Production of value added products from biodiesel-derived crude glycerine

K Somdaka

24878510

Dissertation submitted in partial fulfilment of the requirements
For the degree *Magister* in Chemical Engineering at the
Potchefstroom Campus of the North-West University

Supervisor: Prof S Marx
Co-Supervisor: Dr J Bezuidenhout

November 2015

Abstract

The increasing growth of the biodiesel industry has resulted in the increase of crude glycerine generated as a by-product. Typically, for every 100 kg of biodiesel produced, 5-10 kg of crude glycerine is produced. The disposal of crude glycerine, which contains impurities such an unreacted methanol, triglycerides and catalysts, creates a number of challenges to the environment and, added to this, glycerine purification processes are not economically feasible. Thus, the process of the conversion of crude glycerine into valuable products is promising for the safe disposal of biodiesel production wastes. Crude glycerine can be converted into a variety of products through catalytic or biological conversion pathways. Research indicated that biological pathways showed advantages over catalytic pathways in terms of product selectivity and because glycerine can be fermented to a number of fuels and products. A number of microorganisms are able to utilise glycerol as a sole carbon source despite the presence of impurities in the feed mixture. The exploitation of pretreatment methods would improve the conversion of and product yields from crude glycerine fermentations.

The objective of the study was to evaluate the possible routes/pathways for producing value-added products from the fermentation of crude glycerine and washed crude glycerine with *Clostridium diolis*. Batch fermentations were performed at 37^{o}C, a pH of 6.8 and an agitation at 150 rpm under N_2 sparging and anaerobic conditions. The concentration of glycerol used ranged from 50 g.L^{-1} to150 g.L^{-1}. Fermentations were conducted for 48 hours with samples taken every 3 hours for analyses. The crude glycerine used in this study had a relatively high pH (10.84) and contained methanol (309.9 g/L). Moreover, the crude glycerine was then washed with petroleum ether which reduced the methanol content (0.23 g/L) and the pH (8.94).

An analysis of the fermentation products resulted in the production of 1,3-propanediol, lactic acid, butyric acid and acetic acid after analysis by high-performance liquid chromatography (HPLC). The most prominent product was 1,3-propanediol with a concentration of 17.83 g/L after 24 hours of fermentation with 50 g/L of pure glycerol as carbon source and 18.89 g/L of 1,3-propanediol when fermented with 50 g/L of washed crude glycerine. Impurities in the crude glycerine

inhibited the growth of *C. diolis* with a maximum inhibition rate of 92% in crude glycerine and a maximum inhibition rate of 73 % in washed crude glycerine.

Subsequently, the effect of ultrasound on the growth of *Clostridium diolis* and the production of 1,3-propanediol from pure glycerol at a concentration of 50 g/L was investigated. The use of ultrasound irradiation showed that the fermentation time could be reduced from 24 hours to 20 hours when fermented on 50 g/L pure glycerol. An activation time of 2 minutes prior to fermentation resulted in high concentrations of 1,3-propanediol (16.89 g/L), decreased fermentation time and increased bacterial growth. The exposure of *Clostridium diolis* to ultrasound for 10 minutes did not significantly produce 1,3-propanediol formation and slow bacteria growth was observed. The study indicates that crude glycerine inhibits the growth of *C. diolis* and that washed glycerol has the potential of being used as a sole carbon source for anaerobic fermentation to produce value-added products.

Key words: Glycerine, *Clostridium diolis,* 1,3-propanediol, Butyric acid, Lactic acid, Acetic acid, Ultrasound, Glycerol

Opsomming

Die toenemende groei van die biodieselbedryf het tot ‘n toename in die produksie van ru-gliserien as ‘n newe-produk gelei. Vir elke 100 kg biodiesel wat geproduseer word, word daar tipies 5-10 kg ru-gliserien geproduseer. Die wegdoen van ru-gliserien, wat onsuiwerhede soos ‘n ongereageerde metanol, trigliseriede en katalisators bevat, skep ‘n aantal uitdagings vir die omgewing en daarby is gliseriensuiweringsprosesse nie ekonomies haalbaar nie. Daarom is die proses van die omskakeling van ru-gliserien na waardevolle produkte belowend vir die veilige wegdoen van biodieselproduksieafval. Ru-gliserien kan in ‘n verskeidenheid van produkte omgeskakel word deur katalitiese of biologiese omskakelingspaaie. Navorsing het getoon dat biologiese paaie meer voordele as katalitiese paaie het in terme van produkselektiwiteit en gliserien wat gefermenteer kan word in ‘n aantal brandstowwe en produkte. ‘n Aantal mikroorganismes kan gliserol as ‘n uitsluitlike koolstofbron gebruik ten spite van die teenwoordigheid van onsuiwerhede in die voermengsel. Die ontginning van voorbehandelingsmetodes sal die omskakeling van en produkopbrengste uit ru-gliserien fermentasies verbeter.

Die doel van hierdie studie was om die moontlike roetes/paaie vir die produksie van waarde-toegevoegde produkte uit die fermentasie van ru-gliserien en gewasde ru-gliserien met *Clostridium diolis* te evalueer. Groepfermentasies is by 37^{o}C, ‘n pH van 6.8 en ‘n agitasie van 150 rpm onder N_2-besprinkeling en anaerobiese toesrande uitgevoer. Die konsentrasie van gliserol wat gebruik is het gewissel van 50 g.L^{-1} tot 150 g.L^{-1}. Die fermentasies is vir 48 uur uitgevoer waartydens monsters elke 3 uur vir analise geneem is. Die ru-gliserien wat in hierdie studie gebruik is het ‘n relatief hoë pH (10.84) gehad en het metanol (309.9 g/L) bevat. Daarna is die ru-gliserien met petroleum eter gewas wat die metanol inhoud (0.23 g/L) en die pH (8.94) verlaag het.

‘n Analise van die fermentasieprodukte het die produksie van 1,3-propaandiol, melksuur, bottersuur en asynsuur na analise met hoë-prestasie vloeistofchromatografie (HPVC) aangetoon. Die prominentste produk was 1,3-propaandiol met ‘n konsentrasie van 17.83 g/L na 24 uur se fermentasie met 50 g/L suiwer gliserol as koolstofbron en 18.89 g/L 1,3-propaandiol wat gefermenteer is met 50 g/L gewasde ru-gliserien. Onsuiwerhede in die ru-gliserien het die groei van *C.*

diolis geïnhibeer met ‘n maksimum inhibisiekoers van 92% in ru-gliserien en ‘n maksimum inhibisiekoers van 73 % in gewasde ru-gliserien.

Vervolgens is die effek van ultraklank op die groei van *Clostridium diolis* en die produksie van 1,3-propaandiol uit suiwer gliserol by ‘n konsentrasie van 50 g/L ondersoek. Die gebruik van ultraklankbestraling het aangetoon dat die fermentasietyd van 24 uur na 20 uur verminder kan word wanneer dit gefermenteer word op 50 g/L suiwer gliserol. ‘n Aktiveringstyd van 2 minute voor die aanvang van fermentasie het hoë konsentrasies van 1,3-propaandiol (16.89 g/L), ‘n verlaagde fermentasietyd en verhoogde bakteriële groei tot gevolg gehad . Die blootstelling van *Clostridium diolis* aan ultraklank vir 10 minute het nie beduidende hoeveelhede 1,3-propaandiol geproduseer nie en stadige bakteriële groei is waargeneem. Die studie toon aan dat ru-gliserien die groei van *C. diolis* inhibeer en dat gewasde gliserol die potensiaal het om as uitsluitlike koolstofbron vir anaerobiese fermentasie gebruik te kan word om toegevoegde-waarde produkte te lewer.

Sleutelwoorde: Gliserien, *Clostridium diolis,* 1,3-propaandiol, Bottersuur, Melksuur, Asynsuur, Ultraklank, Gliserol

Declaration

I, Somdaka Khuthala hereby declare that this dissertation is my own work and I am the sole author. It is being submitted for the degree Master of Science, at the North-West University, Potchefstroom, South Africa. The work has not been submitted for any degree at any other institution.

Somdaka Khuthala

Acknowledgements

First and foremost, I would like to express my sincere thanks and appreciation to God almighty.

I would like to express my deep appreciation to Prof. Sanette Marx. Thank you for giving me the opportunity to be part of the team and for your continued guidance throughout the study. I thank you for support and providing an intellectually stimulating environment.

I am especially thankful to Dr. Jaco Benzuidenhout, who was really willing to share his wealthy experience in microbiology and fermentation technology with me. I also would like to thank the entire North-West University microbiology group.

I would like to express my deep appreciation to Dr. Idan Chiyanzu for his guidance, support and valuable suggestions throughout the project.

A distinctive thank you goes to the Chemical Engineering Department, the laboratory technician and staff in the Bioenergy group.

To all my post graduate colleagues who shared my moments of frustration and joy, words alone cannot express how grateful I am.

A special thanks to Coega Development Cooperation for financial assistance and motivation with regard to my studies.

To my family; I can find no words to express how much I appreciate your endless support. Without your love and support, this journey would not have been possible.

Table of Content

ABSTRACT **2**
DECLARATION **6**
ACKNOWLEDGEMENTS **7**
TABLE OF CONTENT **8**
LIST OF FIGURES **11**
LIST OF TABLES **13**
TABLE OF ABBREVIATIONS **14**
1 INTRODUCTION **16**
1.1 Aim **17**
1.2 Objectives **18**
1.3 Scope of the study **18**
1.4 References **19**
2 LITERATURE REVIEW **21**
2.1 Biofuel background **21**
2.2 Biodiesel **22**
2.2.1 Blending of oil with diesel 23
2.2.2 Micro-emulsion of oil 23
2.2.3 Pyrolysis 23
2.2.4 Transesterification 24
2.3 Biodiesel production by transesterification **24**
2.3.1 Base catalysed transesterification 25
2.3.2 Acid catalysed transesterification 26
2.3.3 Biological catalysed transesterification 26
2.4 Glycerol structure and properties **26**
2.4.1 Purification of crude glycerine 28
2.5 Production of value added products from crude glycerine **29**
2.6 Biological conversion of glycerol **30**
2.6.1 *Clostridium* genus 32
2.6.2 Fermenting crude glycerine by *Clostridium* species 33
2.6.3 Effect of crude glycerine in fermentation 34
2.6.4 Removal of impurities for biological conversion 36
2.6.5 Mechanism of ultrasound 36
2.7 Possible products from biological conversion of glycerol **38**
2.7.1 Ethanol 38
2.7.2 Butanol 39

2.7.3 Succinic acid 39
2.7.4 Lactic acid 39
2.7.5 Citric acid 40
2.7.6 Acetic Acid 40
2.7.7 1, 3-Propanediol 40
2.8 Concluding remarks 41
2.9 References 42
3 EXPERIMENTAL 50
3.1 Source of Crude glycerine 50
3.2 Wash of crude glycerine by petroleum ether 50
3.3 Physical analysis 51
3.3.1 Viscosity 52
3.3.2 pH 52
3.3.3 Density 52
3.4 Microorganism and culture conditions 52
3.4.1 Microorganism 52
3.4.2 Reinforce Clostridium Media (RCM) 53
3.4.3 Fermentation medium 54
3.5 Culture condition 54
3.6 Enhancement by ultrasound 55
3.7 Analysis 56
3.7.1 Ultraviolet spectroscopy 56
3.7.2 High-performance liquid chromatograph (HPLC) 57
3.7.3 Fourier transform infrared spectroscopy (FTIR) 58
3.7.4 Microscope analysis 59
3.7.5 Gas chromatograph 60
3.8 References 62
4 RESULTS AND DISCUSSION 63
4.1 Characterization of glycerol feedstock 63
4.1.1 Analysis of physical properties of glycerol sources 63
4.1.2 Quantification of methanol and glycerol content 64
4.2 Analysis of functional groups using Fourier transform infrared spectroscopy (FTIR) 65
4.3 Growth potential of *C. diolis* on different concentrations of glycerol 68
4.4 Fermentation results 72
4.4.1 Production of 1,3-propanediol from glycerol fermentation 73
4.4.2 Production of other products from glycerol fermentation 76

4.5 Effect of ultrasound radiation on the production of 1, 3-propanediol 79
4.5.1 The influence of time in ultrasound assisted fermentation 79
4.5.2 Production of 1,3-propanediol from ultrasound assisted fermentation 80
4.5.3 Growth morphology 82
4.6 References 84
5 CONCLUSION AND RECOMMENDATIONS 85
5.1 Conclusion 85
5.2 Recommendations 85
A1 INTRODUCTION 87
A2 HPLC PRODUCT ANALYSIS 87
A3 GC ANALYSIS 91
B1 INTRODUCTION 95
B2 MOLECULAR WEIGHT OF OIL 95
B3 CONCENTRATION CALCULATIONS 96
B4 PRODUCTIVITY 100
B5 ERROR CALCULATION 100
C1 INTRODUCTION 102
C2 INFORMATION ABOUT *C. DIOLIS*. 102

LIST OF FIGURES

Figure 2.1: The entire reaction process of the transesterification of triglycerides (Ratmat et al., 2010) 25
Figure 2.2: Microbial fermentation of glycerol (Leja *et al*., 2011). 31
Figure 3.1: Flow diagram of the crude glycerine wash by petroleum ether. 51
Figure 3.2: *Clostridium diolis* colonies grown at 37°C for 72 hours in an anaerobic chamber 53
Figure 3.3 : Modified Erlenmeyer flask that is used for the fermentation of glycerol to maintain an anaerobic environment. 55
Figure 3.4: Ultrasonic bath and flask used for ultasound assisted fermentation of glycerol by *Clostridium diolis*.....56
Figure 3.5: Ultraviolet spectroscopy to monitor cell growth. 57
Figure 3.6: High-performance liquid chromatograph used to quantify product concentrations 58
Figure 3.7: Fourier transform infrared spectroscopy 59
Figure 3.8: Light microscope used to identify the effect of ultrasound-assisted fermentation. 60
Figure 3.9: Gas chromatography to analyse fatty acids for the production of biodiesel 61
Figure 4.1: FTIR spectrum for pure glycerol. 66
Figure 4.2: FTIR spectrum for crude glycerine 67
Figure 4.3: FTIR spectrum for washed crude glycerine. 68
Figure 4.4: Growth curves of *C. diolis* on pure glycerol at 600 nm, (♦ - 50 g/L ■ - 100 g/L, ▲ - 150 g/L).....69
Figure 4.5: Growth potential of *C. diolis* when grown in crude glycerine, ♦ - 50 g/L ■ - 100 g/L, ▲ - 150 g/L.....70
Figure 4.6: Growth potential of *C. diolis* when grown in washed crude glycerine, ♦ - 50 g/L ■ - 100 g/L, ▲ - 150 g/L.....71
Figure 4.7: The growth inhibition of crude glycerine (■) and washed crude glycerine (■) on *C.diolis* at 50 g/L, 100 g/L and 150 g/L.....72
Figure 4.8: Production of 1,3-propanediol from pure glycerol (♦ - 50 g/L, ■ - 100 g/L, ▲ - 150 g/L 73

Figure 4.9: Production of 1,3-propanediol from washed crude glycerine (♦ - 50 g/L, ■ - 100 g/L, ▲ - 150 g/L 75
Figure 4.10: Production of acetic acid, butyric acid and lactic acid from pure ??? at different feed concentrations 50 g/L (■ - Lactic acid, ■ - Butyric acid, ■ - Acetic acid) and 100 g/L (■ - Lactic acid, ■ - Butyric acid , ■ - Acetic acid).....76
Figure 4.11: Production of acetic acid, butyric acid and lactic acid from washed crude glycerine at different feed concentrations 50 g/L (■ - Acetic acid, ■ - Butyric acid, ■ - Lactic acid), 100 g/L (■ - Acetic acid, ■ - Lactic acid, ■ - Butyric acid) and 150 g/L (■ - Butyric acid, , ■ - Lactic acid, ■ - Acetic acid). 78
Figure 4.12: Growth of *C.diolis* on media subjected to ultrasound for 2 minutes (■) and 10 minutes (▲)compared to the control (♦) 79
Figure 4.13: Production of 1,3-propanediol from 50 g/L of pure glycerol that was subjected to ultrasound irradiation for 2 minutes (■) and 10 minutes (▲) compared to no ultrasound treatment (♦).....81
Figure 4.14 : Growth morphology of *C diolis* grown at 50 g/L of pure glycerol on media subjected to ultrasound for 2 minutes, 10 minutes and control, (**A** - 3 hours of 2 minutes of ultrasound, **B** - 20 hours of 2 minutes of ultrasound, **C** - 3 hours of 10 minutes of ultrasound, **D** - 20 hours of 10 minutes ultrasound, **E** - 3 hours with no untrasound , **F** - 20 hours with no ultrasound).....82
Figure A.0.1: 1,3-propanediol calibration curve.....88
Figure A.0.2: Butyric acid calibration curve 89
Figure A2.20.3:???.....89
Figure A.0.4 ???.....89
Figure A.0.5: Methanol calibration curve.....90
Figure A.6: Acetic acid calibration curve 90
Figure A.0.9: Calibration curve of C18:2. 93
Figure A.0.10: Calibration curve of C16:0 93
Figure A.0.11: Calibration curve of C18:0. 94
Figure A.0.12: Calibration curve of C18:1 94
Figure C.0.1: pH levels when fermenting with 50 g/L of glycerol sources, (■ - crude glycerine), (▲ - pure glycerol) and (♦ - washed crude glycerine).....103
Figure C.0.2: pH levels when fermenting with 100 g/L of glycerol sources, (■ - crude glycerine), (▲ - pure glycerol) and (♦ - washed crude glycerine).....103

Figure C.0.3: pH levels when fermenting with 150 g/L of glycerol sources (■ - washed, crude glycerine), (▲ - pure glycerol) and (♦ - crude glycerine).....104

List of tables

Table 4.1: The physical characterization of glycerol sources.....63
Table 4.2: Glycerol and methanol content of glycerol sources used in this study.....64
Table 4.3: Glycerol content in each different source of glycerol at different concentrations (g/L) 64
Table 4.4: Possible functional groups present in pure glycerol used in the current study.....65
Table A2.0.1: Components obtained and their corresponding symbols used in the calculations 87
Table A.0.2: Data used for the determination of the sunflower oil molecular weight 92
Table B.0.3: Concentration of 1,3-propanediol when fermenting with washed crude glycerine. 98
Table B.0.4: Concentration of acid when fermenting with washed crude glycerine: acetic acid (AA), lactic acid (LA) and butyric acid (BA).....99
Table B.0.5: Concentration of acid when fermenting with pure glycerol: acetic acid (AA), lactic acid (LA) and butyric acid (BA).....99
Table C.0.2: The information about *Clostridium diolis* DSM 15410.....102
Table C.3: List of the chemicals used in the study 104

List of abbreviations

ADH - alcohol dehydrogenase

ADP - adenosine diphosphate

ATP - adenosine triphosphate

CoA - coenzyme A

DAK - dihydroxyacetone kinase

DHAP - dihydroxyacetone phosphate

GPD - glycerol phosphate dehydrogenase

3-HPA - 3-hydroxypropionaldehyde

HPLC - High-performance liquid chromatography

GC - Gas chromatography

FTIR- Fourier transform infrared spectroscopy

OD - Optical density

1,3-PDO - 1,3-propanediol

LA –Lactic acid

AA – Acetic acid

BA –Butyric acid

PET - polyethylene terephthalate

PYK - pyruvate kinase

C. diolis - *Clostridium diolis* DSM 15410

PTT - polytrimethylene terephthalate

UV - ultraviolet light

Min - Minutes

^{o}C - Degrees Celsius

% - percent

Hz - hertz

CHAPTER 1

1 Introduction

Global energy sources mainly depend on fossil fuels such as coal, natural gas and petroleum. Due to high usage rates, these resources are now depleting faster and research indicates that the combustion of fossil fuels contributes to global warming (Nigam & Singh, 2011). The high demand for energy, together with environmental concerns related to fossil fuel usage, have motivated the need to investigate alternative sources of energy that can supplement fossil fuels and reduce carbon dioxide (CO_2) emissions. Proposed alternative sources of energy should at least be economically viable, technically feasible to produce, environmentally friendly to use and renewable. While wind, solar, geothermal and hydro energy are recognised as sustainable alternative energy sources, these alternative energies cannot replace fossil based carbon (Yusuf et *al.*, 2011). Biofuels produced from biomass feedstock are attractive alternatives or can substitute petroleum fuels (Sarma *et al.*, 2012).

Biofuels can be classified as solid (biochar), liquid (bioethanol, vegetable oil and biodiesel) or gas (biogas, biosyngas and biohydrogen) fuels and are primarily produced from biomass. Biomass is the largest renewable energy source; approximately 77.4 % of global energy supply and 10.4% of the total primary energy supply are from biomass (Yordanov *et al*., 2013). There are two world-renowned biofuels that show the potential to replace diesel and petroleum fuels, i.e. biodiesel and bioethanol. Recently, biodiesel attracted attention due to its simple production process; environmental benefits that include reduced harmful emissions, low sulphur content, and improved lubricity; and lower production costs when compared to petroleum diesel (Balat & Balat, 2010; Gui *et al.*, 2008).

Biodiesel is produced from animal or vegetable oil in the presence of an alkali or acid catalyst via the ethanolysis or methonolysis of the triglycerides through transesterification. Glycerol is the main by-product of the biodiesel production process. Stoichiometrically, for every 10 kg of biodiesel produced, 1 kg of crude glycerine is generated (Yadzani & Gonzalez, 2007; Abdullah *et al.*, 2010). Glycerol has many applications, e.g. it is used as feedstock for the food, cosmetic and pharmaceutical industries (Johnson & Taconi, 2007). However, crude glycerine contains impurities, therefore, in order to be used for any industrial processes, it

needs to be purified. This purification usually involves complex and expensive methods (Escapa *et al.*, 2009). Moreover, crude glycerine cannot be deposited to the environment without treatment, and the cost for treatment is excessive (Nmachukwu *et al.*, 2013).

Different methods for the conversion of crude glycerine to industrial chemicals have been developed; these include biological and chemical pathways (Zheng *et al.*, 2008, Pagliaro *et al.*, 2007). When the chemical conversion of glycerol is compared to the biological pathways, it shows many operational disadvantages, especially the need for high pressure or temperature, low production specificity and the inability to use contaminated crude glycerine (Yadzani & Gonzalez, 2007). Anaerobic fermentation, as biological conversion pathway, is preferred to aerobic fermentation since it uses less energy and requires lower capital and operational costs (Jonson & Tanoci, 2007). Various microorganisms are able to grow anaerobically on glycerol as sole carbon source, including *Enterobacter spp, Klebsiela spp, Citrobacter spp* and *Clostridia spp.* This growth process on glycerol produces a range of acids and solvents (Leja *et al.*, 2011). Microorganisms can, for instance, metabolise glycerol though oxidation and reduction pathways. In this process the usage of glycerol is proportional to the production of 1,3-propanediol (Dobson *et al.*, 2012). The *Clostridiaceae* family has been anaerobically tested and assessed for the production of different chemicals and alcohols, such as 1,3-propanediol, butanol, ethanol and others (Almeida *et al.*, 2012; Khanna *et al.*, 2012).

The world's biodiesel market is estimated to generate 140 billion litres of biodiesel by 2016, resulting in the production of approximately 15 billion litres of crude glycerine (Yang *et al.,* 2012). This means that there is a large surplus of glycerol with little commercial value that can negatively affect the economic feasibility of the biodiesel industry (Chaudhary *et al.*, 2011). Thus, the economic viability of biodiesel production could be increased through the conversion of glycerol into value-added products.

1.1 **Aim**

The aim of this project was to evaluate the potential of producing value-added products via fermentation from biodiesel-derived crude glycerine by using *Clostridium diolis.*

1.2 **Objectives**

The objectives of this study were:

- To evaluate the inhibition growth rate of crude glycerine and washed crude glycerol compered to pure glycerol on the growth of *C. diolis* at feeding concentrations of 50 g/L, 100 g/L and 150 g/L glycerol;
- To assess the production of possible value-added products when using raw crude glycerine as carbon source with the growth of *C. diolis* and the production of value-added products and when washed crude glycerine is used as carbon source;
- To identify and characterize possible products formed during anaerobic fermentation which include 1,3- propanediol, acetic acid butyric acid and lactic acid;
- To evaluate the degree to which glycerol bioconversion is enhanced when exposed to ultrasound irradiation prior to fermentation.

1.3 **Scope of the study**

Chapter 1 contains the introduction to renewable energy, biofuels and the utilisation of biodiesel based crude glycerine for the production of valuable products. Furthermore, chapter 1 includes the aim and objectives of the study. Chapter 2 reviews current literature on the production of different products and the different routes for the conversion of crude glycerine. Specific methods and procedures for the production of value-added products, starting with the characteristics of crude glycerine to the extraction of impurities and fermentation conditions, are provided in Chapter 3. Chapter 4 contains the results and discussion of the study. Characterisation of the glycerol samples, microbial growth and the production yields are presented and discussed. Chapter 5, which is the last chapter, provides conclusions and recommendations concerning the entire study.

1.4 **References**

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

Almeida, J.R.M., Favaro, L.C.L. & Quiriro, B.F. 2012. Biodiesel biorefinary: opportunities and challenges for microbial production of fuels and chemicals from glycerol waste. *Biotechnology for Biofuels,* 5:1-16.

Balat, M. & Balat, H. 2010. Progress in biodiesel processing. *Applied Energy*, 50:1817-1835.

Chaudhary, N., Ngadi, M.O., Simpson, B.K. & Kassama, L.S. 2011. Biosynthesis of ethanol and hydrogen by glycerol fermentation using *Escherichia coli*. *Advances in Chemical Engineering and Sciences*,1:83-89.

Dobson, R., Gray, V. & Rumbold, K. 2012. Microbial utilization of crude glycerine for the production of value-added products. *J Ind Microbiol Biotechnol*, 39:217-226.

Escapa, A., Manuel, M.F., Moran, A., Gomez, X., Guist, S.R. & Tatakavsky, B. 2009. Hydrogen production from glycerol in a membraneless microbial electrolysis cell. *Energy fuels*, 23:4612-4616.

Gui, M.M., Lee, K.T. & Bhatia, S. 2008. Feasibility of edible oil vs. non-edible oil vs. waste edible oil as biodiesel feedstock. *Energy,* 33:1646-1653.

Johnson, D.T. & Taconi, K.T. 2007. The glycerine glut: Options for the value-added conversion of crude glycerine resulting from biodiesel production. *Environmental progress*, 26(4):338-348.

Khanna, S., Jaiswal, S., Goyal, A. & Moholkar, V.S. 2012. Ultrasound enhancement of the bioconversion of glycerol by *Clostridium pasterianun*: A mechanistic investigation. *Chemical Engineering Journal*, 200-202:416-425.

Leja, K., Drozdzynska, A. & Czaczyk, K. 2011. Biotechnological production of 1,3-propanediol from crude glycerine. *Journal of biotechnology, computational biology and bionanotechnology*, 92(10):92-100.

Nigam, P.S. & Singh, A. 2011. Production of liquid biofuels from renewable resources. *Progress in energy and combustion science*, 37:52-68.

Nwachukwu, R.E.S., Shahbazi, A., Wang, L., Worku, M., Ibrahim, S. & Schimmel, K. 2013. Optimisation of cultural conditions for conversion of glycerol to ethanol by *Enterobacter aerogens* S012. *AMB Express a Springer Open Journal,* 3(12):1-10.

Pagliaro, M., Ciriminna, R., Kimura, H., Rossi, M. & Pina, C.D. 2007. From glycerol to value-added products. *Angen.Chem Int ed,* 46:4434-4440.

Sarma, S.J., Brar, S.K., Sydney, E.B.. Bihan, Y.L., Buelana, G. & Soccol, C.R. 2012. Microbial hydrogen production by bioconversion of crude glycerine: A review. *International Journal of Hydrogen*, 37:6473-6490.

Yang, F., Hanna, M.A. & Sun, R. 2012. Value added uses for crude glycerine as by-products of biodiesel production. *Biotechnology for Biofuels,* 5:13.

Yazdani, S.S. & Gonzelez, R. 2007. Anaerobic fermentation of glycerol: A path of economic viability for biofuel industries. *Current option in biotechnology,* 18(3):213-219.

