce 1,3-PDO from crude glycerol. In this study 11 bacterial isolates were found in the crude glycerol. In addition three isolates *viz., Klebsiella pneumoniae, Sphingomonas yabuuchiae* and *Sphingomonas*

pseudosanguinis showed significant potential to degrade crude glycerol up to a concentration of 80 g/l.

Due to glycerol having a greater degree of reduction than sugars, it is more a viable option to bioconvert into reduced chemicals such as succinate, hydrogen, proprionate or ethanol resulting in higher yields as compared to sugars (da Silva *et al.,* 2009). Dharmadi *et al.* (2006) showed glycerol fermentation produced ethanol and formate with an overall yield higher than that of glucose fermentation in *E. coli* as the glucose concentration was reduced to 50 % due to its conversion to carbon dioxide. Suhaimi *et al. (*2012) showed *E. coli* SS1 yielded ethanol values three-fold higher in glycerol fermentation than that of glucose fermentation.

In lieu of the above, this study focused on using crude untreated glycerol to produce 1,3-PDO and gellan with *K. pnueumoniae* and two *Sphingomonas,* strains respectively. 1,3-PDO yields were much higher than that of previous reports with glucose supplementation (up to 50% as discussed in chapter 3). 1,3-PDO has been well researched as a by-product of glycerol bioconversion. A tremendous amount of success has been achieved in shake flask experiments. Factors such as media composition and formulation have been reported to play a major role in catalysing the bioconversion of glycerol. The main carbon source used in 1,3-PDO production is glycerol. In the case of 1,3-PDO production using *K. pneumoniae*, glucose is generally used to catalyse degradation of glycerol and thus reduce fermentation time via co-fermentation. Haynie and Wagner (1997) produced up to 10 g of 1,3-PDO using 100 g of glucose as an initial concentration in a patented method of

biotransformation whereby one microorganism ferments the carbon source to glycerol which is then converted to 1,3-PDO by another. Baeza-Jiménez *et al.* (2011) reported 16.81 g/L 1,3-PDO production in shake flask experiments with 100 mM glucose/200 mM glycerol mix. However, the balance between glycerol and glucose in co-fermentation can be a tedious optimisation as the disposal of $NADH^+H^+$ and consequent accumulation of 3-HPA affects the metabolic flux in the cells which can be toxic to the cells. This results in 1,3-PDO being produced at a reduced rate and a lower volume (Baeza-Jiménez *et al.,* 2011). Since glycerol is the natural carbon source for 1,3-PDO production, methods which are based solely on the optimal use of glycerol for fermentation are more sensible and cost-effective to employ.

High 1,3-PDO production volumes have been reported by several researchers due to the following factors (i) supplementation with vitamins B12 and trace metals (Homann *et al.,* 1990; Sattayasamitsathit *et al.,* 2011; Gungormusler *et al.,* 2010; Ji *et al.,* 2009) (ii) genetic modifications (Ashok *et al.,* 2011; Oh *et al.*, 2012; Zhuge *et al.,* 2010), (iii) anaerobic conditions (Homann *et al.,* 1990; Dharmadi *et al.,* 2006; Xiu *et al.,* 2004; Kumar *et al.,* 2012) (iv) crude glycerol purification or pre-treatment (Wilkens *et al.,* 2012).

In this study, up to 37 g/l of 1,3-PDO was produced from 80 g/l untreated crude glycerol under aerobic conditions at pH 8. *K. pneumoniae* does however prefer anaerobic conditions as the GDHt enzyme can be inactivated in the presence of oxygen (http://www.brenda-enzymes.org/php/result_flat.php4?ecno=4.2.1.30). Nonetheless, 1,3-PDO was still

produced at significantly high concentration under aerobic conditions. Industrially this would be the preferred condition as the aerobic fermentation process can not only reduce the production cost but also minimizes the HPA accumulation (Saxena *et al.,* 2009). The high yield at aerobic conditions can be attributed to the GDHt enzyme being at pH 8 which is optimal activity for this enzyme (http://www.brenda-enzymes.org/php/resultflat.php4?ecno =4.2.1.30). The media formulation was basic as it did not require trace metals or the addition of vitamins (B12) or antibiotics. In addition the glycerol was utilised in its raw form thus allowing this study to have economic implications long-term. There is also potential for large scale production since with the fermentation volume being increased to a 5 L reactor (3 L working volume) good levels of 1,3-PDO (18.5 g/l) were attained, although this is only 50% of the maximum production achieved when compared to shake-flask experiments.

