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# Exploring whole cell systems utilising Escherichia coli Nissle on gel carriers for biodiesel production

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**Exploring whole cell systems utilizing *Escherichia coli* Nissle on gel carriers for biodiesel production**

by

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**Dissertation**

Submitted in fulfilment of the requirements for the degree **Master**

**of Science**

in

**Molecular and Cell Biology**

in the Faculty of Science, University of the Witwatersrand, Johannesburg, South Africa

Supervisor: Prof. Karl Rumbold

Co-supervisor: Prof. Kevin Harding

December 2022

# DECLARATION

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



Brendan Sholto Booysen

879906

12th day of December 2022

# Abstract

Worldwide energy shortages are becoming the major factor in curtailing the development of the world economy. Due to this, there has been a sustained increase in research into biofuels such as biodiesel due to the ease of production. However, high amounts of co-produced crude glycerol waste is becoming a critical issue for biodiesel production. A favourable outlet for this crude glycerol waste is its use as a renewable feedstock for microbes such as *Escherichia coli*. In this scenario, the manufacture of biofuels and other chemicals is done through a bioconversion process. This study aims to use processes such as whole cell biocatalysis and biofilm formation on cheap, gel-like carriers to determine whether *Escherichia coli* Nissle can use glycerol as a feedstock to produce biodiesel. The study concluded that, although EcN does not produce enough fatty acids to initiate a reaction to produce biodiesel, it can produce enough bioethanol and hydroxide catalyst to convert outside-added oil into biodiesel, at the same rate as the former, with much cleaner glycerol than the conventional process. The introduction of genetic modification to *Escherichia coli* Nissle to enable increased fatty acid production is an avenue to explore to make this technology more efficient and cost-effective for biodiesel production using *Escherichia coli* Nissle.

# DEDICATION

This work is dedicated to my partner, Sona Mahendra – For dealing with the mess that came with completing this project part time – It means the world to me.

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# Contents

DECLARATION ..... ii

DEDICATION ..... iv

ACKNOWLEDGEMENTS ..... v

List of Tables ..... x

List of Figures ..... xi

Chapter 1 ..... 1

1.1 Introduction ..... 1

1.1.2 Research objectives ..... 4

1.2 Literature review ..... 5

1.2.1 Biofuels ..... 5

1.2.2 Biodiesel ..... 7

1.2.3 Biodiesel production ..... 8

1.2.4 Trans-esterification ..... 8

1.2.5 Enzymatic biodiesel production ..... 9

1.2.6 Glycerol ..... 10

1.2.7 Various value uses for glycerol ..... 11

1.2.8 Glycerol bioconversion ..... 12

1.2.9 *Escherichia coli*-based glycerol fermentation ..... 13

1.2.10 *Escherichia coli* ..... 16

1.2.11 Whole cell biocatalyst system ..... 16

1.2.12 Whole cell biocatalysis compared to chemical catalysis ..... 18

1.2.13 Biofilm and carriers ..... 19

1.2.14 Initial adhesion ..... 19

1.2.15 Early development of biofilm ..... 19
1.2.16 Maturation ..... 20
1.2.17 Dispersion ..... 21
Chapter 2 ..... 22
2.1 Methods ..... 22
2.1.1 Preparation of *Escherichia coli* Nissle bacterial strain ..... 22
2.1.2 *Escherichia coli* Nissle Growth Curve with LB Media ..... 22
2.1.3 Production of competent cells and transformation of *Escherichia coli* Nissle ..... 23
2.1.4 Competent cells ..... 23
2.1.5 Transformation of *Escherichia coli* Nissle ..... 23
2.1.7 Plasmid extraction from the transformed EcN ..... 25
2.1.8 Restriction enzyme digestion of extracted plasmid ..... 25
2.1.9 Agarose gel electrophoresis ..... 26
2.1.10 EcN growth curve with LB media and M9 media ..... 27
2.1.11 Preparation of carriers ..... 27
2.1.11.1 Preparation of the calcium alginate carrier ..... 27
2.1.11.2 Preparation of water pebble carriers ..... 27
2.1.11.3 Preparation of the glass balls carriers ..... 28
2.1.11.4 Preparation of the agarose gel carriers ..... 28
2.1.12 *Escherichia coli* Nissle biofilm growth ..... 28
2.1.13 EcN biofilm imaging ..... 29
2.1.14 Biofuel production and determination ..... 29
2.1.14.1 Growth period of EcN biofilm ..... 29
2.1.14.2 Fractional distillation of microbially produced media ..... 29
2.1.14.3 Proton NMR of distilled ethanol ..... 30
2.1.15 Biodiesel production: ..... 30