Yordanov, D.I., Tsonec, Z.B., Pilichev, T.V. & Mustafa, Z.A. 2013. A new approach for the production of coffee oil from waste coffee residue as feedstock for biodiesel. *Petroleum and Coal*, 55(2):74-81.

Yusuf, N.N.A.N., Kamarudin, S.K. & Yaakub, Z. 2011. Overview of current trends in biodiesel production. *Energy conversion and management,* 52:2741-2751.

Zheng, Y., Chen, X. & Shen, Y. 2008. Commodity chemicals derived from glycerol, an important biorefinary feedstock. *Chem Rev*, 108:5253-5277.

CHAPTER 2

2 Literature Review

This chapter contains a view of current literature on biofuel production and opportunities for the use of biodiesel by-products to produce high value chemicals and fuels. This section also reviews alternative utilisations of crude glycerine and the purification of crude glycerine. Lastly, chemical and biochemical routes to transform crude glycerine into value-added products are proposed according to existing literature.

2.1 Biofuel background

Global energy generation is mainly dependent on non-renewable resources with 82% of energy coming from fossil fuels (Sarma *et al.*, 2012). The transport sector consumes approximately 95% of the energy derived from fossil resources with 39% of energy consumed as petrochemicals (Ajanovic & Hass, 2010). Approximately 20% of the global CO_2 emission is generated by the utilisation of fossil fuels in the transport sector alone (Balat & Balat, 2010). Based on the aforementioned, the continued demand and daily use of fossil fuels for living are contributing to the existing challenges that include acid rain, water pollution, global warming and other ecological and environmental issues. The finite nature of fossil resources and the associated environmental issues have led to continued and increasing research and experiments on biomass based resources to replace or supplement petroleum based fuels. Biofuels are fuels produced from organisms and their metabolic extracts that result from agricultural, garbage and industrial waste products or by-products (Zhang & Zhang, 2012). In other words, biofuels are derived from biomass and biomass originates from the photosynthesis of plants that use energy from the sun to produce their own food. Since the feedstock used to make biofuels can be easily regenerated, it is classified as a renewable energy form.

Biodiesel, which is mainly produced through the transesterification of oils and fats, is one of the globally recognized liquid biofuels that can substitute petroleum diesel (Nigam & Singh, 2011). Some of the major differences between fossil based diesel fuel and biodiesel are that biodiesel has a higher cetane number, lower sulphur levels and lower aromatic content and flashpoint (Bankovic-Ilic *et al*., 2012), which decreases environmental pollution. Low sulphur levels decrease the emissions of

sulphur oxides to the environment. On the other hand, viscosity is one of the disadvantages of biodiesel, as it affects the fuel injection operations, especially in regions with cold temperatures (Madyira *et al*., 2012). Other disadvantages include: a lower energy content, higher cloud point and pour point, higher nitrogen oxide (NOx) emissions, lower power and high price (Demirbas, 2009).

2.2 **Biodiesel**

Biodiesel is mono-alkyl-esters of long chains of fatty acids that can be produced from vegetable oil or animal fat. It is biodegradable, environmentally friendly, renewable, carbon neutral, and possesses the ability to replace fossil diesel and to be utilised in diesel engines (Atadishi *et al.*, 2013). Approximately 350 oil-bearing crops have been identified as potential feedstock for biodiesel, but sunflower, soybean, safflower, palm, cottonseed, rapeseed and peanut oil are considered to be best suited for the production of biodiesel for modern diesel engines (Demirbas, 2007). The choice of feedstock however, depends mostly on the availability of the crop and the political and geographical factors playing a role in a particular region.

The use of vegetable oils in diesel engines dates back to 1894, when Rudolf Diesel tested his new invention using peanut oil. According to Mathur *et al.* (2011) Rudolf wrote in 1912 that the use of oils may become the fuel of choice in the course of time (Mathur *et al.*, 2011). Although vegetable oils and animal fats could be used directly as an alternative fuel, there are some limitations when using neat oils as fuel. It has been proven that a low volatility and high viscosity are the leading obstacles that prevent the direct use of vegetable oils in conventional diesel engines, since they cause problems, such as carbon deposition, ring sticking, injector choking and lubricating oil thickening in engines. These problems result in the formation of high amounts of ash due to incomplete combustion (Yusuf *et al*., 2011). A lower calorific value leads to a higher fuel consumption and lower power output, while a lower volatility and high viscosity lead to an increased delay time. These disadvantages of the direct utilisation of oil as fuel can be overcome by modifying oil/fats through processes such as:

1. Blending

2. Micro emulsion

3. Pyrolysis or thermal cracking

4. Transesterification

2.2.1 **Blending of oil with diesel**

Blending processes involve the mixing of vegetable oils with conventional diesel. The process focuses on minimizing viscosity by mixing the oil with conversional diesel (Balat & Balat, 2010). The idea of blending was initiated when it seemed evident that the utilisation of vegetable oil in a car will require an engine modification, such as changing the pipes and reconstructing the injector material. If the engine was not modified, the biodiesel could damage the engine and the car could then be expensive to maintain (Aworanti *et al.,* 2013**)**. Furthermore, researchers reported that the substitution of 100% vegetable oil for diesel fuel is not practical. Therefore, the blending of 20-25% vegetable oil with diesel has been considered to give good results for utilisation in a diesel engine (Nigam & Singh, 2011). The blending process is known to lower the viscosity and improve the volatiles of the oil, but the oil will remain polyunsaturated in nature, since there is no change in the molecular structure of the vegetable oil. Normally, the direct use of vegetable oils and their blends have been considered to be difficult in both direct and indirect diesel engines (Yusuf *et al* , 2011).

2.2.2 **Micro-emulsion of oil**

Micro-emulsification has been viewed as a reliable method for lowering the viscosity of oil, as it affords a viscosity close to that of petroleum diesel fuel (Atabani *et al*., 2012). The process involves colloidal equilibrium dispersion of optically isotropic fluid microstructures that results spontaneously from two immiscible liquids. The oil is mostly immersed in a solvent such as ethanol, methanol, hexanol and n-butanol. One of the limitations of micro-emulsion is that it yields fuels that have low energy contents (Leung *et al.*, 2010).

2.2.3 **Pyrolysis**

Pyrolysis is the conversion of biomass or coal to fuel by using heat in the presence of a catalyst. The biomass can be animal fat, natural fatty acids, vegetable oil, methyl esters or fatty acids. The pyrolysis of oils produces a number of products such as aromatics, alkenes, alkanes and carboxylic acids, which are suitable to be used in diesel engines (Satyrthi *et al*., 2013). Researchers have shown that the process is

promising for the production of biodiesel and it would be more viable if it could be used in combination with well-developed industrial hydrotreatment processes, because of the similarities of this process to petroleum refining processes (Abbaszaadeh *et al*., 2012). Unfortunately, despite all the advantages of the pyrolysis of oil and the ability to produce biodiesel with chemical similarities to petroleum diesel, the method is expensive and energy extensive (Leung *et al*., 2010).

2.2.4 **Transesterification**

The transesterification of oil to biodiesel is the conversion method most often used, because of the simple process and relatively cheap reagents (Leung *et al.*, 2010). The fatty acid esters produced in the transesterification process have physical characteristics close to those of diesel fuel and the process is relatively environmentally friendly (Demirbas, 2008).

Amongst the mentioned methods, blending and transesterification are the most common methods to reduce the viscosity of vegetable oils. Blending is simple, as it does not contain any chemical modification of the vegetable oil. However, blending is undesirable, because it requires purification of the vegetable oil before the blending process. Transesterification has been regarded as the best method worldwide, and has been preferred to other approaches due to its low cost and simplicity. (Leung *et al*., 2010).

2.3 **Biodiesel production by transesterification**

Transesterification is a process in which animal fat or vegetable oil reacts with alcohol in the presence of a catalyst to produce fatty acid alkyl esters (FAME) and glycerol (Leung *et al.*, 2010). According to Marchetti *et al*. (2007) the reaction mechanisms of transesterification proceed through three reversible reactions. Firstly, the triglyceride is converted to diglycerides, followed by the conversion of diglycerides to monoglycerides and lastly, monoglycerides are converted to glycerol (Marchetti *et al*., 2007). The reaction mechanism is summarised in Figure 2.1 below.

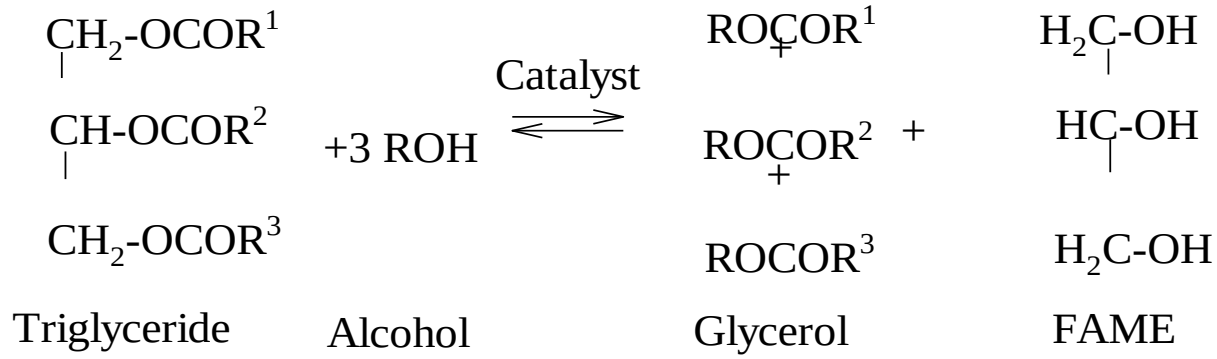

Figure 2.1: The entire reaction process of the transesterification of triglycerides (Ratmat *et al*., 2010)

The production of biodiesel via transesterification is affected by a number of factors, such as the amount of catalyst, type of catalyst, reaction time and oil to alcohol ratio (Shereena & Thangaraj, 2009). The amount of alcohol and catalyst are the main factors that influence the transesterification, since an excess of alcohol is needed to shift the equilibrium to favour the production of FAME and the catalyst splits the oil molecules (Tan *et al*., 2013). Methanol and ethanol are the two main light alcohols used for the transesterification process due to their relatively low cost. However, propanol, isopropanol, tert-butanol, branched alcohols, octanol and butanol can also be employed, but the cost is much higher (Balat & Balat, 2010). Ethanol has often been chosen as alcohol for biodiesel production, because it can be derived from agricultural residues such as starch or sugar. However, methanol is most often use because of its lower cost, high polarity and short alkyl chain. Glycerol is an important by-product of this process that can be burned for heat or be used as feedstock in the cosmetic industry. A range of catalysts can be used, including acid, alkaline and biological catalysts that are chosen based on the amount of free fatty acids present in the oil.

2.3.1 **Base catalysed transesterification**

In a typical transesterification reaction, alkaline catalysts such as sodium hydroxide, calcium hydroxide and potassium hydroxide are most often used as homogenous catalysts. This is due to their fast reaction times and high yields at low molar concentrations. Conversely, the presence of free fatty acids and water produce soap

when using alkaline catalysts (Balat & Balat, 2010). Soap formation decreases the quality and yield of biodiesel and causes difficulties during the purification step when producing biodiesel.

2.3.2 **Acid catalysed transesterification**

When working with oils that have high free fatty acids (FFA) content, acid catalysts are often used as homogenous catalysts for biodiesel production. Moreover, when the acid value of the oils or fats is very high, one-step esterification pretreatment may not reduce the FFA efficiently, due to the high content of water produced during the reaction and thus, acidic catalysts, normally sulphuric acid, are used (Leung *et al*., 2010). Acid catalysed transesterification reactions have several advantages that include an insensitivity to the presence of FFA, making it suitable for use even in the transesterification of waste oil (Tan *et al*., 2013). Unfortunately this reaction requires high methanol to oil ratios that result in slow reaction rates and difficulties with glycerol recovery (Demirbas, 2009).

2.3.3 **Biological catalysed transesterification**

The biological route of transesterification involves the use of enzymes such as lipase for the production of biodiesel. Enzymatic transesterification was developed to overcome the challenges presented by acid and alkali transesterification, such as the presence of water and FFA that result in soap formation. The use of lipases for biodiesel production at an industrial scale has not yet been realised due to high enzyme costs, enzyme deactivation and slow reaction rates (Thanh *et al.*, 2012). Enzymes can be immobilized on a support to obtain a heterogeneous catalyst. However, the approach is only viable if the enzyme costs are reduced as in the case of the enzymes used in detergents, dairy products, textile, and leather processing.

2.4 **Glycerol structure and properties**

Glycerol is the main by-product of biodiesel production through transesterification with an alcohol. It is a highly reactive tri-alcohol containing three hydroxyl groups that is soluble in water, but insoluble in hydrocarbons (Ratmat *et al*., 2010). The presence of the hydroxyl group usually results in the formation of hydrogen networks within the molecules, thus giving the glycerol a viscosity of 10 cP to 25 cP and a boiling point of 290°C. The three hydrogen groups and high viscosity allows for the elasticity of glycerol, which makes it suitable for use as a precursor for many

products such as plastics, resins and lubricants (Da Silva *et al*., 2009). Glycerol is primarily used in the food and beverage, pharmaceutical and cosmetic industries for the production of a variety of chemical products (Johnson & Taconi, 2007). Some properties of glycerol are shown in Table 2.2.

Table 2.2: List of some glycerol properties (Ratmat *et al*., 2010)

Properties	**Values**
Chemical formula	$C_3H_8O_3$
Molecular weight	92.09 (kg/mol)
Physical appearance	Colourless liquid
Boiling point	290°C
Melting point	17.9°C
Viscosity	
At 100% purity	10 cP
At 50% purity	25 cP

Although glycerol is mainly produced through saponification, transesterification and the high pressure splitting of fats and water, it can also be synthesized from the oxidation and chlorination of propylene or by microbial fermentation (Lin, 2013).

Crude, purified and refined glycerol differ with regard to purity, moisture and soap content. The typical glycerol content for crude, purified and refined glycerine is 60% - 80%, 99.1% - 99.8% and 99.2% - 99.98% respectively (Ayoub & Abdullah, 2012). Crude glycerine production resulting from biodiesel production has attracted attention in recent years since it can yield valuable products. However, the glycerine contains impurities that include methanol, proteins, fats, ash, metals, water and sodium (Thompson & He, 2006), which make it difficult to be used for pharmaceutical, food and cosmetic purposes. The composition of crude glycerine may differ, depending on the makeup of the original feedstock as well as the reaction parameters used. The presence of impurities has made crude glycerine a low valued chemical that is not considered for industrial purposes (Quispe *et al*., 2013). Currently, most crude glycerine is burned or deposited into rivers (Anand & Saxena, 2012). Therefore, to prevent the waste of crude glycerine, developments of new,

economically feasible methods are needed to increase the value of crude glycerine to sustain the biodiesel sector.

2.4.1 **Purification of crude glycerine**

For crude glycerine to be used in the food and pharmaceutical sectors, a purity of 99.7% is required. Crude glycerine can be purified to various degrees by using single or multiple methods that include neutralization to adjust the pH, filtration, chemical additives and vacuum distillation (Quispe *et al*., 2013). Moreover, purification can be achieved by employing a combination of techniques, such as heating, evaporation, splitting, decantation, vacuum distillation and adsorption. The combination of these processes can yield glycerol from normal crude glycerine with a purity greater than 99.5%. Ismail *et al*. (2010) reported on the purification of crude glycerine for the preparation of a bio-lubricant by combining neutralization, microfiltration, and ion exchange technologies. This combination of techniques resulted in successful purification. Hajek and Skopal (2010) worked on the saponification, filtration, neutralization and phase separation to obtain a glycerol purity of 86wt%. Manosak *et al*. (2011) also purified crude glycerine by using polar solvent extraction, acidification and adsorption and obtained a glycerol purity of 95.7wt%.

After every purification process, the following technologies can be used to further enhance the purity of the glycerol: fractional distillation, ion exchange, adsorption, precipitation, extraction, crystallization and dialysis (Hunsom *et al.,* 2013). An example of the most common purification pathway would involve this sequential order: soap splitting, combined methanol/water removal, fractional distillation, ion exchange and adsorption. Distillation to separate methanol and water is found to be the most used and researched purification method, but it requires a high energy input (Tan *et al*., 2013). Aiken (2006) developed a novel process that gained popularity, which involves five steps to provide glycerol with a purity of 99.5%. The first step involves the reaction between a methyl ester and glycerol to produce methanol and glycerol, with the remaining methyl ester being converted to glycerol and methanol. The following step entails the separation of the oil layer by decanter where after the remaining glycerol is fed into a distillation column at 180°C and 5 mmHg - 200 mmHg. This is followed by the glycerol being passed through activated

carbon, an ion exchange resin and a moderate sieve to remove trace impurities and maintain colour (Aiken , 2006)

Cost is one of the main issues to be considered when a purification route for crude glycerine is chosen. Posada *et al*. (2011) estimated the cost of crude glycerine purification by a combination of neutralization, centrifugation, evaporation and column distillation that achieved a purity of up to 98wt%. During the purification process, methanol at 99wt% was recovered. The purification of crude glycerine can be economically assessed under two conditions: (1) methanol is considered as waste, or (2) methanol could be reused in the transesterification process .The lowest cost for glycerol purification was obtained for the second condition with an estimated cost of R1.98 per kg of glycerol. Posada *et al*. (2011) further indicated that the adsorption process is the least expensive process with a cost of R6.12 per litre of crude glycerine, producing a slightly coloured 87% pure glycerol. Nonetheless, when the processes are combined with chemical extraction by n-propanol and adsorption, the cost increases rapidly to R227.63 per litre of crude glycerine for an almost clear 99% glycerol. Thus, the cost for the purification of crude glycerine is mostly linked to the quality and purity of crude glycerine that need to be achieved.

2.5 **Production of value added products from crude glycerine**

Glycerol has been a promising precursor for the production of a wide variety of chemicals. Chemicals can be produced from glycerol by either chemical conversion or biological conversion. Chemical conversion uses a metallic catalyst and biological conversion utilises enzyme activities of a microorganism to produce a variety of chemicals. The catalytic conversion of glycerol provides for the production of several products, such as syngas and hydrogen, ethers, propanediol and polyols (Len & Luque, 2014). However, catalytic conversions have limitations that include a high operational cost, low product specificity, inability to utilise glycerol with high impurities, high temperature and pressure conditions (Yazdani & Gonzalez, 2007). The catalytic production of 1,3-propanediol over a catalyst, for instance, uses petroleum based ethylene oxide and acrolein (Kurosaka *et al.,* 2008), which make the reaction dangerous to the environment. Biological conversion involves the use of anaerobic or aerobic microorganisms for the production of fuels and reduced organic chemicals. The biological conversion of glycerol avoids some of the catalytic

conversion disadvantages. Nonetheless, biological conversion needs to improve on production yield and recovery, as these affect the profit and sustainability of the process (Dobson *et al*., 2012).

2.6 **Biological conversion of glycerol**

Clostridia, Klebsiella, Enterobacter and *Citrobacter* are some of the well-known bacteria genus that utilise glycerol via a reduction and oxidative pathway (Wilkens *et al*., 2012). Compared to other microbial carbon sources, glycerol has a higher degree of reduction and an ability to generate reduced nicotinamide adenine dinucleotide ($NADH^{+}$) at a higher rate than xylose or glucose (Li *et al.*, 2013). Mu *et al*. (2006) have worked on the production of ethanol and reported that crude glycerine produced more ethanol (11.9 g/L) than pure glycerol when fermented with *Klebsiella pneumonia.* The pathway for the biochemical conversion of glycerol to valuable chemicals is shown in Figure 2.2.

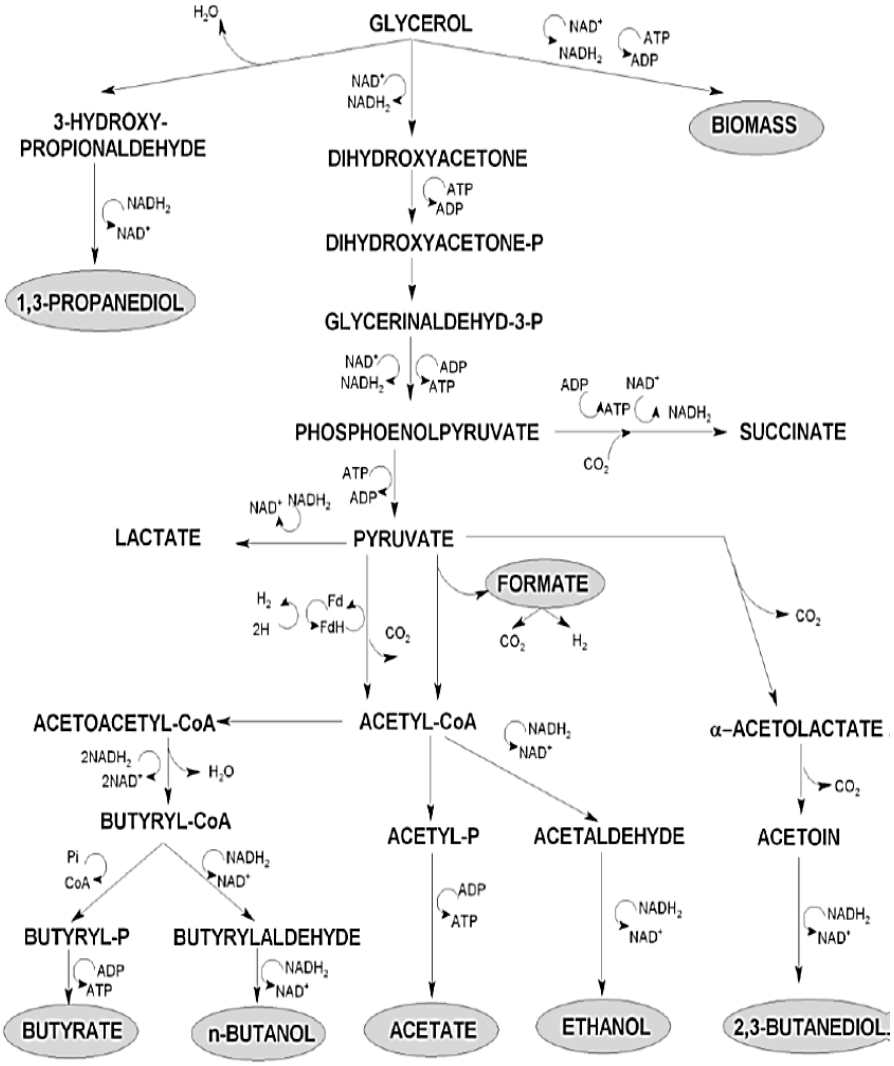

Figure 2.2: Microbial fermentation of glycerol (Leja *et al*., 2011).

The metabolism of glycerol with a microorganism commences with the transportation of glycerol into the cytoplasm by a glycerol facilitator (transporter protein) to be metabolized. During oxidation, glycerol is dehydrogenated to dihydroxyacetate (DHA) by a nicotinamide adenine dinucleotide(NAD)linked glycerol dehydrogenase (gly DH), where after it is phosphorylated to dihydroxyacetate phosphate (DHAP) by phosphoenolpyruvate (PEP) and adenosine triphosphate (ATP) dependent dihydroxyacetone phosphate kinase (DHAK). This is then oxidised further to pyruvate and enter the glycolysis pathway (Yadzani & Gonzalez, 2007). Pyruvate is utilised differently by different strains. Pyruvate is oxidised to acetyl-CoA or reduced to lactic acid (Dobson *et al.*, 2013). Acetyl-CoA form acetic acid and citric acid after acetate kinase and acetyl transferase, which yield ATP. In the meantime, the

presence of ethanol results in the reduction of Acetyl-CoA by acetaldehyde. Simultaneously, Acetyl-CoA is condensed to acetoacetate to form butanol or butyrate (Leja *et al.,* 2011).

During the reduction pathway, glycerol is firstly dehydrated to 3-hydroxypropionaldehyde (3HPH), which later undergoes reduction to 1,3-propanediol in which NAD is regenerated (Dobson *et al*., 2012). The reductive pathway involves a balance of NAD/$NADH_2$. Hydrogen (H_2) is produced through the re-oxidation of ferredoxin, which acts as an electron carrier for the formation of pyruvate to acetyl-CoA. The ferredoxin that is re-oxidized can also be achieved by proton transfer to NAD (P) via the activity of ferredoxin-NAD(P) reductase (Kubiak *et al.*, 2012). All the other products of glycerol catabolism, such as lactate, ethanol, butanol, H_2, and particularly butyrate, compete with 1,3-propanediol for reducing equivalents (NADH) during their synthesis.

2.6.1 *Clostridium* genus

Clostridia are rod-shaped, spore-forming Gram positive bacteria and are typically strict anaerobes. They can naturally produce butanol, acetone, ethanol, isopropanol and 1,3-propanediol. Moreover, *Clostridia* can produce chiral products, which are difficult to obtain through chemical synthesis and they also degrade a number of toxic chemicals. .The nutrient requirements for the growth of *Clostridia* are quite simple with no complex nitrogen sources that are generally required for good growth and solvent production. The non-pathogenic *Clostridia* have a large potential industrial application, since they are mostly utilised for the industrial production of solvents such as butanol, acetone, isopropanol (Ezeji *et al*., 2007), hydrogen and butyric acid (Zhang *et al*., 2012). Different types of enzymes involved in the degradation of starch and hemicellulose have been identified in a number of different strains. Cellulolytic *Clostridia,* such as *Clostridium thermocellum,* produce a multi-enzyme cellulase that is able to degrade cellulose, starch and hemicellulose (González-Pajuelo *et al*., 2006). There have been noticeable developments in the production of *Clostridial* toxins and spores that are being used in the treatment of human diseases. Amongst all *Clostridia* species, the best natural producer of 1,3-propanediol in terms of yield *C. butyricum* . Through a genetic engineering strategy, it was possible to introduce the NADH consuming 1,3-propanediol pathway from *C. butyricum* into *C. acetobutylicum* and obtain the mutant strain *C. acetobutylicum*

DG1 (Li *et al.*, 2013) that is able to grow on glycerol and produce 1,3-propanediol as the main fermentation end-product.

2.6.2 **Fermenting crude glycerine by *Clostridium* species**

The dissimilation of glycerol in microorganisms is strictly linked to the capacity of the microorganisms to synthesize the highly reduced products and 1,3-propanediol (Leja *et al*., 2011) . *Clostridium diolis (*formerly *Clostridium butyricum)* and *Klebsiella pneumonia* are the two well-known bacteria that utilise glycerol naturally and that can produce a variety of chemicals. *Clostridium diolis* is the most studied microorganism for the utilisation of glycerol, since it produces the desired chemicals at a high productivity and high yield (Vlysidis *et al.*, 2011). *Klebsiella pneumonia* is able to tolerate substrate impurities, which makes this organism suitable for the conversion of crude glycerine (Yang *et al.*, 2012). However, the *Klebsiella pneumonia* species are associated with pathogenicity that needs special precaution during use and cultivation.

Clostridium diolis (*C. diolis*) is a gram positive bacterium. The genus was named by Prazmowski in 1880. The characteristics that categorise this type of bacteria include anaerobic metabolism, spore forming, the inability to carry out a dissimilate reduction of sulphate and having a gram positive wall (Lee *et al.*, 2008). In the main, anaerobic fermentation has lower capital and operational costs with cheaper fermenters that require less operational energy than aerobic fermentation. Despite the high reducing nature of glycerol, it requires microorganisms that can metabolise it in the absence of an electron acceptor. In a study conducted by Wang *et al.* (2013), it was reported that *C. diolis* DSM 15410 can produce up to 70.3 g/L of 1,3-propanediol, with a yield of 0.68 mol/mol and a productivity of 1.5 g/L/h. *C. diolis* from the *clostridium* strains is able to naturally synthesise coenzyme B-12 dependent glycerol dehydratase (Leja *et al.*, 2010). This ability opens up economically feasible industrial operations, which mean when fermenting with *C. diolis*, the expensive vitamin B-12 will not be added to the growth media. The biological production of 1,3-propanediol is either vitamin B12 dependent or independent, which is determined by the vitamin B12 dependence characteristics of the glycerol dehydratase that catalyses glycerol dehydration to 3-HPA. Most characterized 1,3-propanediol producers are encoded for vitamin B12-dependent glycerol dehydratase, whereas the enzyme of *C. butyricum* was

characterized as vitamin B12-independent (Lee *et al.*, 2008) . The structure of the two types of glycerol dehydratases varies significantly; the B12-dependent enzyme is encoded by three genes, whereas the B12-independent enzyme is encoded by two genes.