Whilst gellan production was not the primary focus of this study, it did emerge as a major by-product of glycerol bioconversion which indirectly had a significant impact in this research. In addition this find is of extreme commercial value as the current market for biopolymers is very competitive. Synthetic polymers commonly used for packaging, lack the ability to completely biodegrade due to their texture, thickness and transparency which results in negative effects on the environment. Therefore there is much need to replace these materials with more ecologically-friendly biopolymers such as xanthan, alginate, chitosan, levan and gellan (Freitas *et al.,* 2011). Another advantage of biopolymers is that they may be recovered from renewable

resources such as glucose, sucrose, lactose, corn steep liquor and molasses via bacterial fermentation thus reducing energy requirements as well as the addition of chemicals and overall production costs (Alves *et al.,* 2011). Currently the market for biopolymers is very competitive therefore there is a constant need to procure low-cost carbon sources to reduce commercial production expenditure while increasing productivity. One such carbon source is waste glycerol derived from biodiesel production.

Gellan has approval to be used in the USA and EU in the food and medical industries due to its thermal and acid stability, elasticity, rigidity, transparency and flavour release (Fialho *et al.,* 2008). The production of gellan is mainly achieved via fermentation of sugars summarised in Table 3.3. However, the uniqueness in this study was the production of gellan as a result of the bioconversion of crude glycerol. Of further significance was gellan production levels being much higher than that reported with sugars using S. *pseudsanguinis* (50.9 g/l) and *S. yabuuchiae* (52.6 g/l). These factors may be vital in contributing to this exopolysaccaride becoming a possible commodity product in the biopolymer industry.

This find has the potential to make a huge impact in industry as a result of: (i) reduced production costs due to limited dependence on glucose and sucrose; (ii) the gellan yield is almost 3-fold higher and (iii) no dependence on trace elements or amino acids in comparison with other reports (Vanderhoff *et al.,* 2010; Banik and Santhiagu 2006).

Future prospects

Globally the bioconversion of crude glycerol has stirred up sufficient concerns as a potential bottleneck in the biodiesel industry. With the advent of 1,3-PDO and gellan production by unique isolates elucidated in this study, it therefore provides an immense boost to fostering a solution to an environmental concern.

In order to improve the production of 1,3-PDO from this strain of *K. pneumoniae*, further studies maybe conducted to enhance other factors that influence both the GDHt enzyme as well as the enzymes that ensue to produce 1,3-PDO (NADH dependent 1,3-propanediol dehydrogenase and NADPH dependent 1,3-propanediol dehydrogenase). Micro-aerobic conditions can be investigated to increase GDHt activity. Genetic modification of the GDHt gene may also be a viable option at a doctoral level to improve the rate of glycerol degradation as well as the tolerance to higher concentrations of glycerol. However, this may have drawbacks due to the use of antibiotics and the existence of other microorganisms and contaminants in crude glycerol (raw form). Continuous fermentation is also favourable as this process will reduce the concentration of organic acids produced during *K. pneumoniae* metabolism which may be inhibiting 1,3-PDO production due to the decrease in pH. A further improvement would be to investigate the influence of pH. The optimal pH for GDHt activity is generally 8. Regulating the pH to 8 during the entire fermentation process could also promote GDHt activity. Prospectively, this study has shown great potential for this bacteria to be further investigated

to optimise current 1,3-PDO production on a larger scale as well as to be incorporated into industrial application since it is economically able to produce 1,3-PDO in significant concentration from a raw by-product.

The potential for gellan production, to be extended beyond these findings may require genetic modification as previous studies have shown improvement in production by up to 20% by several strains (Fialho *et al.*, 2008). Eighteen genes in four clusters are required for gellan biosynthesis (Ibrahim *et al.,* 2011). It has been inferred that mutations or modifications of these gene clusters may lead to enhanced gellan production. However, as promising as this may seem, there is once again the contaminants in the crude glycerol to consider, the addition of antibiotics when using genetically modified microorganisms as well as a lack of complete understanding of the *Sphingomonas* genome (Fialho *et al.,* 2008). Investigating the genome of this bacterial strain of *Sphingomonas sp.* would be favourable in explaining the ability of this microbe to produce gellan from glycerol. Also the use of statistical analyses (Ludeking-Piret or CCD) to describe fermentation kinetics of optimal conditions could be a good option to further optimise gellan production.