2.1.15.1 Control biodiesel formation using standard procedure: ..... 30
2.1.15.2 Biodiesel formation using media produced from EcN as an ethanol substitute: ..... 30
2.1.15.3 Biodiesel formation using media produced from EcN as an ethanol and catalyst substitute: ..... 30
2.1.15.4 Biodiesel formation using EcN produced media as the oil, ethanol and catalyst substitute: ..... 31
2.1.16 Theoretical up-scaling: Biofuel production ..... 31
Chapter 3 ..... 33
3.1 Results ..... 33
3.1.1 *Escherichia coli* Nissle strain preparation ..... 33
3.1.2 Growth curve of untransformed EcN in LB media ..... 34
3.1.3 Agarose gel electrophoresis ..... 34
3.1.4 Growth curve of transformed EcN in LB media ..... 35
3.1.5 Growth curve of transformed *Escherichia coli* Nissle in M9 media ..... 36
3.1.6 Preparation of carriers for EcN biofilm growth ..... 37
3.1.7 Biofilm growth ..... 37
3.1.8 EcN biofilm imaging ..... 37
3.1.9 Biofuel production and determination ..... 40
2.1.12 Biodiesel formation test ..... 44
3.1.17 Theoretical up-scaling ..... 49
Chapter 4 ..... 51
4.1 Discussion ..... 51
4.1.1 Growth curve (untransformed) ..... 51
4.1.2 Agarose gel electrophoresis ..... 52
4.1.3 Growth curve differences between the transformed EcN and the untransformed EcN ..... 52
4.1.4 Growth curve differences M9 media and LB media for the transformed EcN ..... 53
4.1.5 Differences between the carriers and biofilm formation ..... 54
4.1.6 Difference between biodiesel produced from the carriers ..... 56

4.1.7 Theoretical up-scaling ..... 58

4.1.8 Conclusion ..... 59

4.1.9 Future work ..... 60

Chapter 5 ..... 60

5.1 References ..... 60

# List of Tables

Table 1.1 A comparison of advantages and disadvantages of whole cell biocatalysis compared to chemical catalysis, showing pros and cons of each method (Fukuda etal,2008) ……18

*Table 3.1: The production of ethanol and the calculated results from the fractional distillation to determine the amount of ethanol that was distilled from the fermented sample…… 42*

*Table 3.2: The initial components of biodiesel production was added together to achieve the final biodiesel and glycerine for both olive oil and grapeseed oil……45*

Table 3.3 *The initial components* of biodiesel production *that was added together to achieve the final biodiesel, soap, and glycerine……46*

*Table 3.4: The initial components of biodiesel production that was added together to achieve the final biodiesel and glycerine…… 47*

*Table 3.5: The initial components of biodiesel production that was added together to achieve the final biodiesel and glycerine……48*

# List of Figures

*Figure 1.1. Reaction mechanism of triglyceride transesterification……9*

*Figure 1.2: Aerobic glycerol metabolism in E. coli……14.*

*Figure 1.3: Diagram which displays glycerol metabolism in E. coli as well as other members of Enterobacteriaceae family……15*

*Figure 1.4: Showing an example of how a whole cell biocatalyst system would function and overall ways for optimization……17*

*Figure 2.1. The plasmid pIRES2-EGFP……24*

*Figure 2.2 Restriction enzyme sites on the plasmid pIRES2-EGFP map……26*

*Figure 2.3: CEBER Modeller showing all the inputs that were selected for the CEBER Modeller excel sheet……33*

*Figure 3.1: Growth of Escherichia coli Nissle (EcN) on two different types of growth media for verification……..34*

*Figure 3.2: The growth curve of Escherichia coli Nissle (EcN) in LB media showing the absorbance (OD600) over a period of time(hours)……..34*

*Figure 3.3: A 1% Agarose gel of the extracted plasmid showing two sites that have gone through restriction enzyme digestion……35*

*Figure 3.4: The growth curve of Kanamycin transformed Escherichia coli Nissle (EcN) in LB media showing the absorbance (OD600) over a period of time (hours)……36*

*Figure 3.5: The growth curve of Kanamycin transformed Escherichia coli Nissle (EcN) in M9 media showing the absorbance (OD600) over a period of time (hours)……37*

*Figure 3.6: Microscopic image of the different carriers formulated……37.*

*Figure 3.7: Microscopic image of the Escherichia coli Nissle (EcN) biofilm on the calcium alginate carrier……38*

*Figure 3.8: Microscopic image of the Escherichia coli Nissle (EcN) biofilm on the 1% agarose gel carrier……39*

*Figure 3.9: Microscopic image of the Escherichia coli Nissle (EcN) biofilm on the water pebble carrier……40.*

*Figure 3.10: The proton NMR of fraction distillation obtained from the distilled EcN media utilizing a water pebble as the carrier……43*

*Figure 3.11: The proton NMR of fraction distillation obtained from distilled EcN media utilizing a calcium alginate and 1% agarose as the carrier……..44*