There are four major enzymes involved in the metabolism of glycerol (Kaur *et al.,* 2012). The first is the enzyme vitamin B12-dependent glycerol dehydrogenase (GDHt) that removes a water molecule from glycerol to form 3-hydroxypropionaldehyde (3-HPA), which is then reduced to 1,3-propanediol by a second enzyme, NADH linked 1,3-propanediol oxide reductase. On the other hand, in the oxidative pathway glycerol is dehydrogenated to dihydroxy acetone (DHA) by a NAD+-linked glycerol dehydrogenase (GDH), then to dihydroxyacetone phosphate (DHAP) by an ATP-dependent dihydroxyacetone phosphate kinase (DHAK). Glycerol dehydratase is a coenzyme B12-dependent enzyme composed of three polypeptides that catalyse the free radical mediated conversion of glycerol to 3-HPA. Like any coenzyme, its function is to regulate or manipulate the metabolic reaction. Nakaruna & Whited (2003) explained the role of the B-12 coenzyme. During the normal catalytic cycle with glycerol, the coenzyme B-12 is sometimes reduced inactive (B12-inact). The B12-inact remains tightly bound to the dehydratase and catalysis ceases. An auxiliary enzyme, glycerol dehydratase reactivates the B-12 enzyme and form glycerol dehydratase that is free of cofactor (apoenzyme). The resultant apoenzyme rebinds coenzyme B-12 and glycerol and conversion to 3-HPA restarts.

2.6.3 **Effect of crude glycerine in fermentation**

The biological conversion of crude glycerine is affected by a number of factors, such as low yields, inhibition by both substrate and products formed, medium composition, production of different by-products and pathogenicity of some bacterial strains (Celinska, 2010). In the study by Colin *et al*. (2001), the authors explained how the effect of butyrate and acetate, which are the by-products formed during 1,3-propanediol production, affected the metabolism and growth of *Clostridium butyricum*. Moon *et al*. (2011) evaluated the composition of media nutrients for the production of butanol and 1,3-propanediol from the fermentation of glycerol by

Clostridium pasteurianum. Yeast extract, ammonium sulphate ($NH_4\ SO_4$) and iron sulphate ($FeSO_4$) are noteworthy additives that produce different amounts of butanol and 1,3-propanediol, indicating that the amount of nutrients in the medium influences the glycerol utilisation and the product formation.

As mentioned earlier, crude glycerine from biodiesel production contains a number of impurities which can inhibit the production yield. Chatzifragkou *et al. (*2010) analysed the effect of salts, fatty acids and methanol present in the biodiesel derived crude glycerine on the growth rate of *Clostridium butyricum*. In the study, the authors found that oleic acid was the main inhibitor for *C. butyricum,* since it is a mono-unsaturated acid with double bonds. FFA are also known inhibitors when using biodiesel derived crude glycerine as carbon source for the production of chemicals via a biochemical pathway, therefore, they need to be removed in order to achieve an effective utilisation of crude glycerine (Anand & Saxena, 2012**).** Thus, the presence of the impurities in crude glycerine has a very negative effect on the morphology and biochemical processes of bacterial cells and consequently lower concentrations of metabolites are obtained compared to pure glycerol (Wilkens *et al.,* 2012).

The fermentation of glycerol by *Clostridium pasteurianum* revealed that methanol and salts have no significant effect on the metabolism and growth of the bacteria (Venkataramanan *et al.,* 2012), but that fatty acids (linoleic acid) are strong inhibitors. These inhibitors are the challenges that need to be considered and provided for in the best possible way when using crude glycerine as a carbon source. On average, biodiesel-derived crude glycerine can contain methanol (0.27wt%), potassium (70 $mg.kg^{-1}$), water (0.05wt%), sodium (13.6 $mg.kg^{-1}$), non-glycerol organic matter (17.0wt%), and magnesium (1.9 $mg.kg^{-1}$). Another study showed no decrease in the yield of metabolite production, but the product yield when using crude glycerine was higher than when pure raw glycerol was used (Jun *et al*. ,2010). Samul *et al.* (2014) reported on the effect of the main impurities of crude glycerine on glycerol fermentation. Free fatty acids were shown to influence the metabolites and limit the formation of fermentation products. The authors found high salt concentrations that decreased the Van de Waal's forces on the lipid membrane, which cause swelling of the cell membrane and have a negative effect on biological processes. Alcohol influences cell membrane permeability, but the effect is linked to the concentration and carbon chain of the alcohol present.

2.6.4 **Removal of impurities for biological conversion**

The impurities present in crude glycerine can be reduced by using a number of techniques, which include autoclaving (removes a significant amount of methanol), precipitation by pH adjustment (converts soaps to free fatty acids) and the removal of sodium by crystallization via the co-addition of lime and phosphoric acid (Sarma *et al*., 2012). A number of studies have reported on the successful removal of impurities from biodiesel-derived crude glycerine. For example, Moon *et al*. (2010) investigated the impact of the acid pretreatment of crude glycerine on different *Klebsiella* and *Clostridium* strains for the production of 1,3-propanediol. *Klebsiella* strains were able to grow on crude glycerine, but *clostridium* strains were inhibited by impurities. Acid pretreatment improved the growth rate and minimised the fermentation time, however the origin of the feedstock of different microorganisms determines the utilisation of crude glycerine by that microorganism (Moon *et al*., 2010).

The effect of pretreatment was also evaluated on *Citrobacter freundi* by using a non-polar solvent wash (petroleum ether, heptane, hexane and octane) as pretreatment (Anand & Saxena, 2012). For glycerol derived from sunflower-based biodiesel, washing with petroleum ether produced the best results when compared to washing with other solvents. However, the effectiveness of the removal techniques and solvent is dependent on the feedstock used to produce the biodiesel from which the crude glycerine is obtained.

2.6.5 **Mechanism of ultrasound**

Ultrasound consists of a succession of sounds with a frequency above 20 kHz that is inaudible to the human ear. Ultrasound waves cause cavitation through the generation of gas bubbles in liquids that result in a localised change in pressure that causes a shock wave (Gogate & Kabadi, 2009). The ultrasound technique makes use of a sound wave that is transmitted through the physical medium by waves that then compress and stretch the molecular spacing of the medium through which it passes. While the ultrasound is passing through the medium, the average distance between the molecules will differ while they oscillate about the same position. However, when negative pressure occurs, the distance between the molecules will exceed the minimum molecular distance that holds the liquid intact. This will cause the liquid to break down and create cavitation bubbles. The bubbles will implode and

collapse, causing a shock wave that will propagate through the medium. During implosion, a high pressure and temperature are generated inside the bubble. Ultrasound can be divided into categories according to their frequencies. i.e. ultrasound with low frequency and high power that ranges from 20 kHz -100 kHz is mainly used for welding, cleaning and sonochemistry; and high frequency with low power ultrasounds that ranges from 2 MHz - 10 MHz and are mainly used in medical images and chemical analysis (Rokhina *et al.*, 2009). The choice of frequency mainly depends on the process being considered, the equipment used and the operational power. For instance, ultrasound baths (indirect ultrasound) usually operate at 40 KHz, whereas an ultrasound horn achieves lower frequencies of 20 kHz. This ultrasound frequency assists in the collapse of the bubbles by driving the bubble into resonance.

2.6.5.1 Effect of ultrasound on microbial growth and fermentation

Efficient techniques are required to increase the economic viability of industrial processes by reducing total production costs and improve product quality. Ultrasound can be used to intensify chemical and physiological processes at low energy intensities. This is true, since the shock wave and the mechanical shear produced by the ultrasonic wave in the broth increase the cell pore size, decrease the mass transfer due to gaseous transportation and increase the cellular uptake (Chisti, 2003). This enhancescell permeability which increases the uptake of nutrients by microorganisms. Ultrasound can be used to inactivate or activate microbial growth. The use of low power ultrasound is found to increase microbial growth and high power ultrasound disrupts the cell and can be microbicidal (Pitt & Ross, 2003).

Ultrasound is also used in the extraction of enzymes that can then be applied in various processes**.** Apar & Ozbek (2006) discussed the effect of ultrasound on the hydrolysis of corn, rice and wheat starch using alpha-amylase enzymes produced by *Bacillus* for the production of bioethanol. Sonication of these products showed positive results, but control samples showed high starch hydrolysis and low enzyme activity. Sulaiman *et al*. (2011) used ultrasound to enhance ethanol production (up to 20%) from lactose by fermentation with *Kluyveromyces marxianus*. The ultrasound

enhanced both intracellular and extracellular levels of the enzyme β-galacosidase and the viability of the cell remained lower than 70%.

Khanna *et al. (*2012) worked on the production of 1,3-propanediol and ethanol from the ultrasound fermentation of crude glycerine with *Clostridium pasteurianum*. The results indicated that ultrasound enhanced the production yield of both ethanol and 1,3-propanediol. These authors also found that ultrasound affects the physical nature of the microorganism more than it influences the glycerol metabolism. Another study investigated the viability of yeast cells at a low frequency of 2 MHz. The results showed that ultrasound treatment changes the morphology and agglomeration of the cell, but no loss in viability was observed (Radel *et al.,* 2000). It became evident that ultrasound is an effective alternative method for the activation of cells at a reduced energy input. Moreover, the method possesses great potential for the enhancement of fermentation and microbial growth.

2.7 **Possible products from biological conversion of glycerol**

Many biological conversion studies on glycerol have suggested a number of products obtained during the conversion process, some of which can be used as chemicals. The fermentation of glycerol by *Clostridium* results in the formation of 1,3-propanediol as well as other secondary products such as lactic acid, acetic acid, butyric acid, ethanol, CO_2 and H_2; but yields and product distribution depend on the strain of choice and growth conditions (Clomburg & Gonzalez, 2013). The theoretical maximum product yield from the anaerobic fermentation of glycerol uptake is observed when acetate is formed as a by-product (Saxena *et al.,* 2009). The maximum reported theoretical yield for 1,3-propanediol was 0.72 $mol.mol^{-1}$ glycerol. This yield was calculated for a culture in the absence of H_2 and butyric acid formation (Gonzalez-Pajuelo *et al.,* 2006b).

2.7.1 **Ethanol**

Ethanol is an alcohol that is mostly produced from fermenting yeasts, such as *Saccharomyces cerevisiae,* from sugars (Anton *et al*., 2007). Ethanol is a bio-based alternative fuel for petrol. It can be obtained from the fermentation of glycerol at high productivity and yields. Yazdani and Gonzalez (2007) found that the production of ethanol from sugars is more expensive than using glycerol. The bacteria from the

Enterobacteriaceae and *Clostridiaceae* families can produce ethanol from glycerol, but as the second product of the fermentation process.

2.7.2 **Butanol**

Butanol, an important precursor for the synthesis of products such as paints, plastics and polymers, has been produced via the ABE (acetone, butanol and ethanol) process using microorganisms and via glycerol fermentation (Jang *et al*., 2012). Butanol has gained interest as a potential alternative fuel to fossil fuel that can supplement petrol, because butanol has characteristics similar to those of petrol, such as octane number, vapour pressure, higher heating value and higher miscibility than ethanol (Khanna *et al.*, 2013).

2.7.3 **Succinic acid**

Succinic acid is a di-carboxylic acid that is used as precursor for the production of chemicals such as ethylenediamine disuccinate, adipic acid, diethyl succinate and 1,4-butanediol (Mckinlay *et al.*, 2007) . Succinic acid is mainly produced from petroleum butane via the reduction of maleic anhydride (Vlysidis *et al.*, 2011). The biological production of succinic acid from crude glycerine has gained attention worldwide due to the shortage of petrochemicals. *Klebsiella* and *clostridium* can produce succinic acid as by-product during 1,3-propanediol production. The main route followed for the biological production of succinic acid is the redoxbalanced route with microorganisms such as *Anaerobiospirillum succiniciproducens, Actinobacillus succinogenes, Corynebactecterium glutamicum, E. coli* and *Mannhheimia succiniciproducens* (Blankschein *et al.*, 2010).

2.7.4 **Lactic acid**

Lactic acid is a carboxylic acid that is mostly used in the polymer, pharmaceutical and food industries (Vodnar *et al.*, 2013). Lactic acid can be produced via chemical or biochemical routes. The biochemical routes have more advantages, for example using a low production temperature, low energy use, and improved purity of the product (Choubisa *et al.*, 2012). The demand for lactic acid is growing annually with a growth rate of 5-8% (Abdel-Rahman *et al.*, 2013); therefore the availability of cheap crude glycerine will benefit the microbial fermentation to yield lactic acid.

2.7.5 **Citric acid**

Citric acid is a weak organic acid that is only produced via biochemical processes. Commercially, citric acid is mostly produced by the fermentation of molasses using *A. niger* (Maryam *et al.*, 2011). Citric acid is in great demand with 60% used in the food industry and 10% used in pharmaceuticals. The global production of citric acid is increasing with approximately 1.7 million tons annually (Rywinska *et al.*, 2011). However, glycerol is also an alternative feedstock for citric acid production. The growth of microorganisms and yield of citric acid are affected by crude glycerine impurities, however, repeated batch fermentations are being investigated to improve yields (Almeida *et al.*, 2011).

2.7.6 **Acetic Acid**

Acetic acid is a carboxyl acid that is used in the food and pharmaceutical industries. It is produced via chemical and biological pathways. Chemical pathways entail oxidation and carboxylation reactions, e.g. methanol oxidation and acetaldehyde oxidation (Cheryan, 2009). The biological production of acetic acid is mainly linked to the production of vinegar. The volume of vinegar is composed of 3-5% of acetic acid, in other words vinegar is a dilute acetic acid that is produced by fermentation. The reaction entails the fermentation of ethanol by acetic acid bacteria (AAB) to produce an organic solvent and acetic acid (Mas et al., 2014). From literature, AAB are known for oxidizing alcohols and sugars to produce an organic solvent. *Gluconobacter spp* are able to use glycerol and produce dihydroxyacetate (Segun & Karabiyikli, 2011).

2.7.7 **1,3-Propanediol**

1,3-Propanediol is a colourless viscous liquid that is non-flammable, has a low toxicity, and is miscible in alcohols, water and ethers (Kaur *et al*., 2012). 1,3-Propanediol can be used for the synthesis of heterocyclic compounds and intermediates for the production of polyesters, for instance polymethylene terephthalate (PTT) that has superior mechanical and chemical characteristics when compared to polyethylene terephthalate (PET) (Azbar *et al.*, 2010). Furthermore, Drozdzynska *et al.* (2011) discussed different methods for the synthesis of 1,3-propanediol from petroleum derived acrolein and ethylene ether. However, the method is not environmentally friendly and produces hazardous chemicals. A

number of microorganisms are able to utilise glycerol and produce 1,3-propanediol as main product. Thus, the synthesis of 1,3-propanediol via biochemical route is preferred as it produces less hazardous by-products and is renewable.

2.8 **Concluding remarks**

From the literature review performed in this chapter, the results of the discussed literature can be summarised as follows:

- The high demand for biodiesel results in the production of large quantities of low value crude glycerine.
- Both catalytic and biological conversion offer promising opportunities for the purification of biodiesel derived crude glycerine. However, biological routes have advantages over catalytic conversion routes.
- Compared to other fermentable carbon sources such as glucose and xylose, glycerol has a high prevalence of reduced carbon atoms that result in the production of reduced products.
- *Clostridium diolis* is a promising microorganism for the utilization of crude glycerine and produces a variety of products.
- The presence of impurities results in the limitation of glycerol utilisation by microorganisms, but research and experiments are continually performed to improve glycerol utilisation.
- The production of value-added products from a low value waste product is also an advantage to renewable energy at large.
- The biological conversion of biodiesel based crude glycerine is showing potential for industrial purposes, which will in turn add value to the biodiesel and the biofuel industry.
- Chapter 3 will discuss the methodology used in this study.

2.9 **References**

Abbaszaadeh, A., Ghobadion, B., Omidkhah. M.R. & Najafi, G. 2012. Current biodiesel production technologies: A comparative review. *Energy Conversion and Management*, 63:138-148.

Abdel-Rahman, M.A., Tashiro, Y. & Sononoto, K. 2013. Recent advances in lactic acid production by microbial fermentation processes. *Biotechnology Advances*, 32:877-902.

Abdullah, A.Z. & Ayoub, M. 2012. Critical review on the current scenario and significance of crude glycerine resulting from biodiesel industry towards more sustainable energy industry. *Renewable and Sustainable Energy Review*, 16:2671-2686.

Aiken, J. 2006. Purification of glycerine. (Patent: US 7126032).

Ajanovic, A. & Hass, R. 2010. Economic challenges for the future relence of biofuels in transport in Eu countries. *Energy*, 35:3340-3348.

Almeida, J.R.M., Favaro, L.C.L. & Quiriro, B.F. 2012. Biodiesel biorefinary: opportunities and challenges for microbial production of fuels and chemicals from glycerol waste. *Biotechnology for Biofuels*, 5:1-16.

Anand, P. & Sanexa, R.J. 2012. A comparative study of solvent-assisted pretreatment of biodiesel derived glycerol on growth and 1,3-propanediol production from *Citrobacter freundii*. *New biotechnology*, 29:199-205.

Atadashi, I.M., Aroua, M.K., Abdul-Aziz, A.R. & Sulaiman, N.M.N. 2013. The effects of catalyst in biodiesel production: A review. *Journal of Industrial and Engineering Chemistry*, 19:14-26.

Azbar, N., Gungormuster, M., Gonen, C. & Ozdemir, G. 2010. 1,3-propanediol production potential of *Clostridium saccharobutylicum* NRRL B-643. *New Biotechnology*, 27:782-788.

Atabani, A.E., Silitonga, A.S., Badruddin, I.A., Masjuku, H.H. & Mekilef, S.A. 2012. A comprehensive review on biodiesel as an alternative energy resource and its characteristics. *Renewable and Sustainable Energy Reviews*, 16:2070-2093.

Aworanti, O.A., Agarry, S.E. & Ajani, A.O. 2013. Statistical optimisation of process variables for biodiesel production from waste cooking oil using heterogeneous base catalyst. *British biotechnology Journal*, 3(2):116-132.

Anton, D., Zverlow, V.V. & Schwarz, W.H. 2007. Biofuels from microbes. *Applied* Microbiol*ogical Biotechnology*, 77:23-35.

Apar, D.K. & Özbek, B. 2004. Amylase inactivation by temperature during starch hydrolysis. *Process Biochemistry,* 39:1137-1144.

Balat, M. & Balat, H. 2010. Progress in biodiesel processing. *Applied energy*, 87:1815-1835.

Blankscheim, M.D., Clomburg, J.M. & Gonzalez, R. 2010. Metabolic engineering of *Escherichia coli* for the production of succinate from glycerol. *Metabolic Engineering*, 12: 409-419.

Bankovic-Ilic, I.V., Syamekovic, O.S. & Veljkovis, V.B. 2012. Biodiesel production from oedile plat also. *Renewable and Sustainable Energy vivoiue*, 16:3

Clomburg, J.M. & Gonzalez, R. 2013. Anaerobic fermentation of glycerol: a platform for renewable fuels and chemicals. *Trends In Biotechnology*, 31:20-28

Colin, T., Bories, A. & Moulin, G. 2000. Inhibition of *Clostridium butyricum* by 1,3-propanediol and diols during glycerol fermentation. *Applied Microbiological Biotechnology*, 54:201-205.

Chisti, Y. 2003. Sonobioreactors: using ultrasound for enhanced microbial productivity. *Trends in biotechnology*, 21(2):89-93.

Choubisa,B., Palele, H., Palele, M. & Dholokiya, B. 2012. Microbial production of lactic acid by using crude glycerine from biodiesel. *Journal of Microbiology and Biotechnology Research*, 2(1):90-93.

Celinska, E. 2010. Debottlenecking the 1,3-propanediol pathway by metabolic engineering. *Biotechnology* Advances, 28:519-530.

Chatzifragkou, A., Dictz, D., Komiatis, M., Zeng, A. & Papani-Kollou, S. 2010. Effect of biodiesel-derived waste glycerol impurities on biomass and 1,3-propanediol production by *Clostridium butricum* VPI 1718. *Biotechnology and Bioengineering*, 107:76-84.

Cheryan, M. 2009. Acetic acid production. *Applied Microbiology Industry*, 2:144-149.

Choubisa, B., Palel, M. & Dholakiya, B. 2012. Microbial production of lactic acid by using crude glycerine from biodiesel. *Journal of Microbiological Biotechnology Research*, 2(1):90-93.

Demirbas, A. 2008. The sustainability of renewables. *Energy Sources*, Part 1, 30:1114-1119.

Dermibas, A. 2009. Progress and recent trends in biodiesel fuel. *Energy Conversions and Management*, 50:14-34.

Dermibas, A. 2007. Importance of biodiesel as transport fuel. *Energy Policy*, 35:4661-4670.

Dobson, R., Gray, V. & Rumbold, K. 2012. Microbial utilization of crude glycerine for the production of value-added products. *J. Ind Microbial biotechnol*., 39:217-226.

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

Drozdzynska, A., Leja, K. & Czaczyk, K. 2011. Biotechnological production of 1,3-propanediol from crude glycerine. *Journal of biotechnology, Computational biology and bionanotechnology*, 92(1):92-100.

Ezeji, T.C.L., Qureshi, N. & Blaschek, H.P. 2007. Bio production of butanol from biomass: from genes to bioreactors. *Curr Opin Biotechnol*, 18:220-227.

Gondzalez-pajuelo, M., Menial-Salles, I., Mandes, F., Soucaille, P. & Vasconcelos, I. 2006. Microbial conversion of glycerol to 1,3-propanediol: Physiological comparison of a natural produced, *Clostridium nutyricum* VP13266 and an engineered strain *Clostridium acetonutylicum* DG1. *Applied and Environmental Microbial*, 72(1):96-101.

Gogate, P.R. & Kabadi, A. 2009. A review of application of cavitations in biochemical engineering/biotechnology. *Biochemical Engineering Journal*, 449(1):60-72.

Hajek, M. & Skopal, F. 2010. Treatment of glycerol phase formed by biodiesel production. *Bioresource Technology*, 101:3242-3245

Hunsom, M., Saila, P., Chaiyakom, P. & Kositran, W. 2013. Comparison and combination of solvent extraction and adsorption for crude glycerine enrichment. *Journal of Renewable Energy Research*, 3(2):364-374.

Ismail, M., Yarmo, M.A., Jahim, J.M. & Salimon, J. 2010. Purification of crude glycerine from transesterification RBD palm oil over homogeneous and

heterogeneous catalyst for biolubricant preparation. *Journal of Applied Science*, 10:2590-2595.

Jang, Y., Malaviya, A., Cho, C., Lee, J. & Lee, S.Y. 2012. Butanol production from renewable biomass by clostridia. *Bioresource Technology*, 123:653-663.

Johnson, D.T. & Taconi, K.A. 2007. The glycerine glut: options for the value-added conversion of crude glycerine resulting from biodiesel production. *Environmental progress*.

Jun, S.A., Moon, C., Kang, C.H., Kong, S.W., Sang, B.I. & Um, Y. 2009. Microbial fed-batch production of 1,3-propanediol using raw glycerol with suspended and immobilized *Klebsiella pneumoniae*. *Applied Biochemistry and Biotechnology*, 161(1-8):491-501.

Khanna, S., Jaiswal, S., Goyal, A. & Moholkar, V.S. 2012. Ultrasound enhancement of bioconversion of glycerol by *Clostridium pasterianun*: A mechanistic investigation. *Chemical Engineering Journal,* 416-425.

Khanna, S., Goyd, A. & Molholkar, V.S. 2013. Production of nbutanol from biodiesel derived crude glycerine using *Clostridium pasteurianum* immobilized on Armberlite. *Fuel*, 112:557-5561

Kaur, G., Srivastava, A.K. & Chad, S. 2012. Advances in biotechnological production of 1,3-propanediol. *Biochemical Engineering Journal*, 64:106-11.

Kubiak, P., Leja, K., Myszka, K., Celinska, E., Spychola, M., Szymanowska-Pawolowska, D., Czaczyk, K. & Grajek, W. 2012. Physiological predisposition of various *Clostridium* species to synthesize 1,3-propanediol from glycerol. *Process Biochemistry*, 47:1308-1319.

Kurosaka, T., Maruyama, H., Naribayashi, I. & Yoshiyuki, S. 2008. Production of 1,3-Propanediol by hydrogenolysis of glycerol catalyzed by Pt/WO3/ZrO2. *Catalysis Communication*, 9(6):1360-1363.

Len, C. & Luque, K. 2014. Continuous flow transfer of glycerol to valuable products: An overview. *Sustainable thermical process*, 2:1-10.

Leja, K., Drozdzynska, A. & Czaczyk, K. 2011. Biotechnological production of 1,3-propanediol from crude glycerine. *Journal of biotechnology, computational biology and bionanotechnology*, 92(10):92-100.

Li, C., Lesnik, K.L. & Liu, H. 2013. Microbial conversion of waste glycerol from biodiesel production into value added products. *Energies*, 6:4739-4768.

Li, C. 2013. Catalytic valorisation of glycerol to hydrogen and syngas. *International Journal of hydrogen Energy*, 38:2678-2700.

Lee, S.Y., Park, J.H., Jang, S.H., Nielsen, L.K., Kim, J. & Jung, K.S. 2008. Fermentative butanol production by clostridia. *Biotechnology and Bioengineering*, 101:209-228.

Leja, K., Czaczyk, K. & Myszka, K. 2011. Biotechnological synthesis of 1,3-propanediol using *Clostridium ssp*. *Afr J Biotechnol*, 10:11093-11101.

Leung, D.Y.C., Wu, X. & Leung, M.K.H. 2010. A review on biodiesel production using catalysed transesterification. *Applied Energy*, 57:1083-1095.

Mason, T.J., Panimnyk, L. & Lorimer, J.P. 1996. The uses of ultrasound in food technology. *Ultrasound Sonochemist*, 3:5253-5260.

Mas, A., Torija, M.J., Garcia-Parrilla, M.C. & Troncoso, A.M. 2014. Acetic acid bacteria and the production and quality of wine vinegar. *The Science World Journal*, 1:1-6.

Madyira, D.M., Nkomo, Z. & Akinlabi, E.T. 2012. Characterizing sunflower oil biodiesel blends as alternatives to fossil diesel. *Proceedings of the World Congress on Engineering*, 3:1-6.

Marchetti, J.M., Miguel, V.U. & Errazu, A.F. 2007. Possible methods for biodiesel production. *Renewable and Sustainable Energy Revie*ws, 11:1300-1311.

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

Mathur, Y., Poonia, M.P. & Jethoo, A.S. 2011. Economics, formulation techniques and properties of biodiesel: A review. *Universal Journal of Environmental Research and Technology*, 1(2):124-134.

Moon, C., Lee, C.H., Sang, B. & Um, Y. 2011. Optimization of medium comparisation forming butanol and 1,-propanediol production from glycerol by *clostridium pasteurianum*. *Bioresource Technology*, 102:10561-10568.

Meher, L.C., Sagar, V. & Naik, S.N. 2006. Technical aspects of biodiesel production by transesterification: A review. *Renewables and Sustainable Energy Review*, 10:248-268.

Mu, Y., Teng, H., Zhang, D., Wang, W. & Xiu, Z. 2006. Microbial production of 1,3-propanediol from using crude glycerine from biodiesel preparations. *Biotechnology letter*, 28:1755-1759.

Mckinlay, J.B., Veiile, C. & Zeikus, J.G. 2007. Prospects for a bio-based succinate industry. Apply Microbiol Biotechnol, 76:727-740.

Maryam, M., Iraj, N. & Giti, E. 2011. A comparative study of two *Yarrowia lipolytica* strains for optimum citric acid production. *Research Journal of Microbiology*, 6(6):568-574.

Nigam, P.S. & Singh, A. 2011. Production of liquid biofuels from renewable resources. *Progress in Energy and Combustion Science*, 37:52-68.

Nakamura, C.E. & Whited, G.M. 2003. Metabolic engineering for the microbial production of 1,3-propanediol. *Current Options in biotechnology*, 14:454-459.