Sphingomonas sp. are also gaining wide recognition in research involving the biological removal of waste. Several studies, as mentioned in Chapter 3, have shown the use of these bacteria as well as gellan for the bioremediation of aquifers (Story *et al.,* 2004; White *et al.,* 1996). With the isolation of two novel

Sphingomonas strains, it provides a fresh approach to address the potential of these isolates for bioremediation of biodiesel wastes.

In conclusion it is important to highlight that the objectives of this study have been met with the isolation and identification of novel isolates and the production of 1,3-PDO and gellan as products derived from the bioconversion of crude glycerol. Future work mentioned above will add great value to the realm of the growing biodiesel industry.

References

Abd-El-Aal, S. & Attallah, A. 2007. Induction of highly gellan gum productive *Sphingomonas paucimobilis*. *Research Journal of Agriculture and Biological Sciences,* 3**,** 554-557.

Adhikari, S., Fernando, S. D. & Haryanto, A. 2009. Hydrogen production from glycerol: An update. *Energy Conversion and Management,* 50**,** 2600-2604.

Alves, V. D., Ferreira, A. R., Costa, N., Freitas, F., Reis, M. A. & Coelhoso, I. M. 2011. Characterization of biodegradable films from the extracellular polysaccharide produced by *Pseudomonas oleovorans* grown on glycerol byproduct. *Carbohydrate Polymers,* 83**,** 1582-1590.

Anderson, D., Brydon, W. & Eastwood, M. 1988. The dietary effects of gellan gum in humans. *Food Additives & Contaminants,* 5**,** 237-249.

André, A., Diamantopoulou, P., Philippoussis, A., Sarris, D., Komaitis, M. & Papanikolaou, S. 2010. Biotechnological conversions of bio-diesel derived waste glycerol into added-value compounds by higher fungi: production of biomass, single cell oil and oxalic acid. *Industrial Crops and Products,* 31**,** 407-416.

Ashby, R. D., Solaiman, D. K. & Strahan, G. D. 2011. Efficient utilization of crude glycerol as fermentation substrate in the synthesis of poly (3-hydroxybutyrate) biopolymers. *Journal of the American Oil Chemists' Society,* 88**,** 949-959.

Ashok, S., Raj, S. M., Rathnasingh, C. & Park, S. 2011. Development of recombinant *Klebsiella pneumoniae*Δ dhaT strain for the co-production

of 3-hydroxypropionic acid and 1, 3-propanediol from glycerol. *Applied microbiology and biotechnology,* 90, 1253-1265.

Baeza-Jiménez, R., López-Martinez, L., De La Cruz-Medina, J., Espinosa-De-Los-Monteros, J. & Garcıa-Galindo, H. 2011. Effect of glucose on 1, 3-propanediol production by *Lactobacillus reuteri* efecto de la glucosa sobre la produccion de 1,3-propanodiol por *Lactobacillus reuteri*. *Revista Mexicana de Ingeniería Química,* 10, 39-46.

Bajaj, I. B., Survase, S. A., Saudagar, P. S. & Singhal, R. S. 2007. Gellan gum: fermentative production, downstream processing and applications. *Food Technology and Biotechnology,* 45, 341.

Banik, R. & Santhiagu, A. 2006. Improvement in production and quality of gellan gum by *Sphingomonas paucimobilis* under high dissolved oxygen tension levels. *Biotechnology letters,* 28, 1347-1350.

Banik, R., Santhiagu, A. & Upadhyay, S. 2007. Optimization of nutrients for gellan gum production by *Sphingomonas paucimobilis* ATCC-31461 in molasses based medium using response surface methodology. *Bioresource technology,* 98, 792-797.

Barbirato, F., Himmi, E. H., Conte, T. & Bories, A. 1998. 1, 3-propanediol production by fermentation: An interesting way to valorize glycerin from the ester and ethanol industries. *Industrial Crops and Products,* 7, 281-289.

Barbirato, F., Larguier, A., Conte, T., Astruc, S. & Bories, A. 1997. Sensitivity to pH, product inhibition, and inhibition by NAD+ of 1, 3-propanediol dehydrogenase purified from *Enterobacter agglomerans* CNCM 1210. *Archives of microbiology,* 168, 160-163.

Biebl, H., Menzel, K., Zeng, A.P. & Deckwer, W.D. 1999. Microbial production of 1, 3-propanediol. *Applied Microbiology and Biotechnology,* 52**,** 289-297.