*Figure 3.12: Biodiesel control production via the well-known method of producing biodiesel……..45*

*Figure 3.13: Biodiesel production* **using the media produced from EcN as an ethanol substitute……..47**

*Figure 3.14: Biodiesel production using* **the media produced from EcN as an ethanol and catalyst substitute…….48**

*Figure 3.15: Biodiesel production using* **the media produced from EcN as the oil, ethanol, and catalyst substitute.49**

*Figure 3.16: CEBER modeller of the theoretical product composition, input, output, and waste of materials for the upscaling of ethanol and biodiesel on a commercial scale……..50*

*Figure 4.1: Microscopic image of the Escherichia coli Nissle (EcN) biofilm on the calcium alginate carrier……51*

*Figure 4.2: The growth curve of transformed vs untransformed Escherichia coli Nissle (EcN) in LB media showing the absorbance (OD600) over a period of time (hours)……52*

**Figure 4.3: The growth curve of transformed *v* in LB media and M9 media showing the absorbance (OD600) over a period of time (hours)……53**

*Figure 4 .4: Microscopic image of the Escherichia coli Nissle (EcN) biofilm on the water pebble carrier……54*

*Figure 4.5. The double water-washed results of test 1 and 2 biodiesel production……57*

# Chapter 1
# 1.1 Introduction

There are three main problems plaguing the world when it comes to energy availability: the fight against climate change, air pollution and high fuel prices. A key question is what today's energy crisis means for fossil fuel investments if we are still to achieve our collective climate goals. Are today's sky-high fossil fuel prices a signal to invest in additional supply or a further reason to invest in alternatives?

The current energy sources are divided into either renewable based (such as biomass, solar, hydropower, wind, nuclear or geothermal energy) or fossil based (such as natural gas, coal or petroleum) (Sevim, 2010). Worldwide energy shortages is becoming the major factor in curtailing the development of the world economy. This is especially a problem since the overall global energy demand is growing (Sevim, 2010).

Energy usage is connected to a variety of social issues which include population growth, poverty and urbanization. Although these issues affect the demand for energy, there is a two-part problem: The quantity and quality of energy services, and how they are delivered.

One of the biggest industries that demands a high volume of energy is the transportation industry. There are 1.446 billion on-road vehicles that consume mostly gasoline and diesel fuels, releasing 5.9 gigatons of carbon dioxide into the atmosphere (Frey, 2018). This is clearly a large problem considering the climate change issues we face as humanity.

In the past decade, there has been technological advancements that have attempted to reduce this problem by making vehicles that run on various renewable energy sources, like electric cars (Ambaye et al., 2021). However, due to a high level of anthropogenic activities causing an increase of fossil fuel demand coupled with the need to increase our sources of renewable energy, has attracted scientists and researchers to develop new alternative energy sources such as biofuels (Ambaye et al., 2021). This demand has led to the biofuel and renewable sources global markets to develop in order to support the various challenges being presented by a lack of energy for a growing population (Höök and Tang, 2022).

The greatest benefit of fossil fuel replacement would be the reduced environmental impact of carbon emissions. Biofuels are an attractive alternative since they burn cleaner and faster than fossil fuels (Reijnders, 2006).

Type of biofuels that can be produced are ethanol, biodiesel, and other "green" fuels. This spectrum of biofuels can replace fossil fuels such as petroleum, propane, coal, and natural gas (Datta, Hossain and Roy, 2022). Biofuels can also be classified in another way. This involves separating them into two types: primary and secondary. Biofuels that are part of the primary type and are in reference to organic materials employed for uses like heating and electricity (Mahapatra et al.,2021). This category involves biomass such as wood chips and pellets, mainly used to make fire. These are usually kept in their natural state. Biofuels of the secondary type refer to biomass that needs to be processed in a way to produce liquid biofuels like ethanol and biodiesel. These types of biofuels are mostly used in the transportation industry (Mahapatra et al.,2021).

Biofuels are an attractive option also because these are biomass-based energy sources that are used for a variety of daily task such as transportation, electricity, and heat fuels, which have the potential to be carbon dioxide neutral, as they and recycle carbon atoms (Mahapatra et al.,2021). In addition, this growing demand for biobased fuel could provide economic opportunities to more people than the current fossil fuel industry (Kartha and Larson, 2000).

Since 2019, the total worldwide production of biofuels has increased, especially in key producing regions such as Brazil and the increased supply resulted in significantly reduced market prices for ethanol and biodiesel, which is a critical component for general adoption of the use of biofuels (Bioenergy - Fuels & Technologies - IEA, 2022). The increase in prices in other components needed in biodiesel manufacture like vegetable oils also ate into already small margins. Prices hikes in sugar prices also resulted in a smaller margin for profit for ethanol production. Despite this, government policies played an important role in the reduced pricing of biofuels overall. The governments approach to assist the biofuel sector was through enabling policies like subsidies, taxes incentives and regulated mandates. These government policies have enabled the sustained demand for biofuels by also incorporating policies like obligatory blending (Bioenergy - Fuels & Technologies - IEA, 2022).