Posada, J. A., Higuita, J.C. & Cardona, C.A. 2011. Optimization on the use of crude glycerol from the biodiesel to obtain poly-3-hydroxybutyrate. *World Renewable Energy Congress*, 8-13.

Pitt, W.G. & Ross, S.A. 2003. Ultrasound increases the rate of bacterial cell. *Biotechnology Prog*, 19(3):1038-1044.

Quispe, C.A.G., Coronado, C. Jr. & Carvalho, A.J. Jr. 2012. Glycerol: production, consumption, prices, characterisation and new trends in consumption. *Renewable and Sustainable Energy Reviews*, 27:475-493.

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

Rokhina, E.V., Lens, P. & Virtutyte, J. 2009. Low frequency ultrasound in iotechnology: State of the art. *Trends in biotechnology*, 27(5):298-306.

Rywinska, A., Juszczy, K.P., Wojtatowicz, M. & Rymowics, W. 2011. Chemostat study of citric acid production from glycerol by *Yarromia lipolytica*. *Journal of Biotechnology*, 152:54-57.

Radel, S., McLoughlin, A.J. & Gheradini, L. 200. Viability of yeast cells in well controlled propagating and standing ultrasonic plane waves. *Ultrasonics*, 38:633-637.

Sarma, S.J., Brar, S.K., Sydney, E.B., Bihan, Y.L., Buelana, G. & Soccol, C.R. 2012. Microbial hydrogen production by bioconversion of crude glycerine: A review. *International Journal of Hydrogen*, 37:6473-6490.

Satyarthi, J.K., Chiranjeevi, T., Gokak, D.T. & Viswanatha, P.S. 2013. An overview of catalytic conversion of vegetable oils/fats into middle distillates. *Catalytic Science and Technology*, 3:70-80.

Samul, D., Leja, K. & Grajek, W. 2014. Impurities of crude glycerine and their effect on metabolite production. *Ann Microbiol*, 64:891-898.

Shereena, K.M. & Thangaraj, T. 2009. Biodiesel: an alternative fuel produced from vegetable oils by transesterification. *Electronics Journal of biology*, 3(3):67-74.

Sulaiman, A.Z., Ajt, A., Yunus, R.M. & Chisti, Y. 2011. Ultrasound-assisted fermentation enhances bioethanol productivity. *Biochemical Engineering Journal*, 54:141-150.

Thanh, L.T., Okitsu, K., Boi van, L. & Meda, Y.M. 2012. Catalytic technologies for biodiesel fuel production and utilisation of glycerines: A review. 2:191-222.

Tan, H.W., Abdul-Aziz, A.R. & Aroua, M.K. 2013. Glycerol production and its application as a raw material: A review. *Renewable and Sustainable Energy Reviews*, 27:118-127.

Thompson, J.C. & He, B.B. 2006. Characterisation of crude glycerine from biodiesel production from multiple feedstocks. *Applied Engineering in Agriculture*, 22:261-265.

Vlysidis, A., Binns, S.M., Webb, C. & Theodoropoulus, C. 2011. Glycerol utilization for the production of chemicals: Conversion to succinic acid, a combined study. *Biochemical Engineering Journal*, 58(59)1-11.

Vodnar, D.C., Dulf, F.C., Pop, O.L. & Socaciu, C. 2013. L (+)- Lactic acid production by pellet from *Rhizopus Oryzopus* NRRD 395 on biodiesel crude glycerine. *Microbial Cell Factories*, 12(92):1-9.

Venkataramanan, K.P., Bootman, J.J., Kurniawan, Y., Tanocka, B., Othum, G.D. & Scholz, C. 2012. Impact of impurities in biodiesel derived glycerol on the

fermentation of *clostridium pasteurianum* ATCC 6013. *Appy Microbiol Biotechnol*, 93:1325-1335.

Wang, Y., Tao, F., Tang, H. & Xu, P. 2013. Genome sequence of *Clostridium diolis* strain DSM 15410, a promising natural producer of 1,3-propanediol. *Journal ASM org*, 1:1-2.

Wilkens, E., Ringel, A.K., Hortig, D., Wilke, T. & Vorlop, K. 2012. High level production of 1,3-propanediol from crude glycerine by *Clostridium utyricum* AKR 102a. *Appl Microiol biotechnol*, 93:1053-1063.

Fangxia Yang1,2, Milford A Hanna2* & Runcang Sun B 2012. Value-added uses for crude glycerol – a byproduct of biodiesel production. *Biotechnology for Biofuels,* 5(13):1-10.

Yazdani, S.S. & Gondzelez, R. 2007. Anaerobic fermentation of glycerol: A path of economic viability for biofuels industries. *Current options in biotechnology*, 18(3):213-219.

Yususf, N.N.A.N., Kamarudin, S.K. & Yaakub, Z. 2011. Overviews of the current trend in biodiesel production. *Energy Conversion and Management*, 52:2741-2751.

Zhang, J. & Zhang, W. 2012. Controversies, development and trend of biofuel Industry in the world. *Environmental Sceptics and Critics*, 1(3):48-55.

CHAPTER 3

3 Experimental

This chapter contains the design and structure of the experimental procedure followed for the biological conversion of glycerol to value-added products. The preparation and the wash of crude glycerine is described in section 3.2. The characteristics of the glycerol sources that were used in the experiments, i.e. pure glycerol, washed-crude glycerine or raw crude glycerine are provided in section 3.3. Bacterial growth and maintenance techniques are presented in section 3.4 and methods used to analyse liquid products from fermentation are described in section 3.7.

3.1 Source of Crude glycerine

The crude glycerine used in this study was generated as a by-product of biodiesel production produced in the Bioenergy research group laboratory at the North-West University, Potchefstroom Campus. Biodiesel was produced from the transesterification of sunflower oil with methanol in a ratio of 1:6 (oil: methanol) using 0.5% (w/v) KOH as a catalyst. The transesterification reaction was conducted in a 200 L stainless steel bioreactor with a monitored temperature of 60°C. The KOH was added to the methanol and stirred with an electronic stirrer until the catalyst was dissolved. The mixture was then added to sunflower oil at 60°C and the reaction was allowed to continue for 90 minutes. After the completion of the reaction, the reaction mixture was left overnight to separate into two phases. Crude glycerine (approximately 18 L) was collected from the bottom phase and stored in a sealed plastic container at room temperature. All experiments were conducted on the same batch.

3.2 Wash of crude glycerine by petroleum ether

The crude glycerine obtained from the biodiesel production was washed with petroleum ether, a non-polar solvent to remove impurities (see Figure 3-1). The method was adapted from Anand and Saxena (2012). The crude glycerine was mixed with petroleum ether in a volume ratio of 1:1 and mixed at room temperature for 3 hours at an agitation speed of 200 rpm. The mixture (crude glycerine and petroleum ether) was then left to separate in a separation funnel. The bottom layer,

which is the washed crude glycerine layer, was separated from the petroleum ether layer (top layer) by decantation.

After separation, the washed crude glycerine (bottom layer) was centrifuged at 4000 rpm for 2 minutes, separating it into two distinct phases; (washed crude glycerine at the bottom and the remaining petroleum ether with other impurities at the top).

The washed crude glycerine was washed three times with the same solvent; the washing continued until the solvent was clear in colour. After the third wash, the bottom phase (washed crude glycerine) was further diluted by distilled water in a volume ratio of 1:1. The mixture was placed in a shaker until a white participate formed, after which the mixture was filtered by ACE 2976 filter paper. The filtered washed crude glycerine was dried in an oven at 95°C for 12 hours to evaporate the free water.

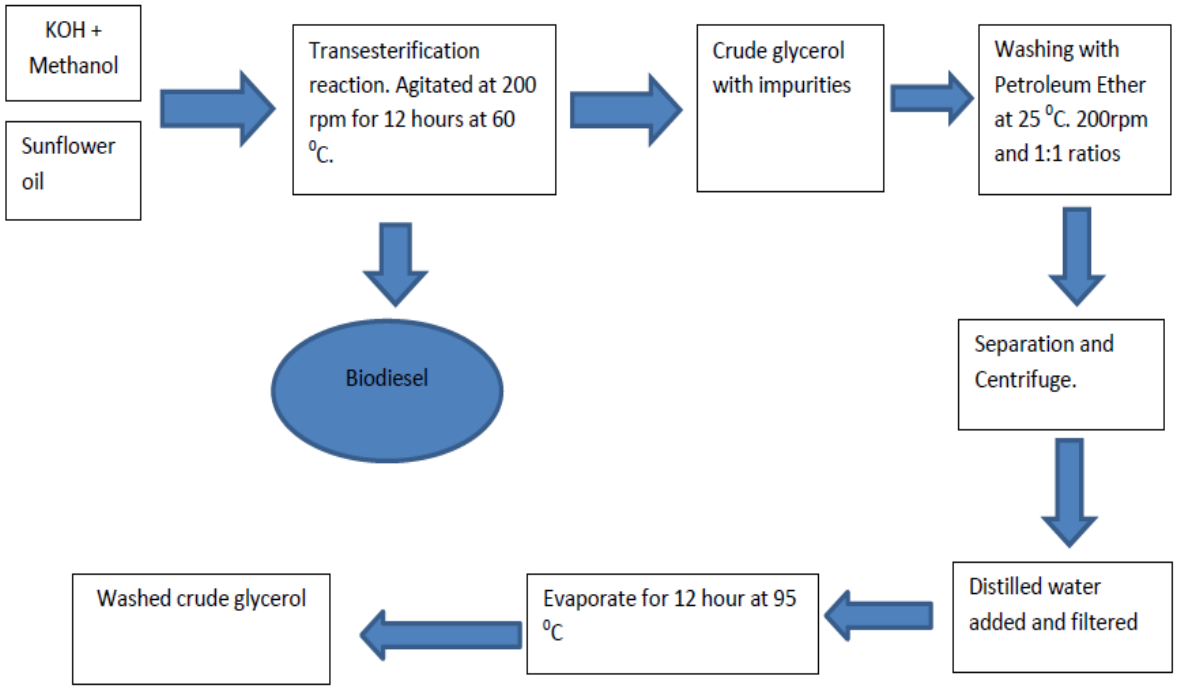

Figure 3.1 Flow diagram of the crude glycerine wash by petroleum ether.

3.3 **Physical analysis**

All glycerol samples (pure glycerol, crude glycerine and washed crude glycerine) were monitored stored at room temperature of 23±1°C prior any experimental setup with the method adopted from (Hu *et al*.,2012). Physical analysis was conducted for each batch of the experiments and all physical analysis was conducted in duplicates.

3.3.1 **Viscosity**

The viscosity of the glycerol samples was determined at 23±1°C by using a Thermo scientific viscometer, Lasec, South Africa with a sample adapter. The method was set to presents the maximum (100% torque) viscosity attainable using the viscosity rages from0.5 mPas – 10^5 mPas (cP) thermo. This value is referred to as full scale range. The allowable error for the viscosity measurement is ± 1% of full scale range.

3.3.2 **pH**

The pH was measured with Metrohm instruments 692 pH/Ion meters that was calibrated with buffers at 4.0 and 7.0 at room temperature (23±1°C). For pH determination, glycerol samples of 1.00 ± 0.1 g were dissolved in 50 ml of deionised water.

3.3.3 **Density**

The density of the glycerol samples was determined gravimetrically by measuring the weight (g) and the volume (ml) of the sample at room temperature (23±1°C).

3.4 **Microorganism and culture conditions**

3.4.1 **Microorganism**

Clostridium diolis (*C.diolis*) DSM 1540 was obtained from the German Collection of Microorganisms culture bank, as freeze dried in a sterile environment. The culture was rehydrated for 30 minutes with 0.5 ml of the Reinforced Clostridium Medium (RCM). The content was mixed gently with an inoculation loop and transferred to a test tube containing 5 mL of RCM. Approximately 100 μl of the mixture was streaked onto an agar plate to prepare a stub culture in an anaerobic chamber containing Quantum-biotech anaerobic sachets. Colonies were observed within 72 hours of growth at 37°C in an anaerobic chamber (see Figure 3.2). The short term storage of the cultures was done at 4°C and the long term storage of the microorganisms was done at -80°C by freezing it with 15% (v/v) reagent grade glycerol in distilled water. Colonies were sub-cultured every month.

Figure 3.2: *Clostridium diolis* colonies grown at 37°C for 72 hours in an anaerobic chamber.

3.4.2 **Reinforced Clostridium Media (RCM)**

The reinforced Clostridium media (RCM) used in this study was adapted from Khanna *et al.* (2012). The media contained the following components per 1 litre (L):

Amount	Chemical
10 g	Beef Extract
10 g	Peptone
5 g	Sodium Chloride
5 g	Dextrose
3 g	Yeast Extract
3 g	Sodium Acetate
1 g	Soluble Starch
0.5 g	Cysteine-HCl
0.5 g	Agar

The final pH of the media was 6.8±0.2 at 25°C. The agar medium contained the same components than the growth medium with 13.5 g of agar.

3.4.3 **Fermentation medium**

The fermentation of glycerol was carried out in fermentation media containing the following per litre (L): Growth media components and culture conditions were adapted from Kaur *et al.* (2012) and Khanna *et al.* (2012).

Amount	Chemical
3.21 g	KH_2PO4
2.75 g	K_2HPO4
2 g	$CaCO_3$
0.2 g	$MgSO_4 \cdot 7H_2O$
0.02 g	$CaCl_2 \cdot 2H_2O$
0.005 g	$FeSO_4 \cdot 7H_2O$
2g	$(NH_4)_2SO_4$
2 g	yeast extract
2 mL	SL7 trace element solution
The composition of SL7 as prepared as follows :	
0.1 g	$MnCl_2 \cdot 4H_2O$
0.07 g	$ZnCl_2$
0.06 g	H_3BO_3
0.04 g	$CoCl_2 \cdot 2H_2O$
0.02 g	$CuCl_2 \cdot 2H_2O$
0.02 g	$NiCl_2 \cdot 2H_2O$
1 mL	25% HCl

3.4.4 **Culture condition**

Experiments were done using a modified Erlenmeyer flask, sparged with nitrogen gas to maintain anaerobic conditions (see Figure 3.3). The Erlenmeyer flask was stoppered with a 4 cm removable rubber stopper. The rubber stopper had two openings, one for the air exit filter and the other for the multi-purpose glass tubing. The multi-purpose glass tubing extended to the bottom of the flask and was used for inoculation, nitrogen sparging and sample collection. The purpose of the air exit filter was to ensure that air leaving the flash does not contaminate the laboratory. Single

colonies were transferred into the growth media and grown at 37°C and 150 rpm agitation for 24 hours prior to fermentation (Kaur *et al.* 2012)

Figure 3.3 : Modified Erlenmeyer flask that is used for the fermentation of glycerol to maintain an anaerobic environment.

Fermentation was done by the inoculation of 10% (v/v) of the pre-cultured media to the fermentation media. All experiments were performed in 250 mL modified Erlenmeyer flasks with a working volume of 100 mL, which were sparged with nitrogen gas. Incubation continued for 48 hours and 5ml of samples were taken for analysis at 3 hour intervals. The glycerol was autoclaved separately. The glycerol sources (pure glycerol, crude glycerine and washed crude glycerine) of concentrations of 50 g/L, 100 g/L, and 150 g/L. were added to the fermentation medium with 10% (v/v) of the inoculum culture.

3.5 **Enhancement by ultrasound**

The ultrasound-assisted fermentation of the glycerol mixtures was conducted in an Elma sonic bath (see Figure 3.4) at a frequency of 35 kHz and a 35 W power input for 2 or 10 minutes prior to fermentation. This was done according to a method adopted from Khanna *et al*. (2012). During sonication, the bath was filled with water for the transmission of waves. Ultra-sonication experiments were performed using

250 mL flasks with 100 mL of the fermentation media. The growth conditions and nutrients were the same than those used for fermentations done without ultra-sonication.

Figure 3.4**:** Ultrasonic bath and flask used for ultasound-assisted fermentation of glycerol by *Clostridium diolis*.

3.6 **Analysis**

3.6.1 **Ultraviolet spectroscopy**

An ultraviolet spectroscopy (SHIMADZU) (Figure 3.5) at a wavelength of 600 nm was used to quantify cell growth during all the fermentation process. Distilled water was used as the blank. The absorbance for each fermentation sample of 5 ml at an interval of 3 hr of fermentation times (Kuar et al.,2012). All samples were analysed in duplicates. The inhibition effect of the impurities in the crude glycerine and washed crude glycerine on the growth of *Clostridium diolis* was quantified by determining the extent to which growth was inhibited and to then compare it to the growth in pure glycerol. Inhibition due to impurities in crude and washed crude glycerine was studied by determining the percentage % inhibition using test tubes containing 9 mL of culture medium with different grades of glycerol. The medium was inoculated with 1 mL of inoculum and incubated at 37°C. The optical density at 600 nm was measured in each tube initially and after 6 hours. The percentage inhibition was determined using Equation 1.

$$\text{Growth inhibition } (\%) = \left(1 - \frac{OD_{E(t=6h)} - OD_{E\,(t=0h)}}{OD_{C(t=6h)} - OD_{C(t=0h)}}\right) \times 100 \qquad (1)$$

where $OD_{E(t=6h)}$-$OD_{E(t=0)}$ is the recorded increase in optical density at 600 nm in the presence of crude or washed crude glycerine; and $OD_{C(t=6h)}$-$OD_{C(t=0)}$ is the recorded increase in optical density at 600 nm in the presence of pure glycerol.

Figure 3.5: Ultraviolet spectroscopy to monitor cell growth.

3.6.2 **High-performance liquid chromatograph (HPLC)**

The glycerol degradation and product formation were detected and quantified using high-performance liquid chromatography (Agilent 1200 Series, see Figure 3.6) .The high-performance liquid chromatograph was equipped with an HPX-87H column (100 x 7.7 mm, Bio-Rad), which is known to detect glycerol, sugars, certain organic acids and alcohols with a refractive-index (RI) detector (Khanna *et al.* (2012). The mobile phase used was at 5 mM H_2SO4 with a flow rate of 0.7 ml/min and a temperature of 65°C. Detailed methods for the HPLC analysis is fully illustrated in section A2 of the appendix.

Figure 3.6: High-performance liquid chromatograph used to quantify product concentrations.

3.6.3 **Fourier transform infrared spectroscopy (FTIR)**

The functional groups of glycerol sources were estimated with a FTIR spectrophotometer (SHIMADZU, see Figure 3.7). Disc preparation was done using sample of glycerol that was spread into the disc with no bubbles or overflow. The transmittance of the sample was measured in the range of 400 cm^{-1} to 4000 cm^{-1} with 40-4000 cm^{-1} scans per sample.

Figure 3.7: Fourier transform infrared spectroscopy

3.6.4 **Microscope analysis**

The microbial microstructure was analysed by using a Primo Star light microscope with a Zeizz camera to take images of the microorganisms (see Figure 3.8). Materials includes glass microscope slides, Plastic cover slips, sample culture with live culture, loop, paper towels or tissues ad Methylene blue solution (0.5 to 1%) Take a very small drop of *C.diolis* with the loop and smear it for 2 to 3 seconds on the slide. A small drop of methylene blue solution is placed on a microscope slide. Place a coverslip on top. Remove excess solution around the coverslip with a paper towel or tissue. View in the compound microscope at 4 x or 10 x initially, before moving to higher magnification. The pictures were analysed at a magnitude of magnifications to compare the effect of ultrasound during the ultrasound assisted fermentation.

Figure 3.8: Light microscope used to identify the effect of ultrasound-assisted fermentation.

3.6.5 **Gas Chromatograph**

Gas chromatography (Agilent 7890A) with an Agilent 5975C auto-injector, HP-88 (100 m) column and a flame ionization detector (FID) was used to determine the composition of fatty acid methyl esters (FAME) of the oil in order to quantify the molecular weight of the oil for the transesterification reaction (the production of crude glycerol).The full procedure is explained on section A3 of the appendix,

Figure 3.9: Gas chromatography to analyse fatty acids for the production of biodiesel.

3.7 **References**

Anad, P. & Sanexa, R.J. 2012. A comparative study of solvent-assisted pretreatment of biodiesel derived glycerol on growth and 1,3-propanediol production from *Citrobacter freundii*. *New biotechnology*, 29:199-205.

Hu, S., Luo, X., Wan, C. & Li,Y. 2012. Characterization of crude glycerol from biodiesel plants. Agriculture Food and Chemistry 60: 5915-5921.

Kaur, G., Srivastava, A.K. & Chand, S. 2012. Advances in biotechnological production of 1, 3-propanediol. *Biochemical Engineering Journal*, 64:106-118.

Khanna, S., Jaiswal, S., Goyal, A. & Moholkar, V.S. 2012. Ultrasound enhancement of bioconversion of glycerol by *Clostridium pasteurianum*: A mechanistic investigation. *Chemical Engineering Journal*, 416-425.

CHAPTER 4

4 Results and discussion

In this chapter, the characterization and composition of raw crude glycerine and washed crude glycerine are compared to that of pure glycerol. The effect of substrate concentration, growth of *Clostridium diolis (C. diolis)* and production of value-added products are discussed in section 4.4. Moreover, the effect of ultrasound radiation on the bacterial morphology and production yield is explained comprehensively in section 4.5.

4.1 Characterization of glycerol feedstock

The glycerol sources used in this study included raw crude glycerine (which is a by-product from the production of biodiesel from sunflower oil using KOH with methanol), washed crude glycerine (obtained by washing raw crude glycerine with petroleum ether with the aim of decreasing the impurities) and commercial pure glycerol.

4.1.1 Analysis of physical properties of glycerol sources

Some physical properties of the glycerol sources utilised in this study are presented in Table 4.1.

Table 4.1: Some physical properties of glycerol sources used in this study

Parameter	Crude glycerine	Washed crude glycerine	Pure glycerol
pH	10.84	8.94	6.41
Viscosity (mPa.s)*	967	1002	1191
Density (g/mL)	1.05	1.15	1.28

The high alkalinity of the raw crude glycerine is due to the presence of the KOH residues remaining following the biodiesel production process. The density of the raw crude glycerine was mainly influenced by the presence of impurities that include

fatty acids, soap, ester, water and methanol resulting from the biodiesel production process. Washed crude glycerine has a pH of 8.94 that is lower than that of raw crude glycerine, but higher than that of pure glycerol. Likewise, the viscosity and density of washed glycerine is lower than that of crude glycerine, but higher than that of pure glycerol.

4.1.2 **Quantification of methanol and glycerol content**

The total glycerol and methanol content of raw crude glycerine and washed crude glycerine were determined by high-performance liquid chromatography (HPLC). The results of this test are provided in Table 4.2. The results indicate that the methanol was removed from the crude glycerine by washing it with petroleum ether.

Table 4.2: Glycerol and methanol content of glycerol sources used in this study

Parameter	Crude glycerine	Washed crude glycerine
Glycerol (g/L)	296.24	370.46
Methanol (g/L)	309.9	0.23

The concentration of glycerol in each of the glycerol sources used was determined for each volume of the source used and is given in Table 4.3.

Table 4.3: Glycerol content in each different source of glycerol at different concentrations (g/L)

Glycerol sources	Glycerol Concentration ($g.L^{-1}$)		
	50 g	100 g	150 g
Crude glycerine	24.673	49.267	78.030
Washed crude glycerine	27.648	55.979	81.777
Pure glycerol	46.910	92.9139	138.786

4.2 **Analysis of functional groups using Fourier transform infrared spectroscopy (FTIR)**

The functional groups present in raw crude glycerine and washed crude glycerine were analysed using FTIR and compared to the functional groups present in pure glycerol (See Figures 4.1 to 4.3). The most prominent functional groups observed from the spectra of pure glycerol are listed in Table 4.4.

Table 4.4: Functional groups present in pure glycerol

Wavelength (cm^{-1})	Functional Group
3350	O-H group stretching
2930 to 2880	C-H bond stretching
1450 to 1100	C-O bond stretching
1400 to 1460	C-O-H bond bending
920	O-H bond bending

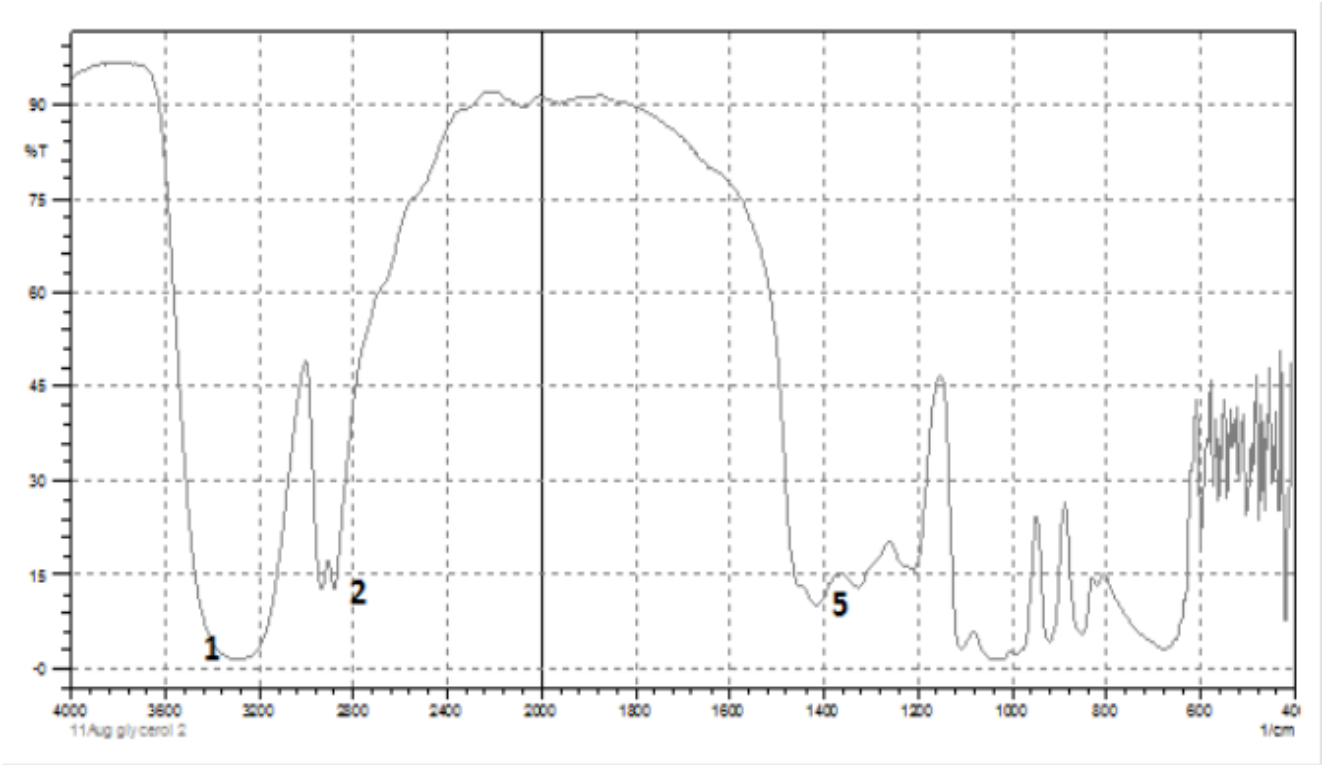

Figure 4.1 : FTIR spectrum for pure glycerol. Numbers 3 and 4 are missing on the spectra

From Figure 4.1 the two asymmetrical CH_2 groups and one symmetrical CH group of glycerol can be observed at 2880 cm^{-1} and 2930 cm^{-1} respectively. The glycerol structure contains three –OH groups as is evident from the sharp, broad OH stretching at 3350 cm^{-1}. The OH groups in the glycerol structure are all connected to carbons, which would explain the CO bond stretching at 2100 cm^{-1}, 1450 cm^{-1} and 1100 cm^{-1} and the COH bending from 1400 cm^{-1} to 1460 cm^{-1}.