Busse, H.-J., Denner, E. B., Buczolits, S., Salkinoja-Salonen, M., Bennasar, A. & Kämpfer, P. 2003. *Sphingomonas aurantiaca* sp. nov., *Sphingomonas aerolata sp.* nov. and *Sphingomonas faeni sp.* nov., air- and dustborne and Antarctic, orange-pigmented, psychrotolerant bacteria, and emended description of the genus *Sphingomonas. International journal of systematic and evolutionary microbiology,* 53**,** 1253-1260.

Celińska, E. 2010. Debottlenecking the 1, 3-propanediol pathway by metabolic engineering. *Biotechnology advances,* 28**,** 519-530.

Chatzifragkou, A., Dietz, D., Komaitis, M., Zeng, A. P. & Papanikolaou, S. 2010. Effect of biodiesel-derived waste glycerol impurities on biomass and 1, 3-propanediol production of *Clostridium butyricum* VPI 1718. *Biotechnology and bioengineering,* 107**,** 76-84.

Chatzifragkou, A., Papanikolaou, S., Dietz, D., Doulgeraki, A. I., Nychas, G.-J. E. & Zeng, A.-P. 2011. Production of 1, 3-propanediol by *Clostridium butyricum* growing on biodiesel-derived crude glycerol through a non-sterilized fermentation process. *Applied microbiology and biotechnology,* 91**,** 101-112.

Choi, W. J., Hartono, M. R., Chan, W. H. & Yeo, S. S. 2011. Ethanol production from biodiesel-derived crude glycerol by newly isolated *Kluyvera cryocrescens. Applied microbiology and biotechnology,* 89**,** 1255-1264.

Čipinytė, V., Grigiškis, S. & Baškys, E. 2009. Selection of fat-degrading microorganisms for the treatment of lipid-contaminated environment. *Biologija,* 55**,** 84-92.

da Silva, G. P., Mack, M. & Contiero, J. 2009. Glycerol: a promising and abundant carbon source for industrial microbiology. *Biotechnology advances,* 27**,** 30-39.

Dąbrowski, S., Zabłotna, E., Pietrewicz-Kubicz, D. & Długołęcka, A. 2012. Screening of environmental samples for bacteria producing 1, 3-propanediol from glycerol. *Acta Biochim Pol,* 59**,** 000-000.

Dharmadi, Y., Murarka, A. & Gonzalez, R. 2006. Anaerobic fermentation of glycerol by *Escherichia coli*: a new platform for metabolic engineering. *Biotechnology and bioengineering,* 94**,** 821-829.

Dobson, R., Gray, V. & Rumbold, K. 2012. Microbial utilization of crude glycerol for the production of value-added products. *Journal of industrial microbiology & biotechnology,* 39**,** 217-226.

Duxbury, D. 1993. Fat reduction without adding fat replacers. *Food Process,* 68**,** 70.

Fialho, A. M., Martins, L. O., Donval, M.-L., Leitão, J. H., Ridout, M. J., Jay, A. J., Morris, V. J. & Sá-Correia, I. 1999. Structures and Properties of Gellan Polymers Produced by *Sphingomonas paucimobilis* ATCC 31461 from lactose compared with those produced from glucose and from cheese whey. *Applied and environmental microbiology,* 65**,** 2485-2491.

Fialho, A. M., Moreira, L. M., Granja, A. T., Popescu, A. O., Hoffmann, K. & Sá-Correia, I. 2008. Occurrence, production, and applications of gellan:

current state and perspectives. *Applied microbiology and biotechnology,* 79**,** 889-900.

Flickinger, M. C. 1980. Current biological research in conversion of cellulosic carbohydrates into liquid fuels: how far have we come. *Biotechnology Bioengineering.;(United States),* 22.

Fredrickson, J., Balkwill, D., Drake, G., Romine, M., Ringelberg, D. & White, D. 1995. Aromatic-degrading *Sphingomonas* isolates from the deep subsurface. *Applied and environmental microbiology,* 61**,** 1917-1922.

Freitas, F., Alves, V. D., Pais, J., Carvalheira, M., Costa, N., Oliveira, R. & Reis, M. A. 2010. Production of a new exopolysaccharide (EPS) by *Pseudomonas oleovorans* NRRL B-14682 grown on glycerol. *Process Biochemistry,* 45**,** 297-305.

Freitas, F., Alves, V. D., Pais, J., Costa, N., Oliveira, C., Mafra, L., Hilliou, L., Oliveira, R. & Reis, M. A. 2009. Characterization of an extracellular polysaccharide produced by a *Pseudomonas* strain grown on glycerol. *Bioresource technology,* 100**,** 859-865.

Freitas, F., Alves, V. D. & Reis, M. A. 2011. Advances in bacterial exopolysaccharides: from production to biotechnological applications. *Trends in biotechnology,* 29**,** 388-398.

Gonzalez-Pajuelo, M., Andrade, J. & Vasconcelos, I. 2004. Production of 1, 3-propanediol by *Clostridium butyricum* VPI 3266 using a synthetic medium and raw glycerol. *Journal of industrial microbiology & biotechnology,* 31**,** 442-446.

González-Pajuelo, M., Meynial-Salles, I., Mendes, F., Andrade, J. C., Vasconcelos, I. & Soucaille, P. 2005. Metabolic engineering of

Clostridium acetobutylicum for the industrial production of 1, 3-propanediol from glycerol. *Metabolic Engineering,* 7**,** 329-336.

Govender, A. & Pillay, B. 2011. Characterization of 1, 2-dichloroethane(DCA) degrading bacteria isolated from South African waste water. *African Journal of Biotechnology,* 10**,** 11567-11573.

Gungormusler, M., Gonen, C., Ozdemir, G. & Azbar, N. 2010. 1, 3-Propanediol production potential of *Clostridium saccharobutylicum* NRRL B-643. *New biotechnology,* 27**,** 782-788.

Hao, J., Lin, R., Zheng, Z., Liu, H. & Liu, D. 2008. Isolation and characterization of microorganisms able to produce 1, 3-propanediol under aerobic conditions. *World Journal of Microbiology and Biotechnology,* 24**,** 1731-1740.

Haynie, S. L. & Wagner, L. W. 1997. Process for making 1, 3-propanediol from carbohydrates using mixed microbial cultures: US, 5599689. Google Patents.

Homann, T., Tag, C., Biebl, H., Deckwer, W.-D. & Schink, B. 1990. Fermentation of glycerol to 1, 3-propanediol by *Klebsiella* and *Citrobacter* strains. *Applied Microbiology and Biotechnology,* 33**,** 121-126.

Hu, H. & Wood, T. K. 2010. An evolved *Escherichia coli* strain for producing hydrogen and ethanol from glycerol. *Biochemical and biophysical research communications,* 391**,** 1033-1038.

Ibrahim S.A, F. M. I. B., S.K. Abd-El-Aal, A.G. Attallah, M.A. El-Sayd 2011. Isolation and molecular genetic identification of some *Sphingomonas*

paucimobilis local strains. *Australian Journal of Basic and Applied Sciences,* 5**,** 2847-2854.

Jalasutram, V., Jetty, A. & Anupoju, G. R. 2011. Fermentative conversion of raw glycerol into 1, 3-propanediol by isolated *Klebsiella pneumoniae* 141 B stain: Optimization of culture variables. *African Journal of Biotechnology,* 10**,** 18493-18502.

Ji, X.-J., Huang, H., Du, J., Zhu, J.-G., Ren, L.-J., Hu, N. & Li, S. 2009a. Enhanced 2, 3-butanediol production by *Klebsiella oxytoca* using a two-stage agitation speed control strategy. *Bioresource technology,* 100**,** 3410-3414.

Ji, X.-J., Huang, H., Zhu, J.-G., Hu, N. & Li, S. 2009b. Efficient 1, 3-propanediol production by fed-batch culture of *Klebsiella pneumoniae*: The role of pH fluctuation. *Applied biochemistry and biotechnology,* 159**,** 605-613.

Johnson, D. T. & Taconi, K. A. 2007. The glycerin glut: Options for the value-added conversion of crude glycerol resulting from biodiesel production. *Environmental Progress,* 26**,** 338-348.

Jun, S.-A., Moon, C., Kang, C.-H., Kong, S. W., Sang, B.-I. & Um, Y. 2010. Microbial fed-batch production of 1, 3-propanediol using raw glycerol with suspended and immobilized Klebsiella pneumoniae. *Applied biochemistry and biotechnology,* 161**,** 491-501.

Karinen, R. & Krause, A. 2006. New biocomponents from glycerol. *Applied Catalysis A: General,* 306**,** 128-133.