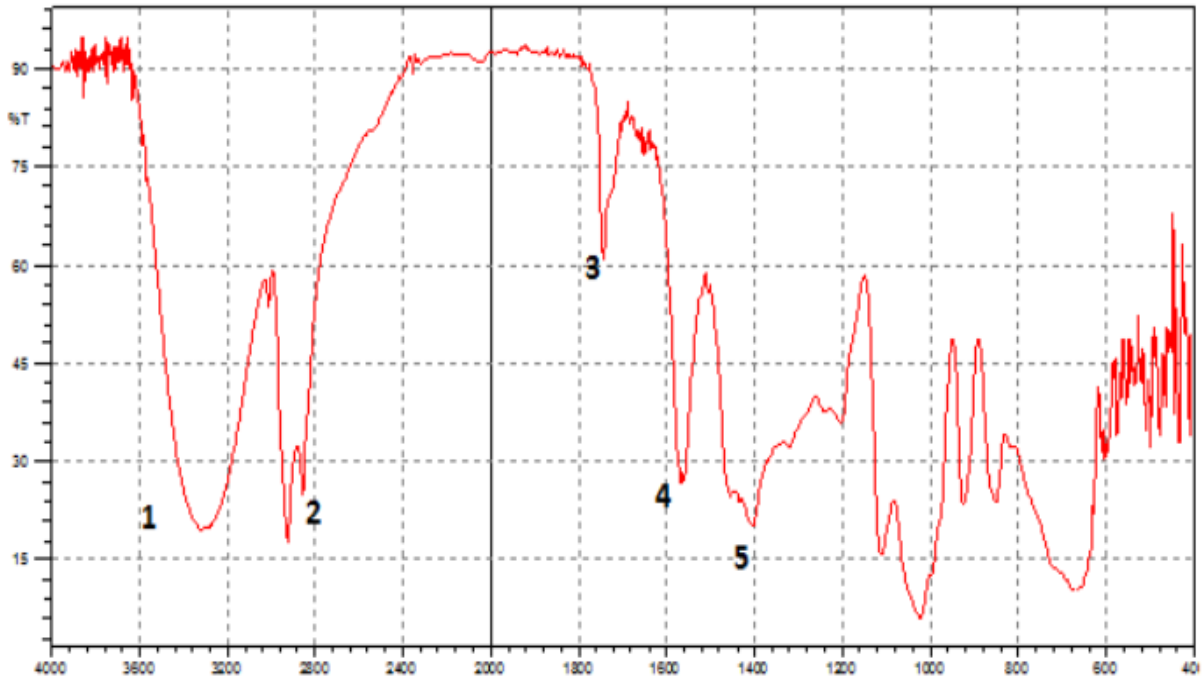

Figure 4.2: FTIR spectrum for crude glycerine

The spectrum of the crude glycerine in Figure 4.2 reflects additional peaks when compared to that of pure glycerol. The major visible peaks are at 1560 cm^{-1}, 1760 cm^{-1} and 3000 cm^{-1}. The peaks at 1560 cm^{-1} indicate the presence of impurities containing carboxylate ions, which are associated with the presence of soap (COO-) in the crude glycerine. The peak at 1760 cm^{-1} represents the carbonyl group (C=O) of an ester, carboxylic acid or fatty acid. Furthermore, the peak at 3000 cm^{-1} indicates the presence of compounds with unsaturated -C=C- bonds. The large broad peak between 920 cm^{-1} and 1120 cm^{-1} was split into two distinct peaks at 1020 cm^{-1} and 1220 cm^{-1}. The peaks from 1020 cm^{-1} to 1060 cm^{-1} are associated with –C-O stretching and O-CH_3 vibrations. The presence of these peaks can be linked to the presence of FAME or unreacted oils. The peak at 1160 cm^{-1} - 1180 cm^{-1} is a C-O-C stretching and is associated with unreacted oils. A small peak that can be assigned to a C-C stretching appeared at 3000 cm^{-1}. Since this peak was not present in the pure glycerol, it is believed to represent the –CH stretching in methanol.

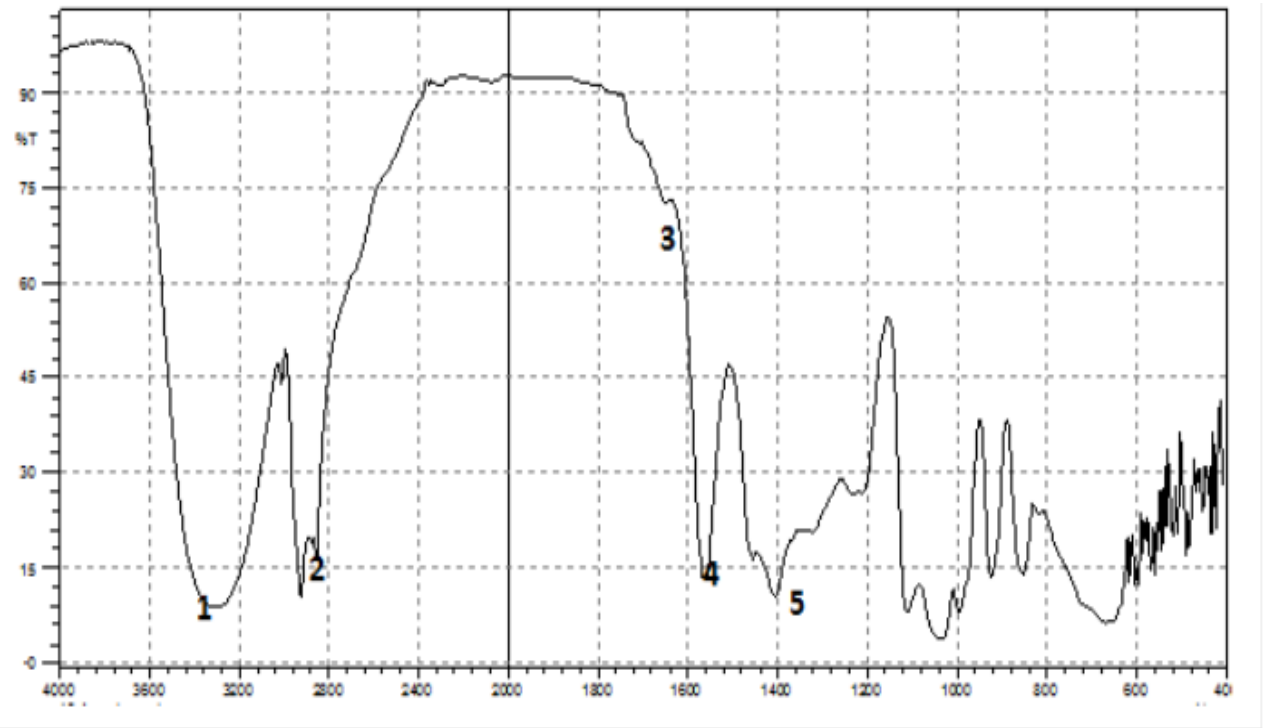

Figure 4.3 : FTIR spectrum for washed crude glycerine.

Figure 4.3 represents the spectrum of washed crude glycerine that has a small C-H stretching at 3000 cm^{-1}, which is assigned to the carboxyl or ester peak. The peak at 1760 cm^{-1} is much smaller compared to the same peak present in the crude glycerine spectra, which means that most of the FAMEs were probably washed out. The peaks at 1560 cm^{-1}, 1400 cm^{-1} and 1000 1160 cm^{-1} are much smaller in comparison to those present in the crude glycerine spectra. These peaks were associated with impurities in the crude glycerine, and the fact that they are reduced after washing means that most of the impurities present in the crude glycerine were removed. The absence of the methanol peak observed at 3000 cm^{-1} in the crude glycerine spectra indicates the complete removal of methanol from the crude glycerol.

4.3 **Growth potential of C. diolis on different concentrations of glycerol**

In this experiment pure glycerol, washed crude glycerine and raw crude glycerine were used as sole carbon sources in a fermentation medium for the growth of *Clostridium diolis*. The pure glycerol was used as a control source to understand the

effect of the different glycerol sources on the growth potential of *C. diolis*. All experiments were conducted in an anaerobic environment at 37 ^{o}C and a shaking speed of 150 rpm. Figure 4.4 represents the growth curves of *C. diolis* when grown on pure glycerol at concentrations of 50 g/L, 100 g/L and 150 g/L.

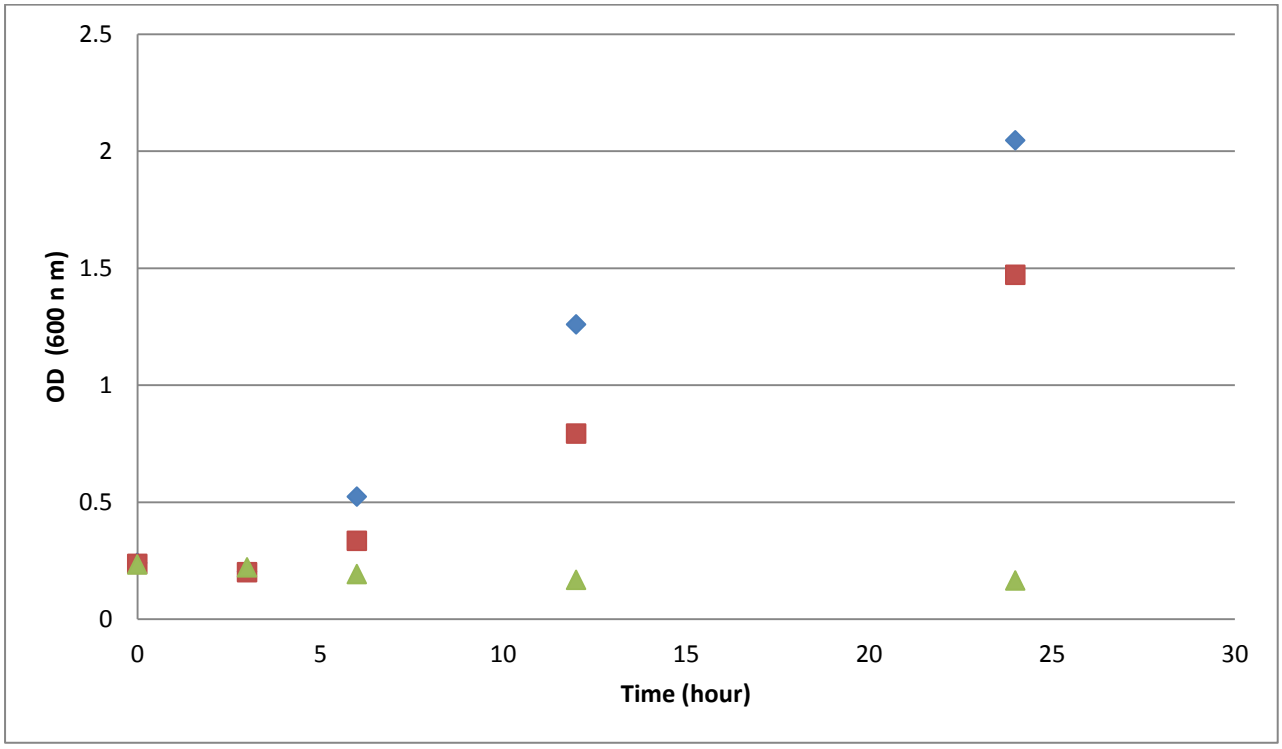

Figure 4.4 : Growth curves of *C. diolis* on pure glycerol, (♦ - 50 g/L ■ - 100 g/L, ▲ - 150 g/L)

When compared to 100 g/L and 150g/L of pure glycerol, pure glycerol of 50 g/L showed a high growth potential for *C. diolis*. Thus, the growth potential decreased with an increase in concentration of glycerol. This phenomenon is associated with the osmolite characteristics of glycerol that change the bacterial cell when grown in a media with high concentrations of glycerol, which results from the changes in the osmotic pressure within the cell. Fundamentally, when the concentrations on the outside of cell walls are too high, the resultant osmotic pressure on the cell wall becomes too great and the cell ruptures, which then inhibits the growth of the organism. When the glycerol concentration was 100 g/L, growth decreased from 1.97 nm to 1.48 nm when compared to the amount of cells present when fermenting with 50 g/L of pure glycerol. At the highest concentration of glycerol used in this

study (150 g/L), the bacterial growth shows no increase, indicating that concentrations of glycerol that are too high inhibited the growth of the organism.

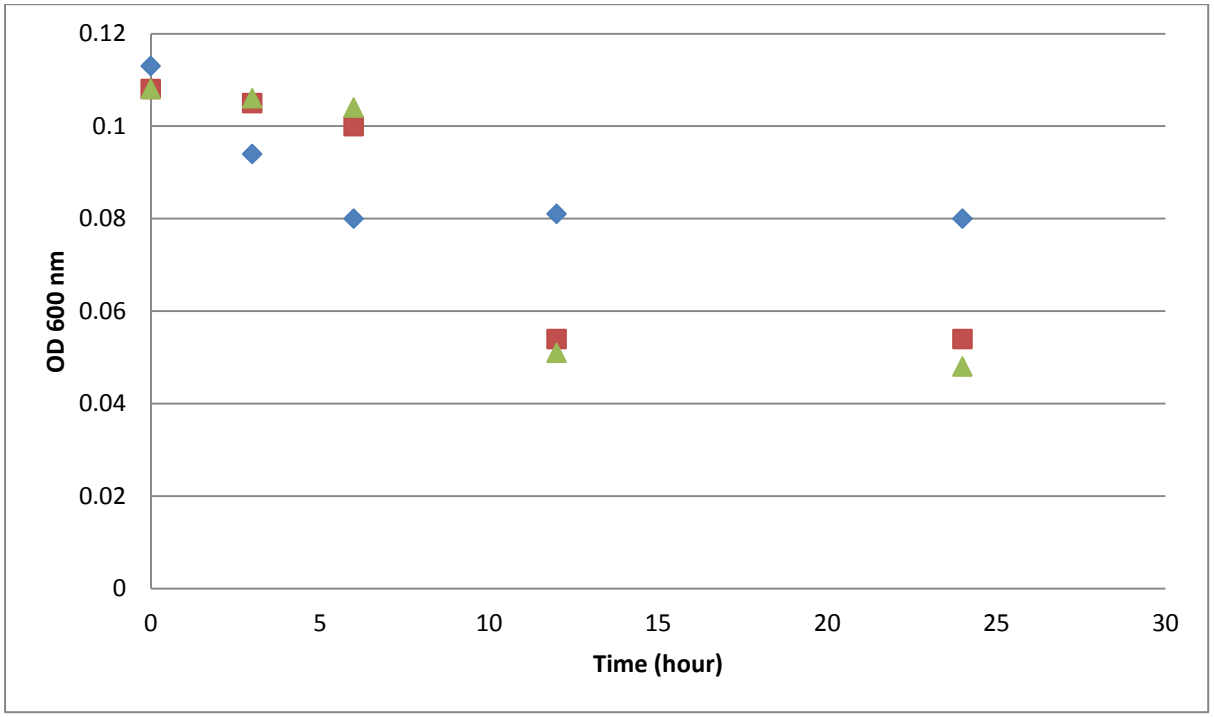

Figure 4.5: Growth potential of *C. diolis* when grown in crude glycerine (♦ - 50 g/L ■ - 100 g/L, ▲ - 150 g/L**)**

When *C.diolis* was grown on crude glycerine (Figure 4.5), the growth potential was low and the microorganism growth decreased as the fermentation time increased. The inhibited growth was due to the presence of the impurities in the crude glycerine. The crude glycerine used in this study contained 309.9 g/L methanol and the FTIR spectra (Figure 4.3) show that crude glycerine contained both soap and fatty acids. These impurities in crude glycerine tend to interact with each other and this interaction results in a synergic effect that affects the growth of *C.diolis*. Alcohols are known to influence cell membranes by increasing permeability, but this effect is strictly linked to the crude glycerine concentration and to the length of the alcohol carbon chain. For instance, the crude glycerine contains methanol, which is an aliphatic alcohol with one carbon atom that decreases the metabolite production in the cell membrane when present in high concentrations. Additionally, fatty acids are components of the cell membrane and are incorporated into the chains of cellular lipids, but can disturb the process of metabolite synthesis when present in concentrations that are too high. Thus, raw glycerol growth inhibition most likely

originates from the high concentrations of methanol or soaps present in the crude glycerine.

Unsaturated fatty acids also influence microbial metabolism and viability and the heavy metal ions retained in the glycerol after transesterification may have a negative effect on the viability of microbial cells. Venkataramanan *et al*. (2012) indicated that fatty acids that are present as contaminants in crude glycerine had the highest inhibitory effect on the growth of *Clostridium pasteurianum* together with methanol and alkaline salts. The growth curve for *C.diolis* on washed crude glycerine is provided in Figure 4.6.

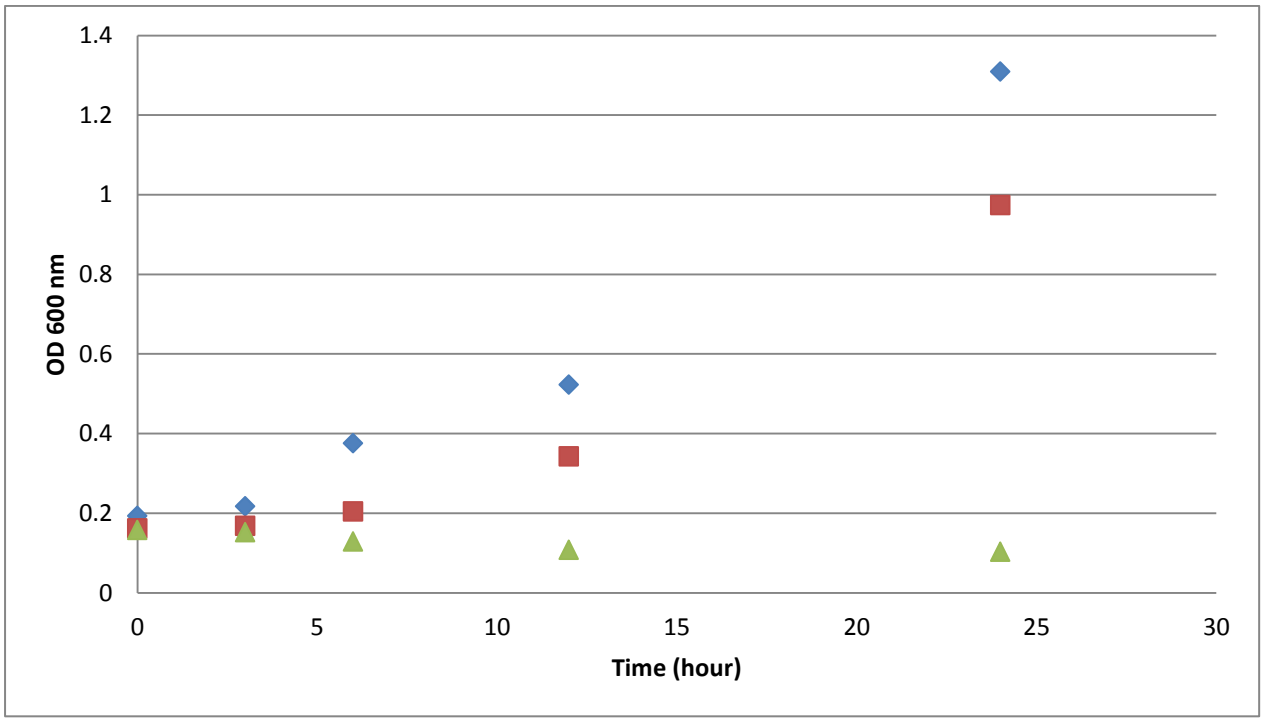

Figure 4.6 : Growth potential of *C. diolis* when grown on washed crude glycerine (♦ - 50 g/L ■ - 100 g/L, ▲ - 150 g/L).

From Figure 4.6 it can be seen that the growth of *C. diolis* in washed crude glycerine had increased when compared to the growth in crude glycerine, but it is lower than when grown on pure glycerol. This indicates that the washing of crude glycerine with petroleum ether removed the impurities and increased bacteria growth. The washed crude glycerine used in this study contained 0.23 g/L of methanol and the FTIR spectra show the decrease in soap and unreacted fatty acids content (Figure 4.3). These results reflect that the effect of washing the crude glycerine with petroleum

ether is a combination of extraction and mechanical washing. In this case, petroleum ether acts as a solvent used to recover glycerol content from the impurities contained in the crude glycerine. For this study, petroleum ether was used, since it has a boiling point lower than that of glycerol and could thus be removed relatively easily from the washed glycerine after washing.

The percentage growth inhibition for the crude glycerine and washed crude glycerine is provided in Figure 4.7.

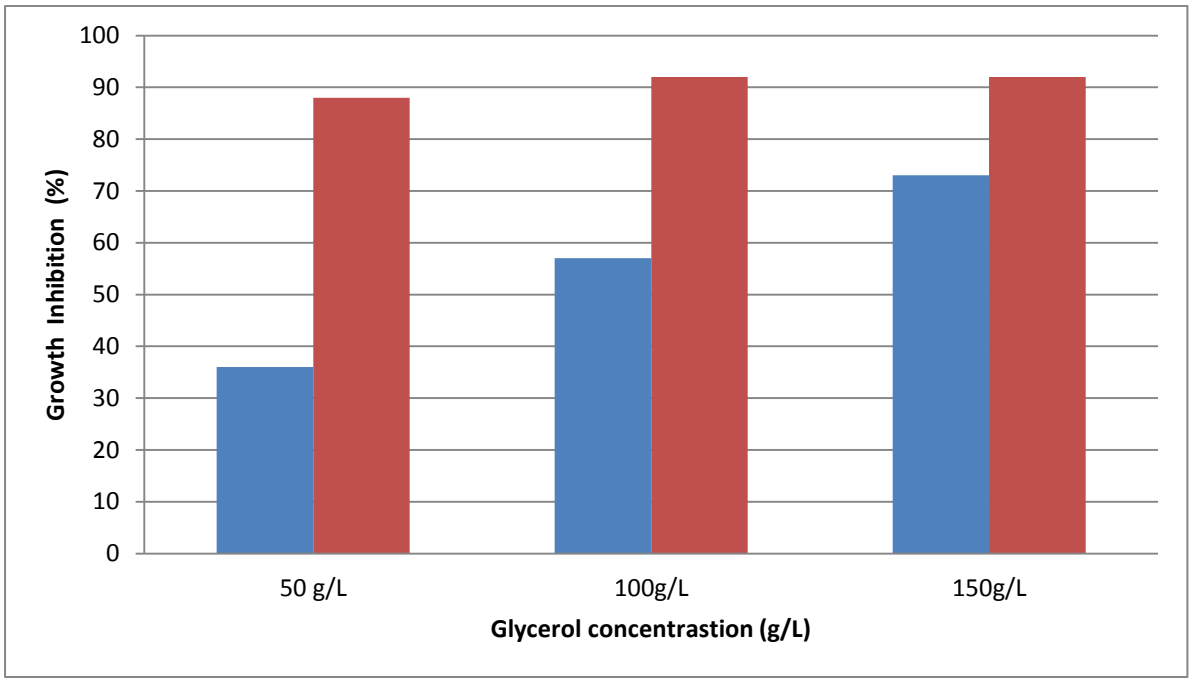

Figure 4.7 The growth inhibition of crude glycerine (■) and washed crude glycerine (■) on *C. diolis* at 50 g/L, 100 g/L and 150 g/L

Crude glycerine showed the highest growth inhibition of *C. diolis*. It was found that the maximum growth inhibition for washed crude glycerine and crude glycerine was 73% and 94 % respectively when compared to pure glycerol. Inhibition increased with an increase in concentration for both crude glycerine and washed crude glycerine, but the effect was more significant for crude glycerine.

4.4 **Fermentation results**

The production of value-added products from crude glycerine washed crude glycerine and pure glycerol was investigated. However, since no significant products

could be obtained from crude glycerine, only the results obtained for pure and washed crude glycerine will be provided in this section.

4.4.1 **Production of 1,3-propanediol from glycerol fermentation**

The production of 1,3-propanediol using *C. diolis* on washed crude glycerine and pure glycerol was investigated under controlled anaerobic conditions in a flask that was modified for the production of possible products. Washed crude glycerine and pure glycerine were used as the sole carbon sources at concentrations of 50 g/L, 100 g/L and 150 g/L. The 1, 3-propanediol produced by *C. diolis* on pure glycerol is indicated in Figure 4.8.

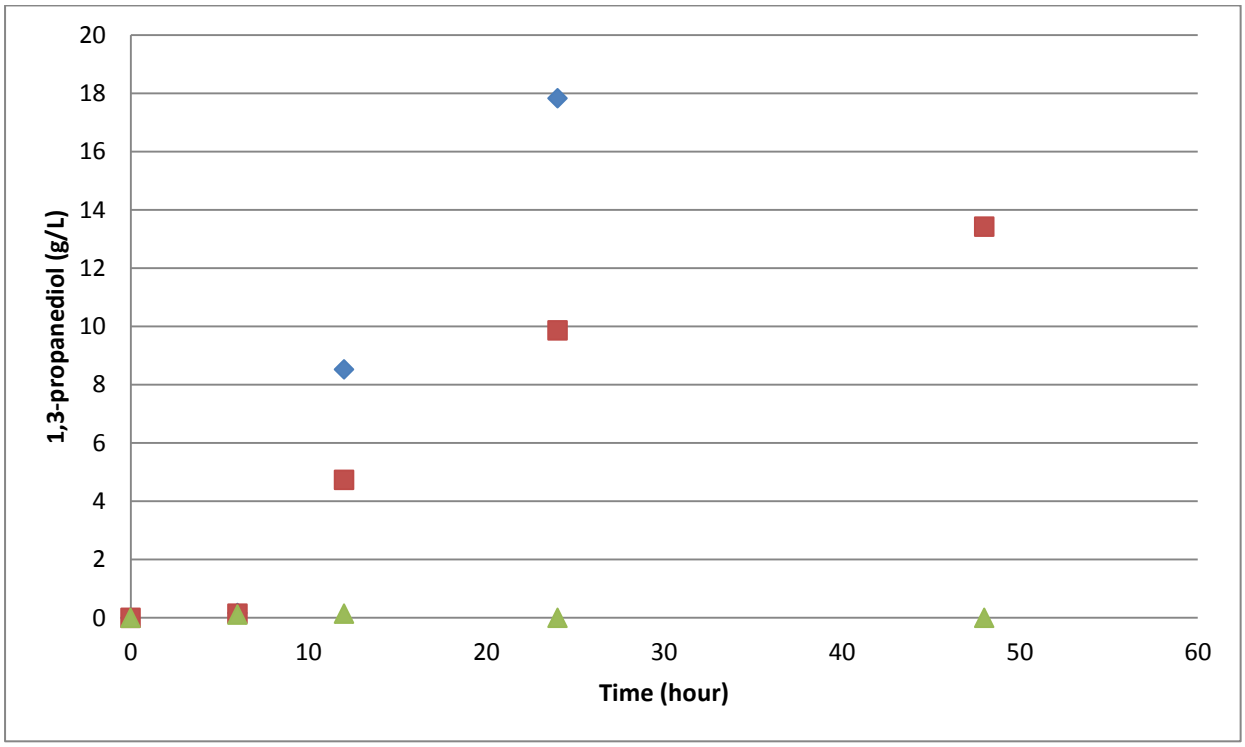

Figure 4.8: Production of 1,3-propanediol from pure glycerol (♦ - 50 g/L, ■ - 100 g/L, ▲ - 150 g/L

From Figure 4.8 it can be seen that the concentration of 1,3-propanediol decreases with an increase in substrate concentrations. The highest concentration of 1,3-propanediol (17,83 g/L) was produced after 24 hours of fermentation on a pure glycerol concentration with a productivity of 0.74 g $l^{-1}h^{-1}$. The highest concentration of 1,3-propanediol produced on a pure glycerol concentration of 100 g/L (13.46 g/L) was obtained after 48 hours of fermentation at a productivity of 0.28 g $l^{-1}h^{-1}$. Very little 1,3-propanediol was produced on a pure glycerol concentration of 150 g/L.

Commonly, a number of stresses can be present during fermentation, which include osmotic stress that results from high substrate concentrations. It has already been shown in Figure 4.4 that *C. diolis* does not grow in glycerol concentrations of 150 g/L, thus it was expected that no 1,3-propanediol would be produced. High glycerol concentrations lowers the utilisation of the substrate and results in a low production of 1,3-propanediol. Biodegradation kinetics could indicate how much glycerol is consumed via catalysed reactions only by the *C diolis* combined with the required enzymes. However, glycerol degradation is largely proportional to the concentration of organisms able to degrade the substrate and is mostly influenced by the substrate concentration. Moreover, saturation kinetics suggests that at a substrate concentration of 50 g/L, the saturation rates are approximately proportional to the substrate concentration, but at the higher concentrations (100 g/L and 150 g/L), the saturation rates are independent of substrate concentration.