Kiatkittipong, W., Suwanmanee, S., Laosiripojana, N., Praserthdam, P. & Assabumrungrat, S. 2010. Cleaner gasoline production by using glycerol as fuel extender. *Fuel Processing Technology,* 91**,** 456-460.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of molecular evolution,* 16**,** 111-120.

Kumar, A. & Sharma, S. 2011. Potential non-edible oil resources as biodiesel feedstock: An Indian perspective. *Renewable and Sustainable Energy Reviews,* 15**,** 1791-1800.

Kumar, S., Tamura, K. & Nei, M. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in bioinformatics,* 5**,** 150-163.

Kumar, V., Sankaranarayanan, M., Jae, K.-E., Durgapal, M., Ashok, S., Ko, Y., Sarkar, R. & Park, S. 2012. Co-production of 3-hydroxypropionic acid and 1, 3-propanediol from glycerol using resting cells of recombinant *Klebsiella pneumoniae* J2B strain overexpressing aldehyde dehydrogenase. *Applied Microbiology and Biotechnology***,** 1-11.

Kurian, J. V. 2005. A new polymer platform for the future—Sorona® from corn derived 1, 3-propanediol. *Journal of Polymers and the Environment,* 13**,** 159-167.

Liu, H.-J., Zhang, D.-J., Xu, Y.-H., Mu, Y., Sun, Y.-Q. & Xiu, Z.-L. 2007. Microbial production of 1, 3-propanediol from glycerol by *Klebsiella pneumoniae* under micro-aerobic conditions up to a pilot scale. *Biotechnology letters,* 29**,** 1281-1285.

Lobas, D., Schumpe, S. & Deckwer, W.-D. 1992. The production of gellan exopolysaccharide with *Sphingomonas paucimobilis* E2 (DSM 6314). *Applied microbiology and biotechnology,* 37**,** 411-415.

Ma, B. B., Xu, X. L., Zhang, G. L., Wang, L. W., Wu, M. & Li, C. 2009. Microbial production of 1, 3-propanediol by *Klebsiella pneumoniae* XJPD-Li under different aeration strategies. *Applied biochemistry and biotechnology,* 152**,** 127-134.

Manosak, R., Limpattayanate, S. & Hunsom, M. 2011. Sequential-refining of crude glycerol derived from waste used-oil methyl ester plant via a combined process of chemical and adsorption. *Fuel Processing Technology,* 92**,** 92-99.

Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J., & Wade, W. G. 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Applied and Environmental Microbiology*, *64*(2), 795-799.

Martins, L. & Sa-Correia, I. 1991. Gellan gum biosynthetic enzymes in producing and nonproducing variants of *Pseudomonas elodea*. *Biotechnology and applied biochemistry,* 14**,** 357-364.

Matsumura, M., Nomura, N. & Sato, S. 2008. Growth and 1, 3-propanediol production on pre-treated sunflower oil bio-diesel raw glycerol using a strict anaerobe-*Clostridium butyricum. Current Research in Bacteriology,* 1**,** 7-16.

Miller, D. J., Jackson, J. E. & MARINCEAN, S. 2009. Process for the preparation of propylene glycol. Google Patents.

Nampoothiri, K. M., Singhania, R. R., Sabarinath, C. & Pandey, A. 2003. Fermentative production of gellan using *Sphingomonas paucimobilis*. *Process Biochemistry,* 38**,** 1513-1519.

Nimje, V. R., Chen, C.-Y., Chen, C.-C., Chen, H.-R., Tseng, M.-J., Jean, J.-S. & Chang, Y.-F. 2011. Glycerol degradation in single-chamber microbial fuel cells. *Bioresource technology,* 102**,** 2629-2634.

Nolte, M. 2007. *Commercial biodiesel production in South Africa: a preliminary economic feasibility study.* Stellenbosch: University of Stellenbosch.

Oh, B.-R., Seo, J.-W., Heo, S.-Y., Hong, W.-K., Luo, L. H., Joe, M.-H., Park, D.-H. & Kim, C. H. 2011. Efficient production of ethanol from crude glycerol by a *Klebsiella pneumoniae* mutant strain. *Bioresource Technology,* 102**,** 3918-3922.

Pachauri, N. & He, B. 2006. Value-added utilization of crude glycerol from biodiesel production: a survey of current research activities. *ASABE paper***,** 1-16.

Papanikolaou, S., Ruiz-Sanchez, P., Pariset, B., Blanchard, F. & Fick, M. 2000. High production of 1, 3-propanediol from industrial glycerol by a newly isolated *Clostridium butyricum* strain. *Journal of Biotechnology,* 77**,** 191-208.

Pollock, T. J. 1993. Gellan-related polysaccharides and the genus Sphingomonas. *Journal of General Microbiology,* 139**,** 1939-1945.

Premjet, S., Premjet, D. & Ohtani, Y. 2007. The effect of ingredients of sugar cane molasses on bacterial cellulose production by *Acetobacter xylinum* ATCC 10245. *Sen'I Gakkaishi,* 63**,** 193-199.

Rahmat, N., Abdullah, A. Z. & Mohamed, A. R. 2010. Recent progress on innovative and potential technologies for glycerol transformation into fuel additives: a critical review. *Renewable and Sustainable Energy Reviews,* 14**,** 987-1000.

Ringel, A. K., Wilkens, E., Hortig, D., Willke, T. & Vorlop, K.-D. 2012. An improved screening method for microorganisms able to convert crude glycerol to 1, 3-propanediol and to tolerate high product concentrations. *Applied microbiology and biotechnology***,** 1-8.

Rossi, D. M., Da Costa, J. B., De Souza, E. A., Peralba, M. D. C. R. & Ayub, M. A. Z. 2012. Bioconversion of residual glycerol from biodiesel synthesis into 1, 3-propanediol and ethanol by isolated bacteria from environmental consortia. *Renewable Energy,* 39**,** 223-227.

Santibáñez, C., Varnero, M. T. & Bustamante, M. 2011. Residual glycerol from biodiesel manufacturing, waste or potential source of bioenergy: a review. *Chilean journal of agricultural research,* 71**,** 3.

Sattayasamitsathit, S., Methacanon, P. & Prasertsan, P. 2011. Enhance 1, 3-propanediol production from crude glycerol in batch and fed-batch fermentation with two-phase pH-controlled strategy. *Electronic Journal of Biotechnology,* 14**,** 4-4.

Saxena, R., Anand, P., Saran, S. & Isar, J. 2009. Microbial production of 1, 3-propanediol: recent developments and emerging opportunities. *Biotechnology advances,* 27**,** 895-913.

Schmidt, S., Wittich, R., Erdmann, D., Wilkes, H., Francke, W. & Fortnagel, P. 1992. Biodegradation of diphenyl ether and its monohalogenated

derivatives by *Sphingomonas sp.* strain SS3. *Applied and environmental microbiology,* 58**,** 2744-2750.

Shams Yazdani, S. & Gonzalez, R. 2008. Engineering *Escherichia coli* for the efficient conversion of glycerol to ethanol and co-products. *Metabolic engineering,* 10**,** 340-351.

Sparks, G., Ortmann, G. F. & Lagrange, L. An Economic Evaluation of Soybean-Based Biodiesel Production on Commercial Farms in the Soybean-Producing Regions of KwaZulu-Natal: Some Preliminary Results. 2010 AAAE Third Conference/AEASA 48th Conference, September 19-23, 2010, Cape Town, South Africa, 2010. African Association of Agricultural Economists (AAAE) & Agricultural Economics Association of South Africa (AEASA).

Story, S., Kline, E., Hughes, T., Riley, M. & Hayasaka, S. 2004. Degradation of aromatic hydrocarbons by *Sphingomonas paucimobilis* strain EPA505. *Archives of environmental contamination and toxicology,* 47**,** 168-176.

Suhaimi, S. N., Phang, L.-Y., Maeda, T., Abd-Aziz, S., Wakisaka, M., Shirai, Y. & Hassan, M. A. 2012. Bioconversion of glycerol for bioethanol production using isolated *Escherichia coli* SS1. *Brazilian Journal of Microbiology,* 43**,** 506-516.

Sullivan, C. Designing polyester resin performance with 2-methyl-1, 3-propanediol. proceedings SPI/composite ins. 48th annual conference, 1993. The Society of the Plastics Industry, 8-11.

Tao, X.-Q., Lu, G.-N., Dang, Z., Yang, C. & Yi, X.-Y. 2007. A phenanthrene-degrading strain *Sphingomonas sp.* GY2B isolated from contaminated soils. *Process Biochemistry,* 42**,** 401-408.

Thompson, J. & He, B. 2006. Characterization of crude glycerol from biodiesel production from multiple feedstocks. *Applied Engineering in Agriculture,* 22**,** 261.