When fermenting with crude glycerine, no 1,3-propanediol was produced by *C. diolis* due to the impurities present that inhibited the growth of the organism. These impurities are the reason for the slower uptake of crude glycerol by *C. diolis* DSM 15410 on crude glycerol from sunflower oil transesterification. The synthesis of 1,3-propanediol is mainly influenced by the osmotic stress that is associated with high concentrations of glycerol. Toxic stresses, caused by high concentrations of impurities and alcohol, are additional factors to the environmental stresses acting on the microorganisms.

When crude glycerine is washed with petroleum ether, it gives a viable product formation of 1,3-propanediol when compared to crude glycerine (see Figure 4.9). In this case, *C. diolis* was able to produce 1,3-propanediol at all the considered concentrations (50 g/L, 100 g/L and 150 g/L) of washed crude glycerine. The production of 1,3-propanediol is the highest when fermenting with 50 g/L and decreases when fermented with 100 g/L and 150 g/L.

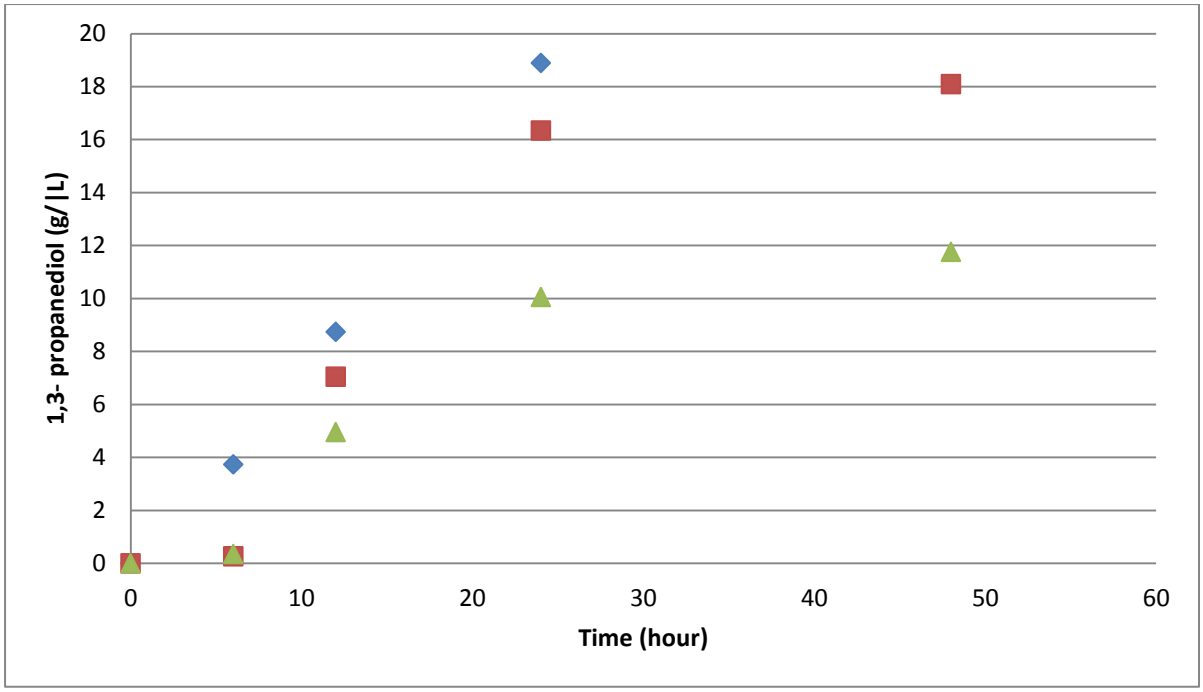

Figure 4.9: Production of 1,3-propanediol from washed crude glycerine (♦ - 50 g/L, ■ 100 g/L, ▲ - 150 g/L)

When C. diolis is grown on washed crude glycerol, it produces 18.89 g/L, 16.34 g/L and 10.08 g/L of 1,3-propanediol from substrate concentrations of 50 g/L, 100 g/L and 150 g/L after 24 hours of fermentation with productivities that ranges from 0.75 g $l^{-1}h^{-1}$ to 0.68 g $l^{-1}h^{-1}$ to 0.42 g $l^{-1}h^{-1}$ respectively. The capability of *C. diolis* to maintain a redox balance is linked to its ability to produce more reduced products when compared to the products produced by glycerol through the increase of redox equivalences. At 50 g/L the washed crude glycerol is totally utilised, however at 100 g/L and 150 g/L it is not totally utilised and thus the productivity decreased as the concentration went beyond 50g/L. From the graphs above, it is evident that the washed crude glycerine consistently produces more 1,3-propanediol than the pure glycerol. This is significant, because it is unexpected, especially since the washed glycerine contains much less glycerol than the pure glycerol. This is in contradiction with the consistent argumentation thus far that the impurities inhibit the growth of *C. diolis*. This contradiction is evident from Figure 4.9, because even the concentration of 150 g/L gave higher yields of 1,3-propanediol. This is a significant result for this study, since it essentially means that it is better to use washed crude glycerol than pure glycerol to produce valuable chemicals

4.4.2 **Production of other products from glycerol fermentation**

In addition to the production of 1,3-propanediol, other end-products from the glycerol fermentation were detected (see Figure 4.10). Samples collected from the effluent showed that glycerol also converted acetic acid, lactic acid and butyric acid.

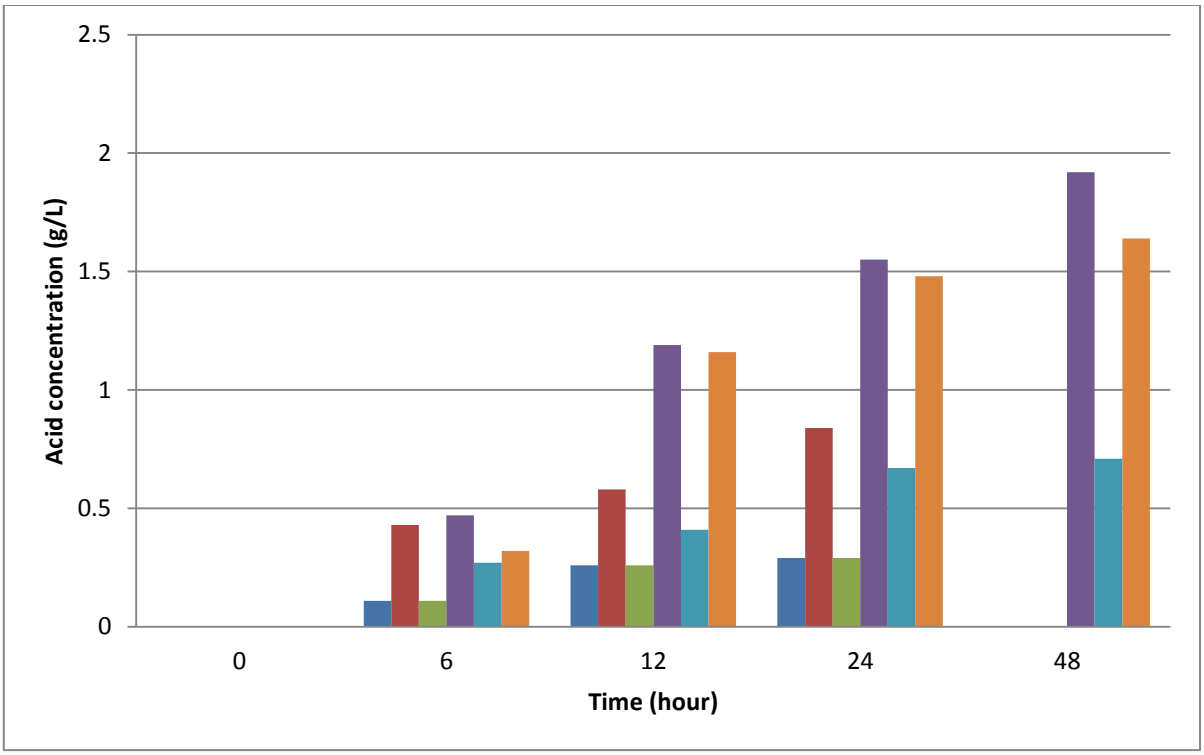

Figure 4.10: Acetic acid, butyric acid and lactic acid obtained from pure glycerol at a feed concentration of 50 g/L (■ - Lactic acid, ■ - Butyric acid, ■ - Acetic acid) and 100 g/L (■ - Lactic acid, ■ - Butyric acid , ■ - Acetic acid)

Figure 4.10 shows that acid production started after 5 hours of fermentation following an instant drop in total glycerol concentration. Acetic acid was produced in higher concentrations than butyric acid and lactic acid when the substrate concentration was 50 g/L of pure glycerol. The concentration of the formed acids slowly increased during the first 12 hours of fermentation. The highest concentration was reached within 24 hours when fermenting with pure glycerol. Ordinarily, during the oxidative pathway, a portion of glycerol is transformed to dihydroxyacetate (DHA) by NAD+-dependent glycerol dehydrogenase. Dihydroxyacetate (DHA) is then phosphorylated by DHA-kinase to enter the Embden-Meyerhof-Parnas (EMP) pathway for glycolysis, which further gains 2 moles of ATP and 1 mole of ADH. After the entrance into the EMP pathway, the reduction and oxidation of pyruvate to lactate and acetyl-CoA

respectively occur. Thereafter, acetyl-CoA is transformed to acetic acid and butyric acid, while lactate is transformed to lactic acid. Although butyric, acetic and lactic acids as well as ethanol are produced during 1,3-propanediol synthesis from glycerol by *C. diolis*, the main by-products of a proper conversion of glycerol to 1,3-propanediol are butyrate and acetate. This means, only under favourable fermentation conditions where these metabolites of acetic acid, lactic acid and butyric acid are practically always found in an anionic form as acetate, lactate and butyrate. The highest concentrations of acetic acid, i.e. 2.11 g/L, 1,64 g/L and 0 g/L were obtained when fermenting with 50 g/L, 100 g/L and 150 g/l of pure glycerol after 24 hours of fermentation. Acetic acid was produced in higher concentrations than butyric acid at a substrate concentration of 50 g/L of glycerol, but at higher substrate concentrations (100 g/L), the acetic acid concentration decreased, while the butyric acid concentration increased. The acetate/butyrate ratio is closely related to the rate of cell growth. As the butyric acid production increased, the growth decreased and as the acetic acid production increased, the cell count increased. It should be noted that the pathway for the synthesis of 1,3-propanediol does not lead to ATP production; thus other pathways are required for the generation of energy. The formation of acetic acid and/or butyric acid leads to ATP production, but is also associated with the generation of reducing equivalents, which are regenerated by the synthesis of 1,3-propanediol.

When fermenting with washed crude glycerine, acetic acid concentrations of 2.05 g/L, 1.65 g/L, and 1.32 g/L were obtained after 24 hours of fermentation on 50 g/L, 100 g/L ad 150 g/L of washed crude glycerol respectively (see Figure 4.11). The production of acetic acid decreased with an increase in the concentration of washed crude glycerine from 50 g/L to 150 g/L.

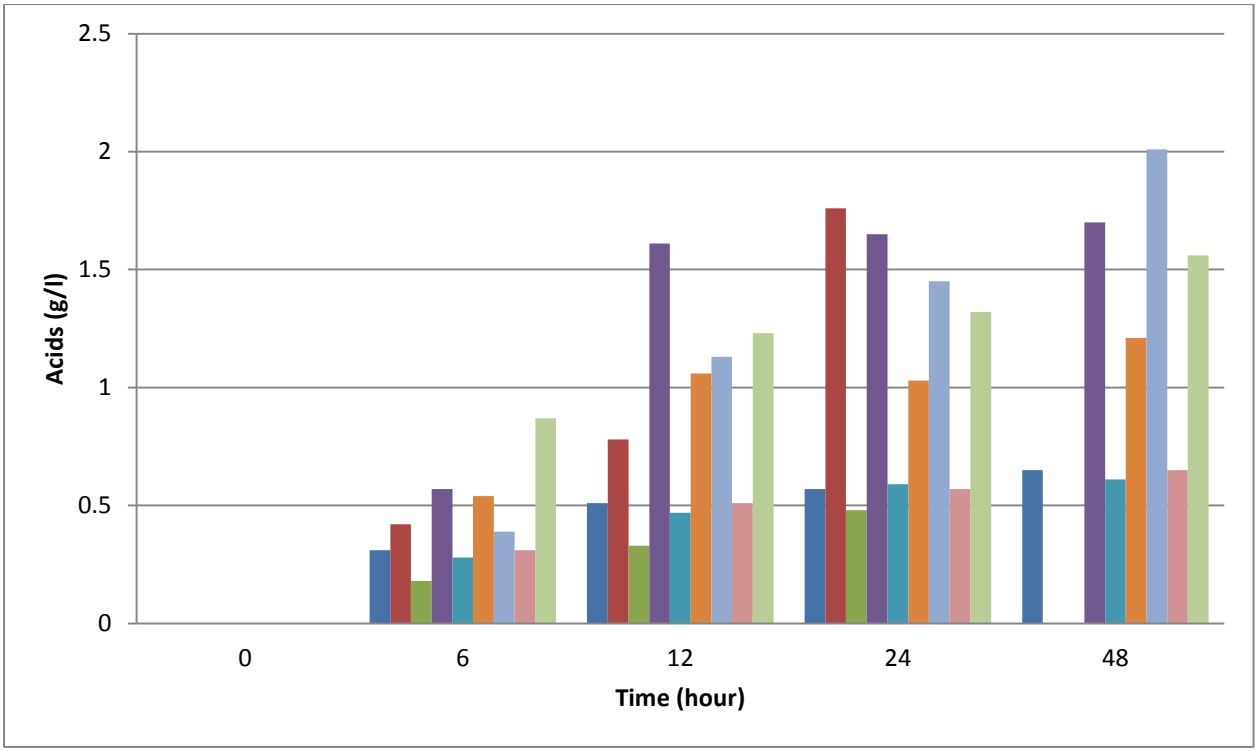

Figure 4.11: Acetic acid, butyric acid and lactic acid concentrations from washed crude glycerine at feed concentrations of 50 g/L (■ - Acetic acid, ■ - Butyric acid, ■ - Lactic acid), 100 g/L (■ - Acetic acid, ■ - Lactic acid, ■ - Butyric acid) and 150 g/L(■ - Butyric acid, ■ - Lactic acid, ■ - Acetic acid).

Butyric acid was produced at lower washed glycerine concentrations than acetic acid. The ratio of acetate to butyrate is mostly associated with the metabolism of the cell, thus the increase in acetic acid over butyric acid reflects the speed or rate of the microbial growth. In the current study, the ratio of acetate to butyrate was 2.34 at 50 g/L, which is more than the ratio of 0.85 at 100 g/L when using pure glycerol as substrate. Additionally, the ratio of acetic acid to butyric acid is lower for washed crude glycerine (1.16) when compared to the ratio obtained in pure glycerol (2.34). From both Figures 4.10 and 4.11 it is evident that the concentration of lactic acid increased as the substrate concentration of glycerol increased, however lactic acid was produced in lower concentrations when compared to the concentrations of both acetic acid and butyric acid. During the synthesis of lactic acid, pyruvate is reduced to lactic acid and under conditions of high glycerol concentrations and severe product inhibition, lactic acid appears in the medium. Thus, an increased content of lactic acid indicates that the process is blocked, due to substrate excess or inhibition at the stage of pyruvate generation.

4.5 **Effect of ultrasound radiation on the production of 1, 3-propanediol**

Fermentation can be enhanced in a number of ways to increase the concentration of the products obtained during fermentation. In this study, the use of ultrasound to enhance 1,3-propanediol production was investigated. A feed concentration of 50 g/L of pure glycerol was used as carbon source.

4.5.1 **The influence of time in ultrasound-assisted fermentation**

The growth curves of *C. diolis* grown on pure glycerol (50 g/L) under ultrasonic treatment for 2 minutes and 10 minutes is compared to the *C. diolis* growth curves without ultrasonic treatment in Figure 4.12 below.

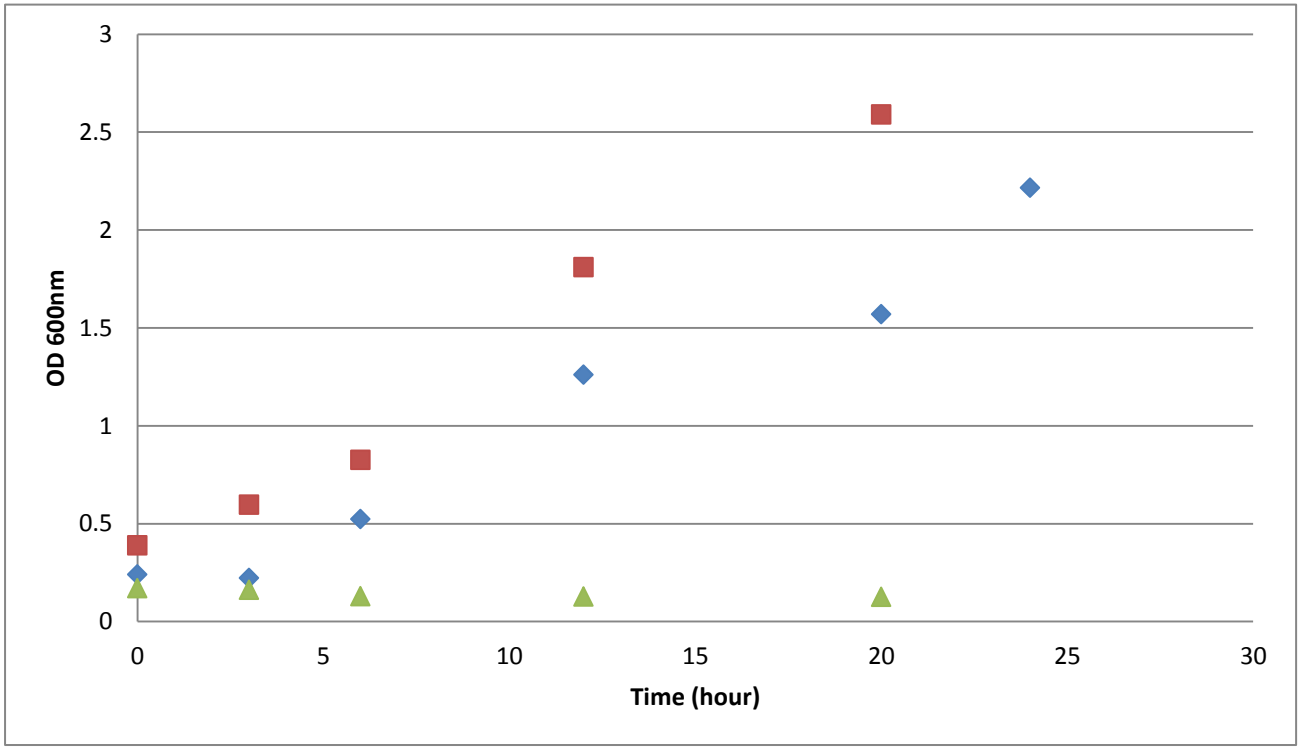

Figure 4.12: Growth curves for *C. diolis* on media subjected to ultrasound for 2 minutes (■) and 10 minutes (▲) compared to the control (♦)

From Figure 4.12 it can be seen that when fermentation is exposed to ultrasound for 10 minutes, the growth of *C diolis* decreases and when exposed to ultrasound radiation for 2 minutes, the growth potential increases. When compared to the fermentation without exposure to ultrasound, ultrasound irradiation for two minutes resulted in complete utilisation of glycerol within 20 hours of fermentation. This

suggests that activation for 2 minutes shortens the fermentation time. The amount of the remaining glycerol and low growth rate when fermentation is activated for 10 minutes, show that the cells have reached death phase. This means ultrasonic radiation at a duration of longer than 2 minutes can disrupt the cell and inhibit growth. For this study, ultrasound-assisted fermentation of glycerol mixtures was conducted at a frequency of 35 kHz and a 35 W power input prior to fermentation to avoid high frequency. This illustrates that moderate frequencies of ultrasound radiations can increase growth activity and modify cell metabolism. In many instances, cavitation by ultrasound has been used to induce the uptake of substances by either inducing the release of agents from liposomes and micelles or transporting agents directly from extracellular sections to the cytosol. Thus, cells are more likely to be exposed to the mechanism created by acoustic cavitation that disrupts the plasma membrane; this results in the transportation of materials to the cell. Moreover, this phenomenon depends on the time the microorganism is exposed to ultrasound radiations and to the frequency of the ultrasound.

4.5.2 **Production of 1,3-propanediol from ultrasound-assisted fermentation**

The production of 1,3-propanediol from pure glycerol (50 g/L) using *C. diolis* under ultrasonic treatment for 2 minutes and 10 minutes is compared to growth without ultrasonic treatment in Figure 4.13.

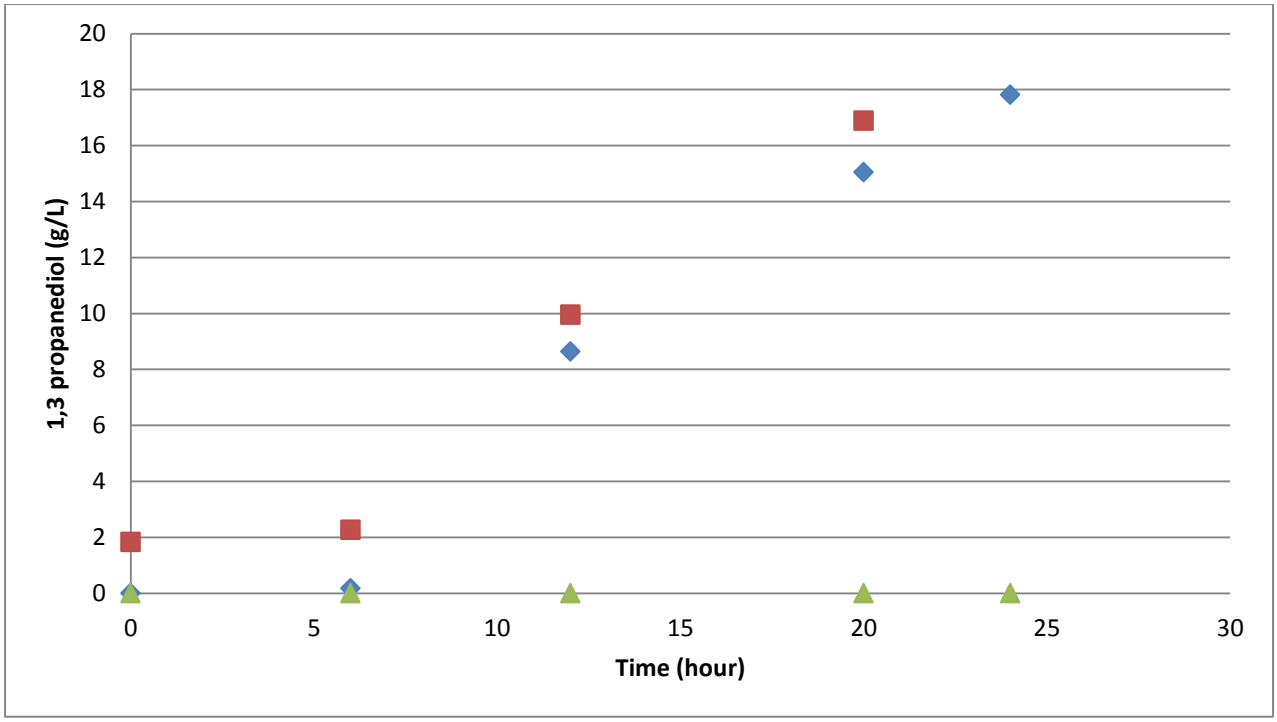

Figure 4.13: Concentration of 1,3-propanediol from 50 g/L of pure glycerol that was subjected to ultrasound irradiation for 2 minutes (■) and 10 minutes (▲) compared to no ultrasound treatment (♦).

Figure 4.13 shows that the highest concentration of 16.89 g/L of 1,3-propanediol could be obtained from *C. diolis* with an ultrasound treatment of 2 minutes after 20 hours of fermentation when glycerol is totally utilised . Furthermore, when *C. diolis* is grown for 2 minutes under ultrasound treatment, the glycerol is consumed completely after 20 hours of fermentation, whereas fermentation without ultrasound treatment requires 24 hours of fermentation for the complete utilisation of glycerol. This effect is linked to the ability of ultrasound to activate the cell growth that includes the utilisation of the substrate. The complete utilisation of glycerol when subjected to ultrasound for 2 minutes suggests that the use of ultrasound decreases the fermentation time when subjected to low intensity indirect ultrasound irradiation. When fermentation is exposed to ultrasound for 10 minutes, there is no significant production of 1,3-propanediol, thus the exposure has inhibited the utilisation of glycerol. Commonly, ultrasound can have two main effects on anaerobic fermentation that include enhancement by physical fragmentation of organic structures or extraction of substances. In this study, the effect of ultrasound is mostly linked to the time that *C. diolis* was exposed to irradiation. This effect can be further

explained in terms of an enhanced suspension of the glycerol by ultrasonication and the ultrasound-induced enhancement of mass transfer within and outside the cell.

4.5.3 **Growth morphology**

The microscopic images of the cell morphology of *C. diolis* while grown on glycerol are shown in Figure 4.13.

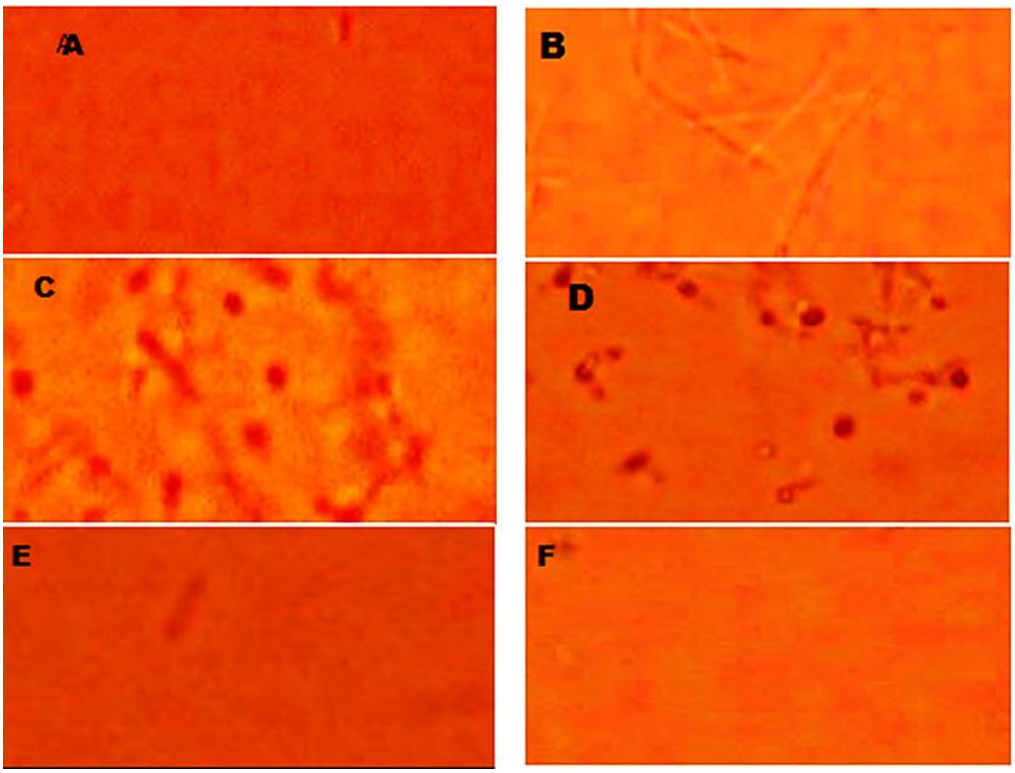

Figure 4.14: Growth morphology of *C diolis* grown at 50 g/L of pure glycerol on media subjected to ultrasound for 2 minutes, 10 minutes and control, (**A** - 3 hours of 2 minutes of ultrasound, **B** - 20 hours of 2 minutes of ultrasound, **C** - 3 hours of 10 minutes of ultrasound, **D** - 20 hours of 10 minutes ultrasound, **E** - 3 hours with no untrasound , **F** - 20 hours with no ultrasound).