Tian, K., Chen, X., Shen, W., Prior, B. A., Shi, G., Singh, S. & Wang, Z. 2012. High-efficiency conversion of glycerol to D-lactic acid with metabolically engineered *Escherichia coli. African Journal of Biotechnology,* 11**,** 4860-4867.

Toraya, T. 2003. Radical Catalysis in Coenzyme B12-Dependent Isomerization (Eliminating) Reactions. *ChemInform,* 34**,** no-no.

Vanderhoff, A., Gibbons, W. R., Bauer, N. & West, T. P. 2010. Development of a low-cost medium for producing gellan from *Sphingomonas paucimobilus. Journal of Biotech Research [ISSN: 1944-3285],* 2**,** 67-78.

Wang, X., Xu, P., Yuan, Y., Liu, C., Zhang, D., Yang, Z., Yang, C. & Ma, C. 2006. Modeling for gellan gum production by *Sphingomonas paucimobilis* ATCC 31461 in a simplified medium. *Applied and environmental microbiology,* 72**,** 3367-3374.

Wang, Z., Zhuge, J., Fang, H. & Prior, B. A. 2001. Glycerol production by microbial fermentation: a review. *Biotechnology Advances,* 19**,** 201-223.

West, T. 2003. Effect of temperature on bacterial gellan production. *World Journal of Microbiology and Biotechnology,* 19**,** 649-652.

West, T., Strohfus, B. & Santiago, M. 2000. A colorimetric assay for gellan elaborated by *Sphingomonas paucimobilis* ATCC 31461. *World Journal of Microbiology and Biotechnology,* 16**,** 529-531.

White, D. C., Sutton, S. D. & Ringelberg, D. B. 1996. The genus *Sphingomonas*: physiology and ecology. *Current Opinion in Biotechnology,* 7**,** 301-306.

Wilkens, E., Ringel, A. K., Hortig, D., Willke, T. & Vorlop, K.-D. 2012. High-level production of 1, 3-propanediol from crude glycerol by *Clostridium butyricum* AKR102a. *Applied microbiology and biotechnology***,** 1-7.

Wilson, S., Matthews, M., Austin, G. & Von Blottnitz, H. 2005. Review of the status of biodiesel related activities in South Africa. *Report for the City of Cape Town, South Africa***,** 76.

Xiu, Z.-L., Song, B.-H., Wang, Z.-T., Sun, L.-H., Feng, E.-M. & Zeng, A.-P. 2004. Optimization of dissimilation of glycerol to 1, 3-propanediol by *Klebsiella pneumoniae* in one-and two-stage anaerobic cultures. *Biochemical Engineering Journal,* 19**,** 189-197.

Yazdani, S. S. & Gonzalez, R. 2007. Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. *Current opinion in biotechnology,* 18**,** 213-219.

Yu, C., Cao, Y., Zou, H. & Xian, M. 2011. Metabolic engineering of *Escherichia coli* for biotechnological production of high-value organic acids and alcohols. *Applied microbiology and biotechnology,* 89**,** 573-583.

Zhang, G., Ma, B., Xu, X., Li, C. & Wang, L. 2007. Fast conversion of glycerol to 1, 3-propanediol by a new strain of *Klebsiella pneumoniae*. *Biochemical Engineering Journal,* 37**,** 256-260.

Zhao, H.-P., Wang, L., Ren, J.-R., Li, Z., Li, M. & Gao, H.-W. 2008. Isolation and characterization of phenanthrene-degrading strains *Sphingomonas sp.* ZP1 and *Tistrella sp.* ZP5. *Journal of hazardous materials,* 152**,** 1293-1300.

Zhuge, B., Zhang, C., Fang, H., Zhuge, J. & Permaul, K. 2010. Expression of 1, 3-propanediol oxidoreductase and its isoenzyme in *Klebsiella pneumoniae* for bioconversion of glycerol into 1, 3-propanediol. *Applied microbiology and biotechnology,* 87**,** 2177-2184.

http://www.brenda-enzymes.org/ php/result_flat.php4?ecno=4.2.1.30

Technische Universität Braunschweig 2013. BRENDA, The comprehensive enzyme information system.

Date accessed: 24 February 2013

http://www.eia.gov/biofuels/biodiesel/production/

US Department of Energy 2013. US Energy Information Administration. Washington DC.

Date accessed: 26 February 2013