Activation for 2 minutes is represented by slides A (3 hours of fermentation) and B (12 hours of fermentation). Slides C (3 hours of fermentation) and D (10 hours of fermentation) represent activation for 10 minutes and slides E and F represent the control with no ultrasonication. Slides A and B show that cells have developed at a high rate, when compared to cell development on slides E and F. Slides E and F show slight cell growth, but there is not much difference in the number of cells, suggesting a slow fermentation growth rate. Slides C and D show that ultrasonic

radiation of 10 minutes placed stress on the growth of the cells as the cells formed spores. One of the most important cellular responses to unfavourable environmental conditions is the production of dormant forms by microorganisms, namely the formation of endospores. The formation of endospores is one of the features that was used to categorize the *Clostridium* species. Predominantly, some microorganisms have the ability to adapt and sense certain modifications in the environment. Thus, cells develop a mechanism to produce spores in order to survive in an unfavourable environment; this is when conditions are of such a nature that cell growth inhibition develops. In other words, slides C and D show that *C. diolis* cells have been inhibited and started producing spores instead of utilising the glycerol for product production. During sporulation, microorganisms are able to lay metabolically dormant waiting for favourable growth conditions to return. The black spots on slides C and D suggest that when *C, diolis* was subjected to the incubator, it already formed spores and that is why glycerol was slowly utilised and no significant production of 1,3-propanediol was observed.

4.6 **References**

Duckhouse, H., Maso, T.J., Phull, S.S. & Lorimer, J.P. 2004. The effect of sonication on microbial disinfection. *Ultrason Sonochem*, 11:173-176.

González-Pajuelo, M., Andrade, J.C. & Vasconcelos, I. 2004. Production of 1,3-propanediol by *Clostrdium butyricum* VPI (3266) using a synthetic medium and raw glycerol. *J. Ind. Microbiol. Biotechnol*, 31:442-446.

Kongjao, S., Damronglend, S. & Hunsom, B. 2010. Purification of crude glycerol derived from waste used-oil methyl ester plant. *Korean Journal Chem Eng*, 27(3):944-949.

Nanda, M.R., Yuan, Z., Qin, W., Poirier, M.A. & Chunbao, X. 2014. Purification of crude glycerine using acidification: Effects of acid types and product characterization. *Austin Journal of Chemical Engineering*, 1:1-7.

Venkataramanan, K.P.1., Boatman, J.J., Kurniawan, Y., Taconi, K.A., Bothun, G.D. & Scholz, C. 2012 . Impact of impurities in biodiesel-derived crude glycerine on the fermentation by *Clostridium pasteurianum* ATCC 6013. Appl Microbiol Biotechnol, 93(3):1325-35, Feb.

CHAPER 5

5 Conclusion and Recommendations

5.1 Conclusion

A summary of the conclusions derived at through the course of this study, will now be provided as follows:

- Crude glycerine inhibits the growth of *C. diolis* with an inhibition percentage as high as 92%;
- Crude glycerine contains 49.267% of glycerol, with 309 g/L methanol, soap and FFA that are associated with growth inhibition;
- The glycerol concentration was shown to affect the production of 1,3-propanediol and the utilisation of the substrate is mostly linked to its content and concentration;
- Improved growth of *C. diolis* and the production of 1,3-propanediol were observed when using a substrate concentration of 50 g/L of pure glycerol and washed glycerol when compared to concentrations of 100 g/L or 150 g/l ;
- The use of ultrasound treatment of *C. diolis* for 2 minutes prior to fermentation had a positive effect on the growth of *C. diolis* and the production of 1,3-propanediol;
- The study has shown that value-added products can be produced from glycerol and that solvent washing and ultrasound irradiating can enhance the production of products such as 1,3-propanediol.

5.2 Recommendations

Recommendations for further research include the following:

- The effect of impurities on the metabolism of *C. diolis* needs to be further investigated to enable the direct use of crude glycerol as substrate.
- An evaluation of the effect of batch fermentation compared to other possible types of fermentation should be performed.

- Future research can also investigate the effect of ultrasonic irradiation on the metabolic pathways of *C. diolis* at different times and frequencies.

APPENDIX A

A1 Introduction

The calibration curves obtained from the experiments are important as they are used to quantify the production of 1,3-propanediol, acetic acid, butyric acid and lactic acid as well as the calibration curves that are used to quantify the glycerol sources used during fermentation as represented in section A2. Additionally, section A3 presents the data used to obtain the gas chromatogram calibration curves.

A2 HPLC product analysis

All the produced products were analysed and quantified by using HPLC. The standard tests were done in order to be able to quantify and get the quality of the expected products. The calibration curves were obtained by mixing pure known amounts of expected organic acids and alcohols with distilled water and this was serially diluted to obtain a decreasing amount of concentrations.Sample preparation for HPLC analysis was done as follows: 1. Adjust the pH of the sample to as close to 7.0 as possible. 2. Using the correct size pipette and a clean pipette tip, transfer 2.0 ml of adjusted sample into a clean polytop. Again using a pipette and clean pipette tip, dilute the sample by adding 4.0 ml distilled water. 3. Filter the sample using a 0.45 µm and 0.2 µm syringe filter into a clean vial. 4. Transfer about 1 ml of filtered sample into a clean HPLC / GC sample vial. 5. Close the vial using a new, unused septum in the cap. 6. Label the vial clearly. 7. The sample is now ready for analysis on the HPLC. The peak areas obtained on the HPLC chromatogram of each standard are shown in Tables A2.2 and Table A2.3. The calibration curves were plotted and the slope (k) of the graph was used to calculate the unknown products of the samples. The k value together with the retention times of each sugar are presented in Table A2.1.

Table A2.1: Components obtained and their corresponding symbols used in the calculations

Component	Retention time (minutes)	K-value
Glycerol	12.94	57600

1,3-Propaanediol	17.17	46766
Lactic acid	12.54	29995
Acetic acid	15.16	30667
Butyric acid	22.52	35273
Methanol	18.49	9257.3

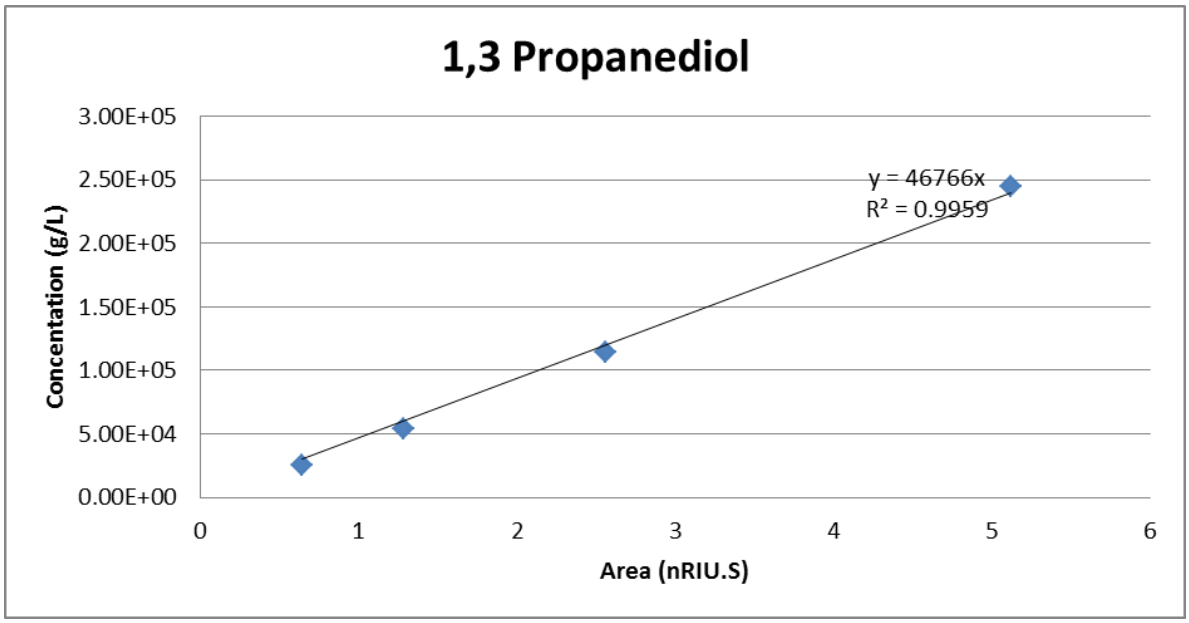

Figure A.0.1: 1,3-propanediol calibration curve

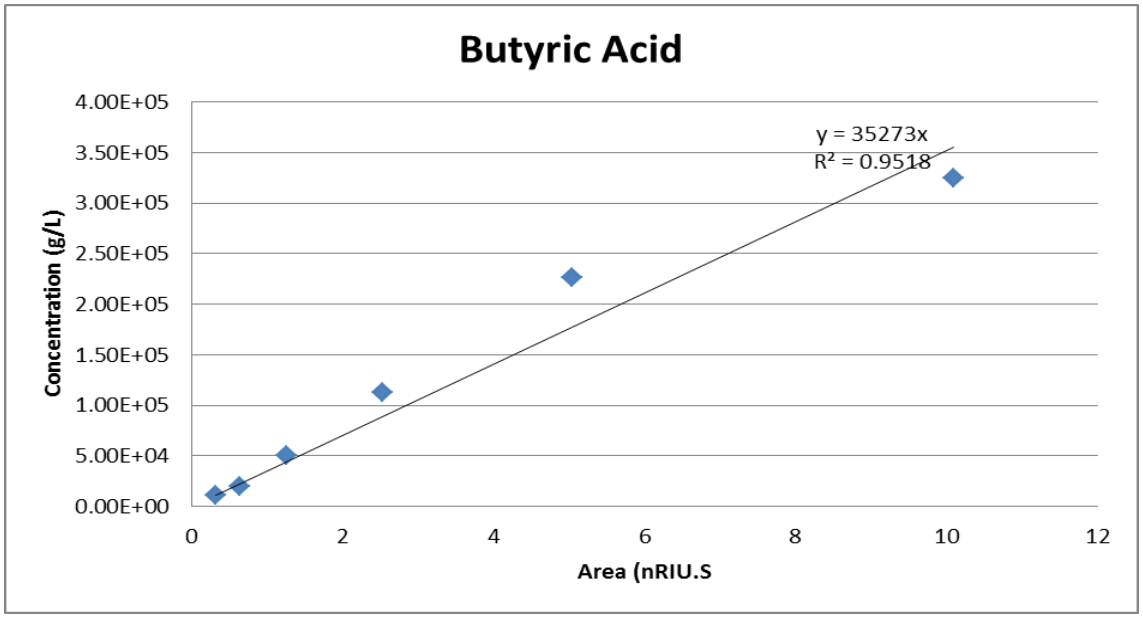

Figure A.0.2: Butyric acid calibration curve

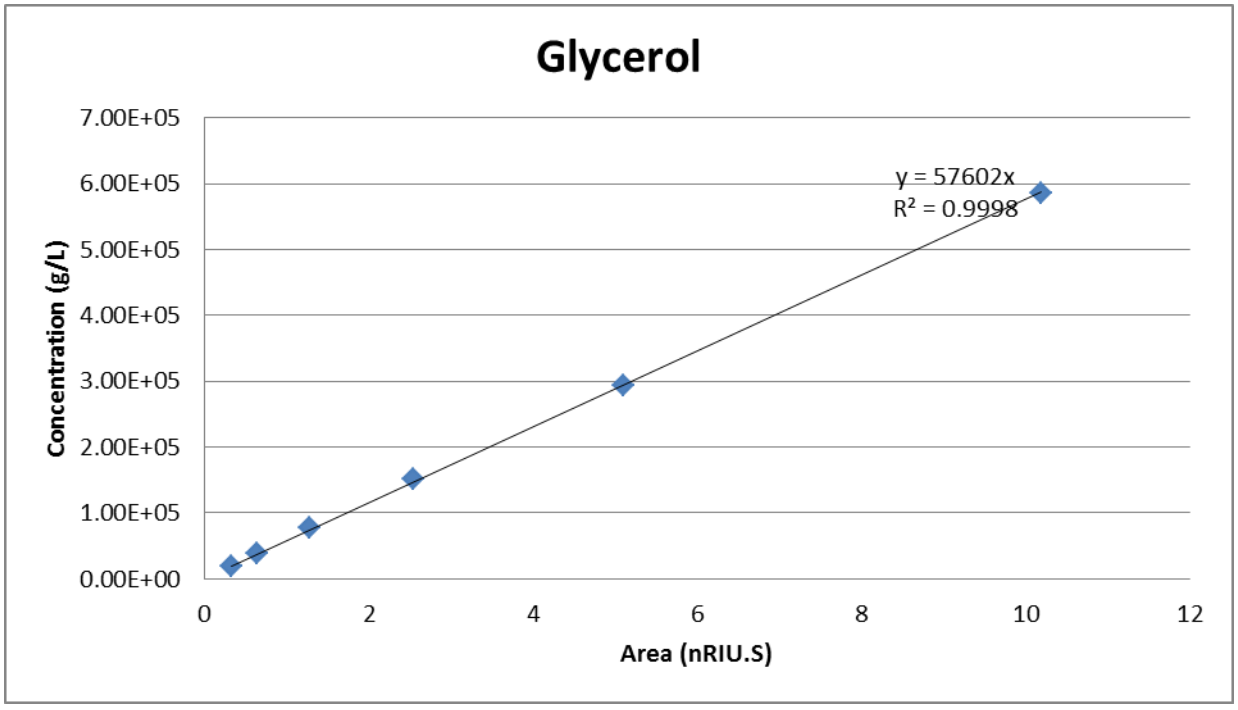

Figure A.0.3: Glycerol calibration curve

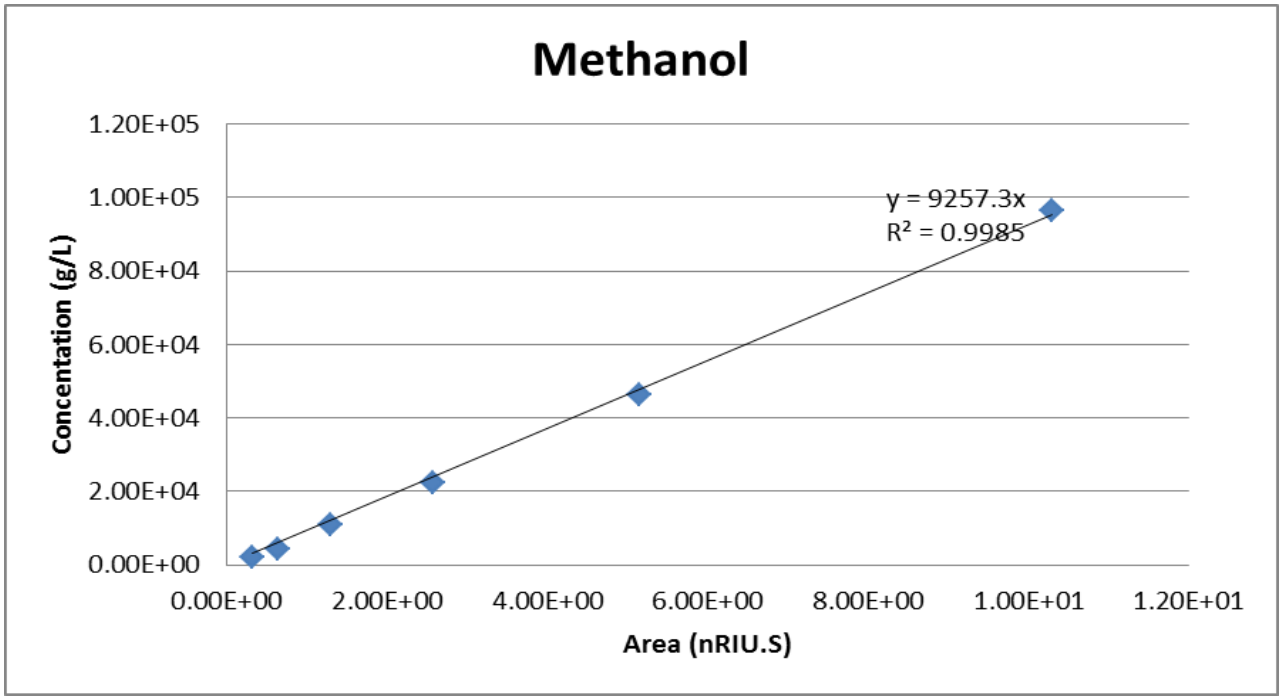

Figure A.0.4: Methanol calibration curve

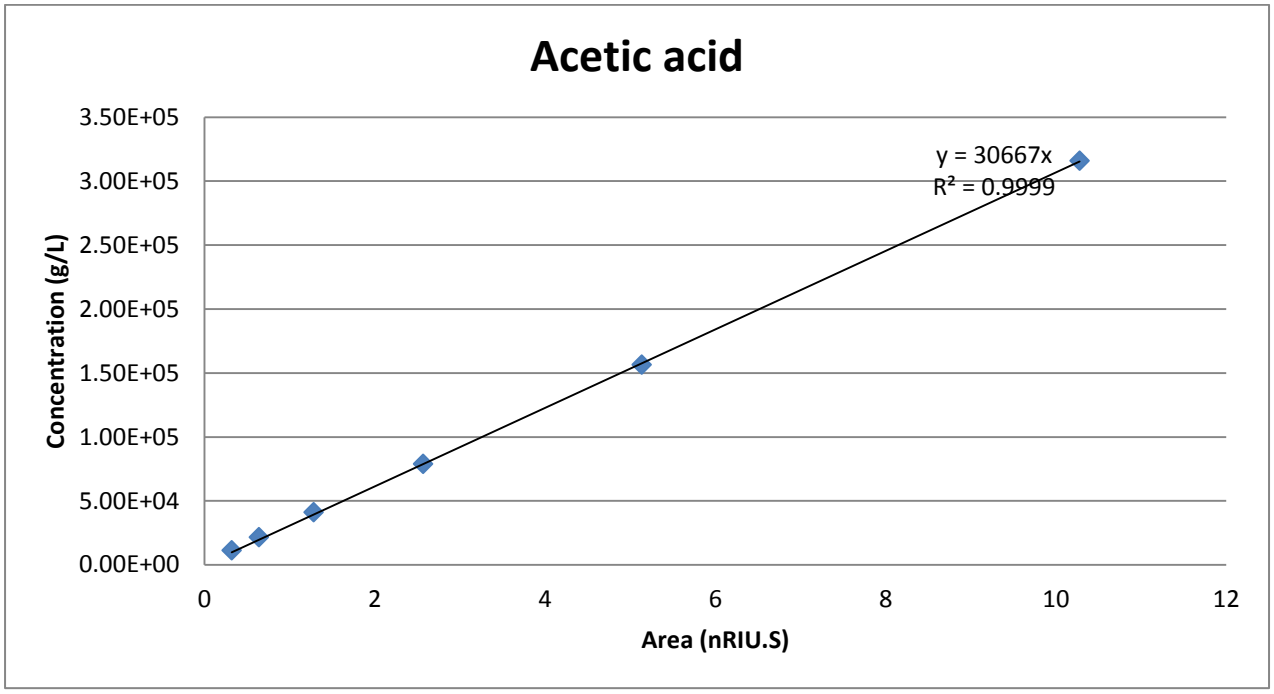

Figure A.5: Acetic acid calibration curve

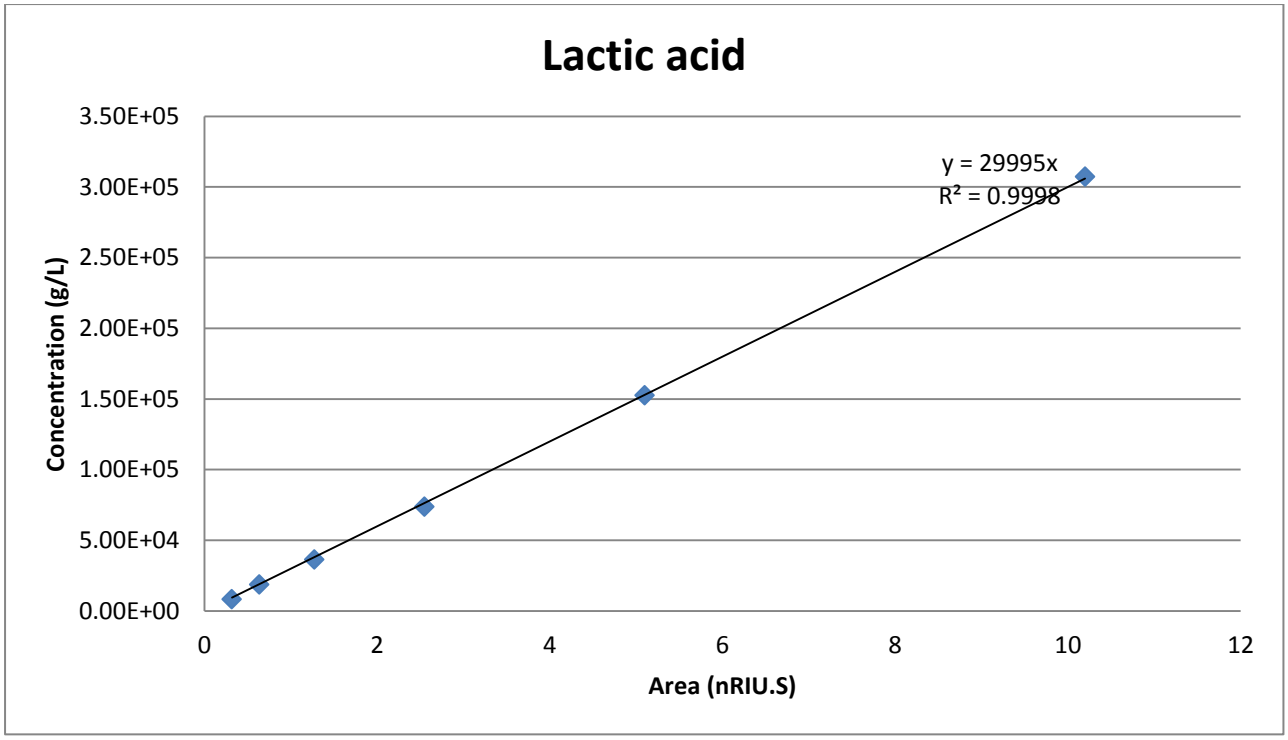

Figure A.0.7: Lactic acid calibration curve

A3 GC analysis

Biodiesel analysis: A 100 µL biodiesel sample was transferred into a sample vial and the mass was recorded. An internal standard (IS)(methyl nonanoate) (20 µL) was added to the biodiesel sample and the mass of the mixture was recorded.

mIS = mcombined - mbiodiesel

The mixture of biodiesel and IS was diluted to approximately one mL using dichloromethane (DCM). The mixture was vortexed and analysed by GC.

Sunflower oil analysis: A 100 µL sunflower oil sample was mixed with a Trimethylsulfonium hydroxide solution (TMSH) (100 µL). After vortexing the mixture, 10 µL of dodecane was added and then the mixture was analysed by GC.

.

Table A3.1 : Data used for the determination of the sunflower oil molecular weight

Name	Formulas	Areas	Mass fraction	Molecular weight $g.mol^{-1}$
Palmitic	C16:0	3019.58081	0.29	256.42
Stearic	C18:0	358.74162	0.02	284.48
Oleic	C18:1	5626.95916	0.31	282.46
Linoleic	C18:2	6.81E+03	0.38	280.45
Unknowns	/	2070.22079	0.00	/
Glycerol				92.09
Water				18.02

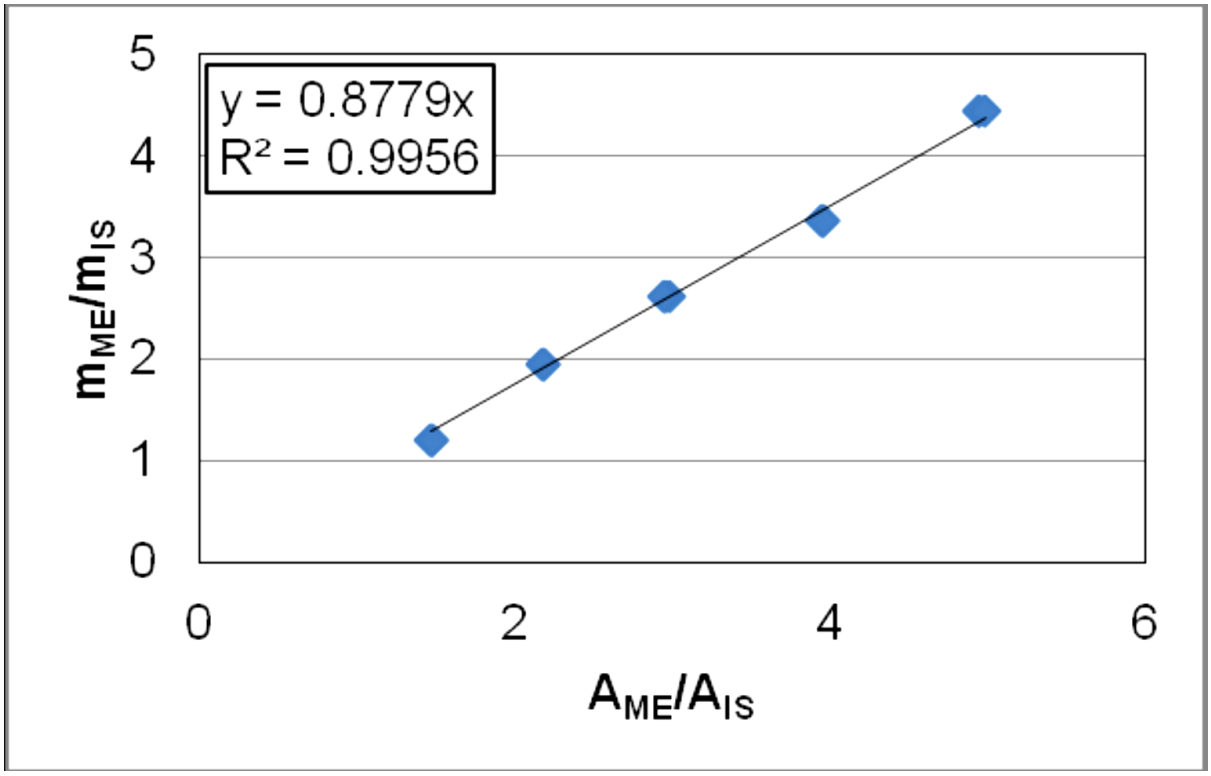

Figure A.0.1: Calibration curve of C18:2.

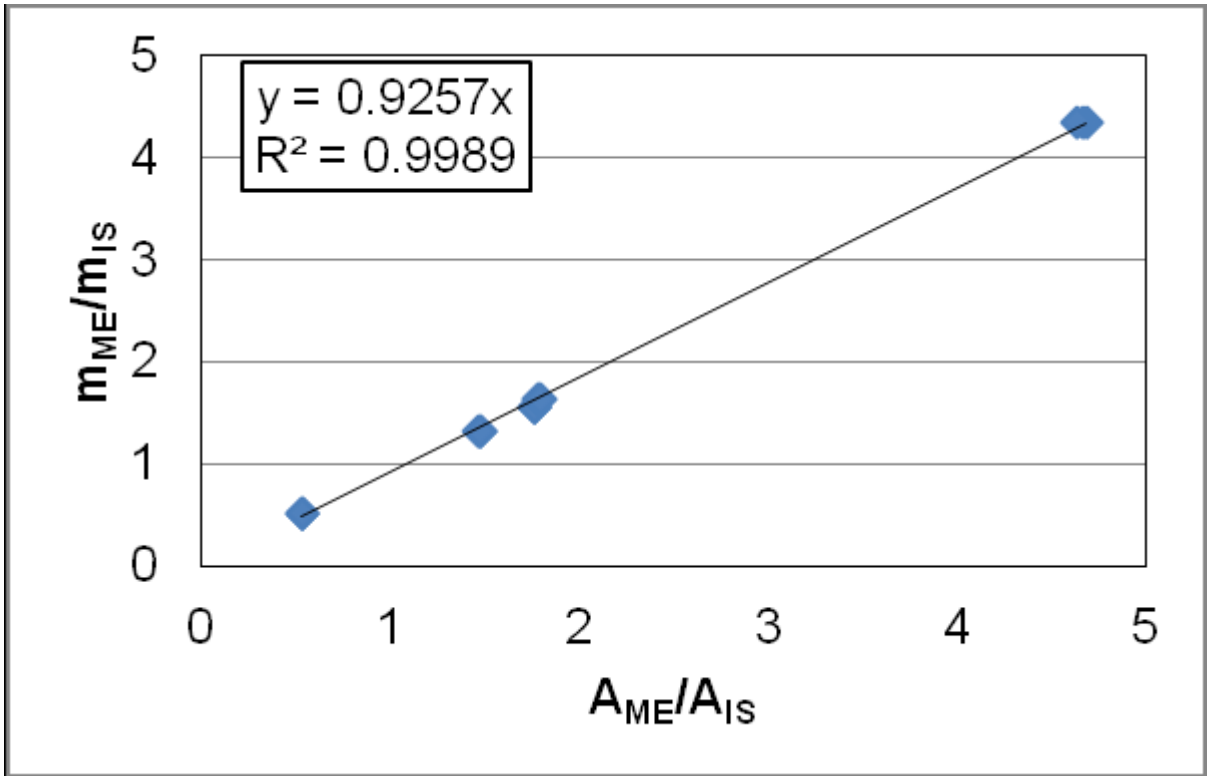

Figure A.0.2: Calibration curve of C16:0

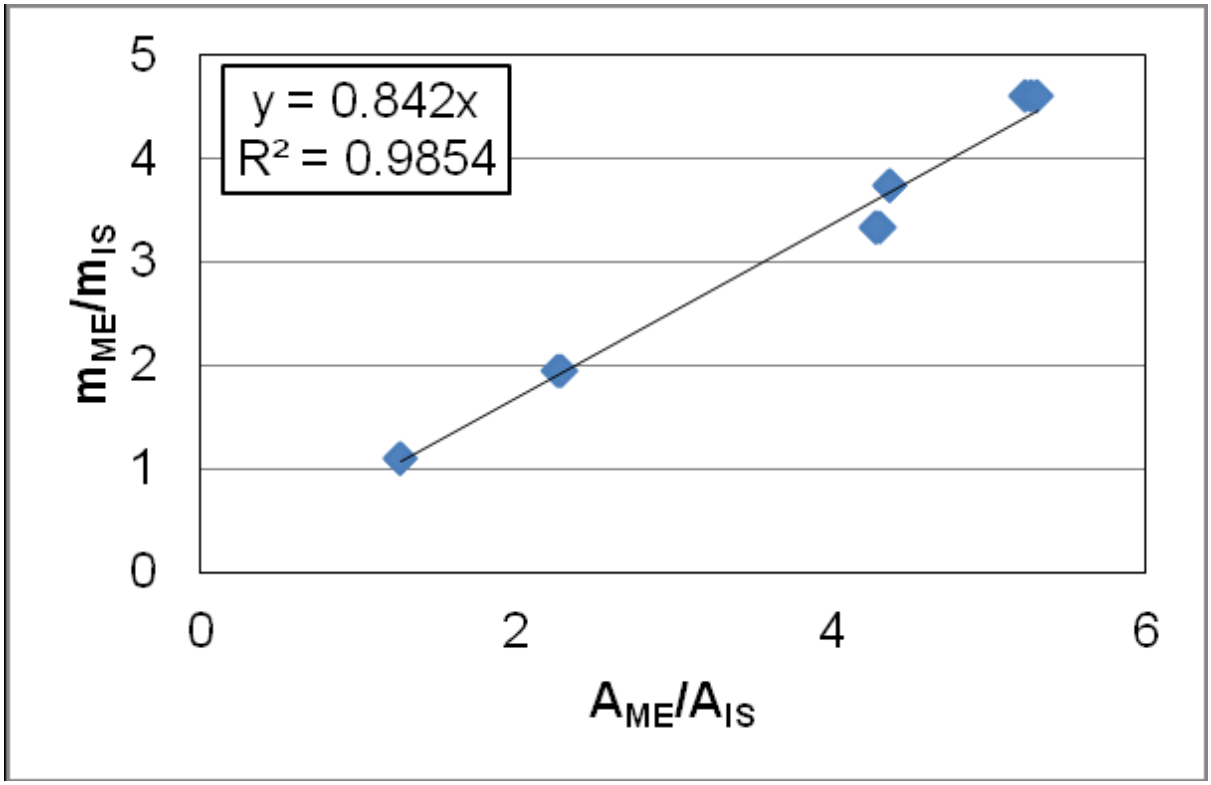

Figure A.0.3: Calibration curve of C18:0.

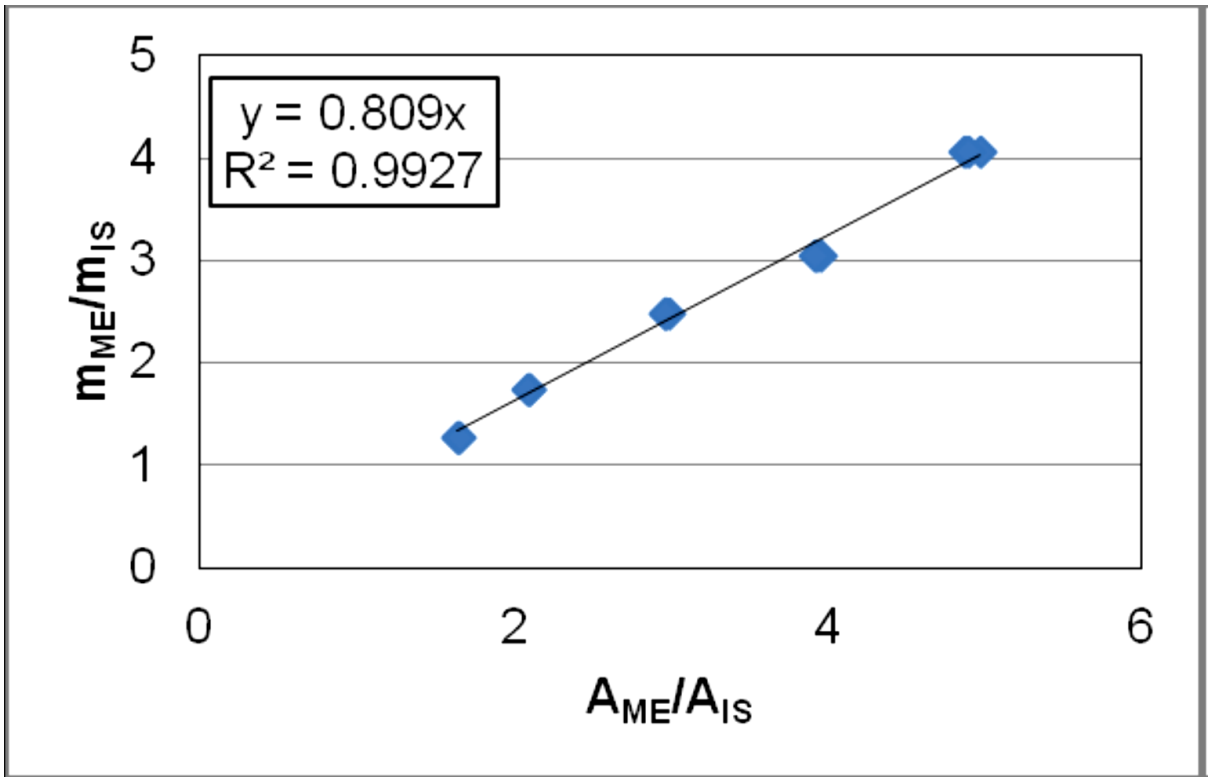

Figure A.0.4: Calibration curve of C18:1

B1 Introduction

All the calculations used in this study are shown in this section. The calculations used to quantify the fatty acids for biodiesel production are shown in Section B2. The calculations to determine the concentration of the produced products are shown in Section B3. The productivity obtained during fermentation is shown in Section B4. The experimental error calculations are described in Section B5.

B2 Molecular weight of Oil

$$X16:0 + X18:0 + X18:1 + X18:2 + Xunk = 1 \quad (1)$$

$$\text{Therefore: } X16:0 = 1 - X18:0 - X18:1 - X18:2 - Xunk \quad (2)$$

The mass fraction is given in terms of areas relative to the internal standard (dodecane);

$$X16:0 = 1/ K16:0 (A16:0 / Adod);$$

$$X18:0 = 1/ K18:0 (A18:0 /Adod);$$

$$X18:1 = 1/ K18:1 (A18:1 / Adod);$$

$$X18:2 = 1/ K18:2 (A18:2 / Adod) \quad (3)$$

According to the calibration curves: K16:0 = 1.2685; K18:0 = 1.2495; K18:1 = 1.185; K18:2 = 1.1952;

Kunk = 1 Therefore $X18:0 = 1/ (1 + X16:0/ X18:0 + X18:1/ X18:0 + X18:2/ X18:0 + Xunk/ X18:0)$

Replacing the mass fractions with the equations in (3) and substituting with values of the areas, X16:0 was obtained: X18:0 = 0.06; X18:1 = 0.17; X18:2 = 0.65; Xunk = 0.00 (can be neglected because it does not contribute to the total mass) and from (2) X16:0 = 0.12 the mass fractions together with molecular weight of each component were used to calculate an average molecular weight of fatty acids triglyceride.

Table B2.1: Areas and molecular weight of sunflower oil

	Area	Molecular weight (g/mol)
C 16:0	357.51917	256.42
C18:0	334.60086	284.48
C18:1	989.25916	282.46
C18:2	3645.51563	280.45

MWoil = 0.12MW16:0 + 0.06MW18:0 + 0.17MW18:1 + 0.65MW18:2 = 278.1499 g/mol

Calculations: The sunflower oil molecular weight was calculated in the following manner: MWoil = 3Avarage molecular weight + molecular weight glycerol – 3 molecular weight of water = 872.33 g/mol

Molar ratio of oil to methanol and catalyst loading

For 50 g of oil, number of moles was calculated using:

n = mass of oil/ MWoil = 80/ 857.9 = 0.058 mol

With noil/ nmeOH = 1/ 9 hence number of moles for methanol is nmeOH = 9 * noil Therefore mass of MeOH = 9 * noil x MWMeOH = 16.7 g with molecular weight of MeOH as 32.04 g/mol

Mass of KOH = 1.5% of mass oil = 0.75 g.

B3 Concentration Calculations

The calculations used to quantify the glycerol and products are shown in this section.

.

Glycerol concentration

$$\left[G_S \frac{g}{L}\right] = \frac{peak\ area}{slope} \times dilution\ factor$$

Where C_S is the concentration of any glycerol used.

1,3-propanediol concentration

$$\left[1{,}3-Pdo_{\,s}\frac{g}{L}\right]=\frac{peak\ area}{K-value}\times dilution\ factor$$

Where $1{,}3-PDO_S$ is the concentration of 1,3-propanediol.

Table B3.1: Concentration of 1,3-propanediol when fermenting with pure glycerol

Time (hours)	**Area (nRIU.s)**	**Concentration (g/L)**	**Productivity (g $l^{-1}h^{-1}$)**
		50 g/L	
6	3.74E+03	0.16	0.02
12	1.99E+05	8.51	0.71
24	4.17E+05	17.83	0.74
48			
		100 /L	
6	3.27E+03	0.14	0.02
12	8.96E+05	3.83	0.31
24	2.31E+05	9.86	0.41
48	3.15E+05	13.48	0.28

Table B3.2: Concentration of 1,3-propanediol when fermenting with washed crude glycerine

Time (hours)	Area (nRIU.s)	Concentration (g/L)	Productivity (g $l^{-1}h^{-1}$)
		50 g/L	
6	8.75E+04	3.74	0.62
12	2.04E+05	8.73	0.72
24	4.43E+05	18.89	0.75
48			
		100 g/L	
6	6.08E+03	0.26	0.04
12	1.65E+05	7.04	0.58
24	3.82E+05	16.34	0.68
48	4.23E+05	18.09	0.37
		150 g/L	
6	7.95E+03	0.34	0.11
12	1.16E+05	4.94	0.41
24	2.35E+05	10.05	0.41
48	2.75E+05	11.76	0.25

Table B3.3: Concentration of acid when fermenting with washed crude glycerine: acetic acid (AA), Lactic acid (LA) and butyric acid (BA)

Time (hours)	150 g/L			100 g/L			50 g/L		
	AA	LA	BA	BA-	LA	AA	LA	BA	AA
o	0	0	0	0	0	0	0	0	0
6	0.87	0.31	0.39	0.54	0.28	0.57	0.18	0.42	0.93
12	1.23	0.51	1.13	1.06	0.47	1.61	0.33	0.78	1.73
24	1.32	0.57	1.45	1.03	0.59	1.65	0.48	1.76	2.05
48	1.56	0.65	2.01	1.21	0.61	1.7	0	0	0

Table B3.4: Concentration of acid when fermenting with pure glycerol: acetic acid (AA), Lactic acid (LA) and butyric acid (BA)

Time (hours)	100 g/L			50 g/L		
	AA	LA	BA	LA-50-	BA-50-	AA-50-
0	0	0	0	0	0	0
6	0.32	0.27	0.47	0.11	0.43	0.69
12	1.16	0.41	1.19	0.26	0.58	1.56
24	1.48	0.67	1.55	0.29	0.84	1.97
48	1.64	0.71	1.92	0	0	0

Table B3.5: Content of glycerol

Glycerol sources	Glycerol Concentration		
	50 (g/L)	100 (g/L)	150 (g/L)
Crude glycerine (g)	24.673	49.267	78.030
Washed crude glycerine (g)	27.648	55.979	81.777
Pure glycerol (g)	49.401	98.802	148.205

B4 Productivity

$$Productivity = \frac{[1{,}3 - propaediol\ g/L]}{Fermentation\ Time}$$

Table B4.1: Productivity values obtained from fermentation of pure glycerol and washed crude glycerine

Time (hour)	Pure glycerol 50 g/L	Pure glycerol 100 g/L	Washed crude glycerine 50 g/L	Washed crude glycerine 100 g/L	Washe glyceri
	(g $l^{-1}h^{-1}$)	(g $l^{-1}h^{-1}$)	(g $l^{-1}h^{-1}$)	(g $l^{-1}h^{-1}$)	(g $l^{-1}h^{-1}$
6	0.02	0.02	0.62	0.04	0.11
12	0.71	0.31	0.72	0.58	0.41
24	0.74	0.41	0.75	0.68	0.41
48		0.28		0.37	0.25

B5 Error Calculation

The experimental error was calculated using the following equations:

$$\%Error = \frac{confidence\ limit}{\bar{x}} \times 100$$

Where $\bar{x}$ is the average?

The confidence limit was calculated using equation:

$$confidence\ limit = \bar{x} \pm \frac{\sigma}{\sqrt{n}}$$

The standard deviation must be determined to calculate the 95% confidence interval using equation:

$$\sigma = \sqrt{\frac{\sum(\bar{x} - x)^2}{n - 1}}$$

Where *x* is the sample mean, $\bar{x}$ is the sample mean and *n* is the sample size.

Table B5.1: Experimental errors associated with fermentation samples of 50 g/L pure glycerol, washed crude glycerine and pure glycerol under 2 minutes of ultrasound for the production of 1,3-propanediol.

Fermentation	Sample 1 (g/L)	Sample 2 (g/L)	Sample 3 (g/L)	Mean	Standard Deviation	Error (%)
50 g/L of	17.63	17.74	17.83	17.73	0.10017	0.5
50 g/L of	18.85	18.89	18.76	18.8	0.06658	0.3
50 g/L 2 min US	16.69	16.93	16.72	16.78	0.011547	0.8

Table B5.2: Experimental errors associated with fermentation of 50 g/L pure glycerol for the production of acetic acid (AA), lactic acid LA and butyric acid (BA) samples

Fermentation	Sample 1 (g/L)	Sample 2 (g/L)	Sample3 (g/L)	Mean	Standard Deviation	Error (%)
50 g/L - BA	0.84	0.82	0.87	0.84	0.02	2.3
50 g/L –AA	1.87	1.98	1.89	1.88	0.01528	0.8
50 g/L –LA	0.29	0.31	0.30	0.31	0.02646	2.5

Table B5.3: Experimental errors associated with fermentation of 50 g/L washed crude glycerine for the production of acetic acid (AA), lactic acid (LA) and butyric acid (BA)

Fermentation	Sample 1 (g/L)	Sample 2 (g/L)	Sample3 (g/L)	Mean	Standard Deviation	Error (%)
50 g/L -AA	2.07	2.05	2.03	2.05	0.02	0.9
50 g/L -LA	0.48	0.47	0.45	0.46	0.01527	2.9
50 g/L – BA	1.78	1.74	1.76	1.76	0.02	0.8

C1 Introduction

This section contains the additional information with respect to fermentation, concerning the microorganism and all chemicals used during fermentation. Information about *C. diolis* is outlined in Section C2 and the change in pH during the fermentation process is also represented in Section C2. All chemicals used for this investigation are outlined in Section C3

C2 Information about *C. diolis*.

Table C2.1: The information about *Clostridium diolis* DSM 15410

Organism information	
Organism name	*Clostridium diolis* DSM 15410
Biosafety level	Level 1
Culture collection ID	DSM 15410
Domain	Bacterial
Genus	Clostridium
NCBI Class	*Clostria*
NCBI Family	*Clostriaceace*
NCBI Kingdom	Bacteria
Oder	*Clostridate*s
Phylum	*Firmicutes*
Species	*Clostridium diolis*
Strain	DSM 15410
Gram Stain	Gram positive
Habitant	Decaying plant
Metabolism	Propionate metabolism

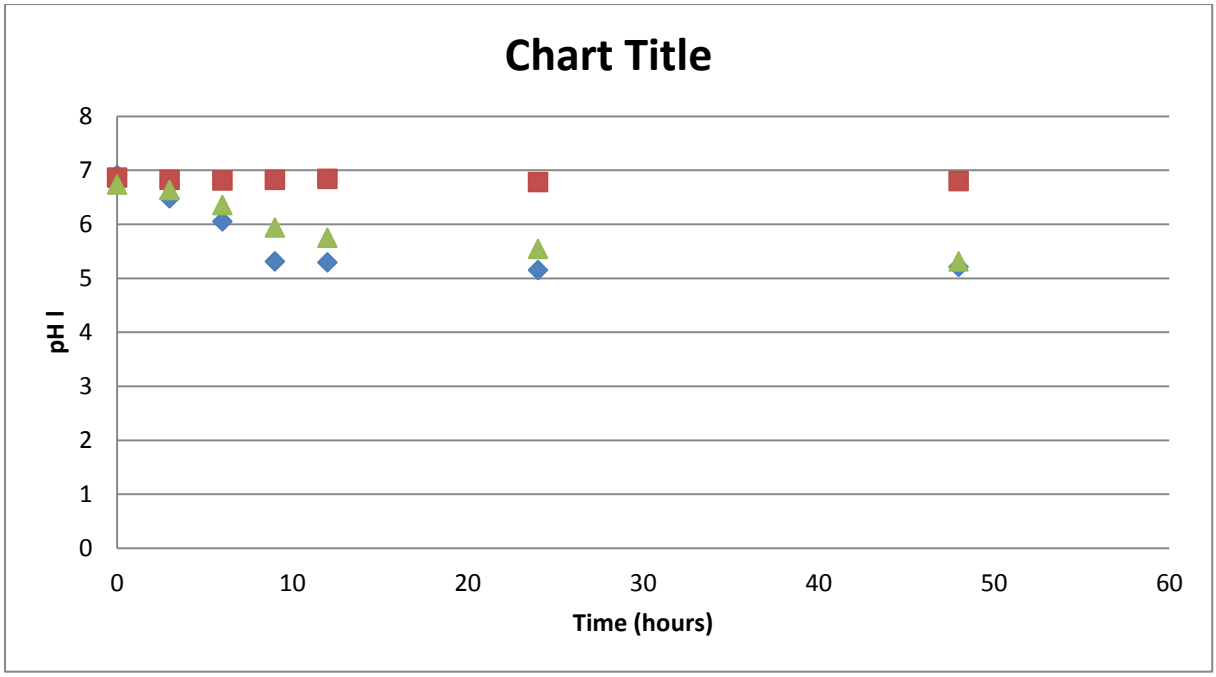

Figure C.0.1: pH levels when fermenting with 50 g/L of glycerol sources, (■ - crude glycerine), (▲ - pure glycerol) and (♦ - washed crude glycerine)

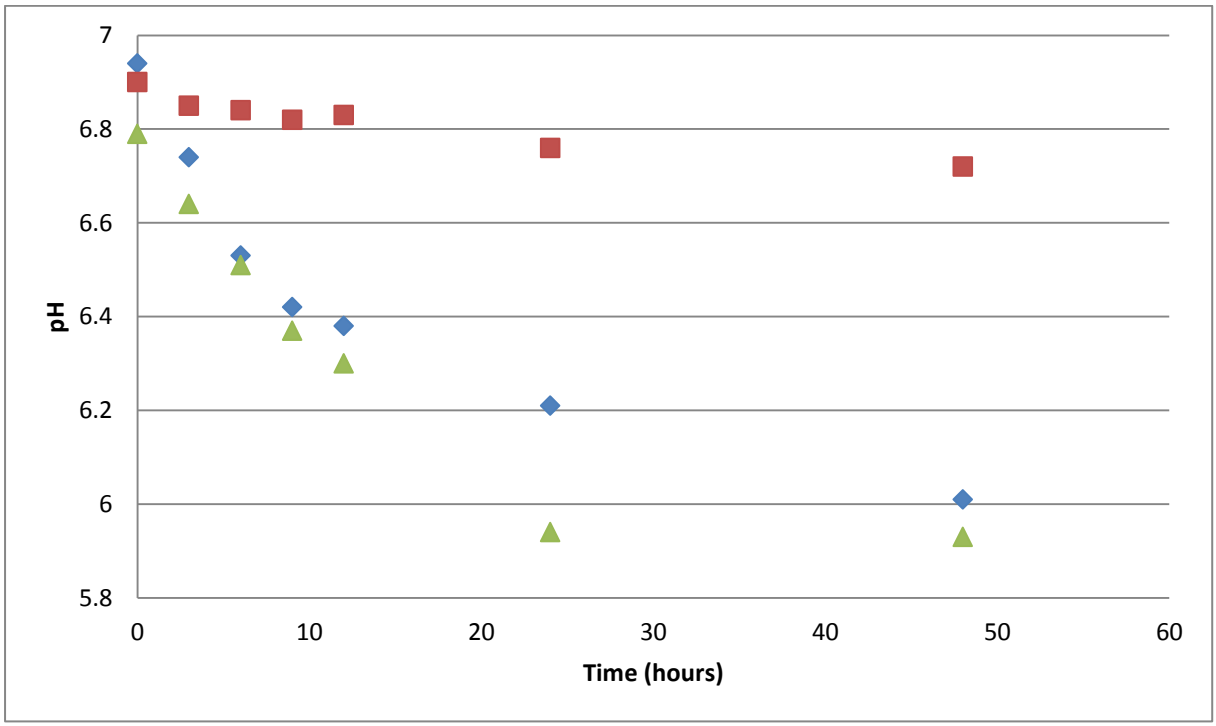

Figure C.0.2: pH levels when fermenting with 100 g/L of glycerol sources, (■ - crude glycerine), (▲ - pure glycerol) and (♦ - washed crude glycerine)

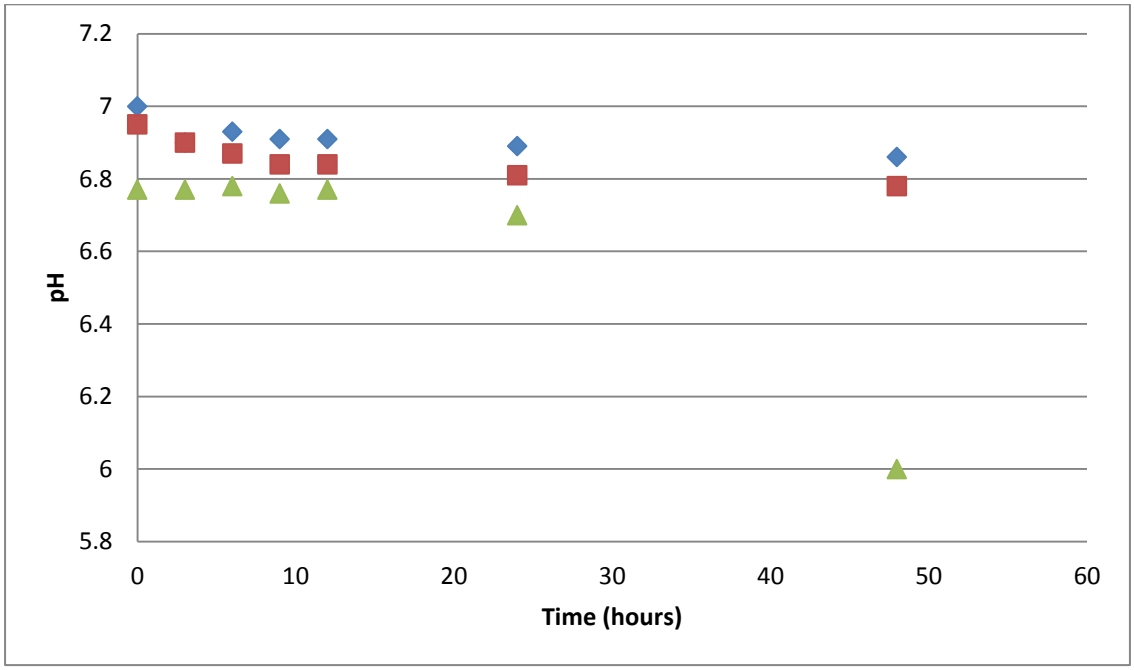

Figure C.0.3: pH levels when fermenting with 150 g/L of glycerol sources (■ - washed, crude glycerine), (▲ - pure glycerol) and (♦ - crude glycerine)

Table C2.2: List of the chemicals used in the study

Component	**Purity**	**Supplier**	**Purpose**
Sunflower oil	N/A	Chrisper	Biodiesel production
Methanol	97	Sigma-aldrish	Biodiesel production
Potassium Hydroxide (KOH)	N/A	Sigma-Aldrish	Biodiesel production
Petroleum ether	95	Sigma-Aldrish	Glycerol pretreatment
Beef extract	N/A	Bio-lab	Growth media nutrient
Peptone powder	N/A	Fluka Analytical	Growth media nutrient
Solid Chloride	N/A	Merck	Growth media nutrient
Dextrose	N/A	Accumedia	Growth media nutrient

Yeast Extract	N/A	Merck	Growth media nutrient
Solid acetate	N/A	Merck	Growth media nutrient
Soluble starch	N/A	Merck	Growth media nutrient
L-Cysteine	N/A	Sigma	Growth media nutrient
Agar	N/A	Merck	Growth media
Potassium phosphate	98	Merck	Growth media nutrient
Dipotassium phosphate	N/A	Fluka Analytical	Growth media nutrient
Calcium carbonate	N/A	Sigma-Aldrish	Growth media nutrient
Magnesium sulphate	98	Sigma-Aldrish	Growth media nutrient
Calcium chloride	N/A	Merck	Growth media nutrient
Iron sulphate	N/A	Sigma-Aldrish	Growth media nutrient
Ammonium sulphate	N/A	Sigma-Aldrish	Growth media nutrient
Zinc chloride	N/A	ACE PTY.LTD	Growth media nutrient
Boron	N/A	ACE PTY.LTD	Growth media nutrient
Cobalt chloride	90	Sigma-Aldrish	Growth media nutrient
Nickel chloride	99	Sigma-Aldrish	Growth media nutrient
Hydrochloric acid	98	Sigma-Aldrish	Growth media nutrient
1,3-propanediol	99.9	Sigma	Product
Ethanol	99.9	Rochelle	Product

		Chemicals	
Citric acid	98	Sigma-Aldrish	Product
Lactic acid	98	Sigma-Aldrish	Product
Butyric acid	97	ACE PTY.LTD	Product
Succinic acid	98	Labchem	Product
Acetic acid	98	Sigma-Aldrish	Product
Glycerol	98	ACE PTY.LTD	Carbon source
Nitrogen gas	98	Afrox	Anaerobic fermentation