The Covid-19 pandemic presented additional problems to the biofuel industry. This added onto other bottlenecks like increasing costs of feedstocks. Despite this however, there are initiatives and policies that are being discussed by the governments of major biofuel markets to support the biofuel

industry. This is not only to promote regional economic growth in the respective countries, but also working towards a more sustainable world. The increasing demand in biofuels can be shown by the fact that between the years 2010 and 2019, global biofuel use increased 5% on average per year. However, the path to 'Net Zero' requires a 14% average annual growth between 2021 and 2030 (Bioenergy - Fuels & Technologies - IEA, 2022). Strong and efficient policies are vital to reach this goal but the adoption of similar milestones and proposals to transition to a more biofuel-based economy is starting to spread across countries. This is highly encouraging. A great example is the consideration or implementation of policies that could accelerate biofuel demand and adoption in regions like North America, Europe and Asia (Bioenergy - Fuels & Technologies - IEA, 2022).

A critical issue facing biofuels in the future is the cost of the various raw feedstocks (such as oil), which are essential to enable large scale industrial manufacture. These raw materials are currently not economical enough and reducing this price is a key way in ensuring the future of biofuels and their ability to be a viable alternative fuel source. Secondly, with the increasing pressure to drop global carbon emissions, the application of biofuels is a plausible alternative to oil in the not-too-distant future (Bioenergy - Fuels & Technologies - IEA, 2022). The use of biofuels as an alternative energy source might be a competitive advantage to companies in the fuel industry by being less-flammable, better lubricating and by being produced from a wide range of materials compared to that of fossil fuels (Cheteni, 2017).

Despite the dire need for alternative fuel solutions, large scale biofuel uptake will only be possible if biofuel prices are in similar ranges of fossil fuels. Being able to undercut the increasing oil prices will become key in driving the demand for biofuels as well. The true positive impacts of biofuels will be felt when countries globally start to use them collectively. This kind of large-scale adoption is key to making the drastic changes needed to ensure the harmful effects of climate change (Bioenergy - Fuels & Technologies - IEA, 2022).

Despite the positive strides being made by biofuel technology, the biggest barrier to faster growth in biodiesel production, is the co-production of crude glycerol waste in the production process. The crude glycerol waste produced is approximately 0.08 kg of crude glycerol per litre of biodiesel, which is around 10% by weight (Bioenergy - Fuels & Technologies - IEA, 2022). However, valorisation of the co-produced crude glycerol waste is becoming a suitable mitigating measure to allow biodiesel to become a more profitable and economical proposition (Konstantinovic et al., 2022).

Glycerol is a renewable raw material with high versatility which allows for its many issues across different industries, with main use in the chemical industry. It can be used as a humectant in sweets, cakes, meats and cheeses, and also as a solvent, sweetener and preservative in other beverages and foods. They are also used to flavour and colour food and soft drinks (Katryniok *et al.*, 2011). The world market for glycerol has been increasing due to the increased demand due to their use as an additive in the food and beverage industry, as well as being produced by the biodiesel industry. This excess production of glycerol has resulted in the search for new applications of crude glycerol since the excess supply is not being absorbed efficiently into the market. Therefore, it is becoming imperative to find alternative uses for glycerol to prevent market saturation and volatility. Many studies have hence been contributing to literature in this space (Katryniok *et al.*, 2011).

A favourable outlet for the crude glycerol waste produced through transesterification, is its use as a renewable feedstock in the bioconversion processes to produce biofuels and other chemicals

This study aims to use systems such as whole cell biocatalyst and biofilm formation to determine whether a particular biomass (*E. coli* Nissle) can use glycerol as a fundamental feedstock to produce biofuels such as biodiesel. Solving for this will benefit the biofuel industry in several ways:

- Reduce market saturation of glycerol, keeping prices reasonable enough to make a profit
- Glycerol being seen less as a waste product in biofuel production and more of a feedstock (thus reducing cost of production)
- Recycling of glycerol back into biofuel production reduces the environmental impact of the process

### 1.1.2 Research objectives

The core focus area of this research will compare the biodiesel produced from *Escherichia coli* Nissle biofilm on different types of carriers to determine the commercialisation aspect of this process.

The objectives of this research:

- Determine the biofilm forming capabilities of *E. coli* Nissle on various carriers to compare level of biomass formation and stability.

- Determine the amount of ethanol and biodiesel that would be produced from the *E. coli* Nissle biofilm on the different carriers when using glycerol as a feedstock.
- Compare the results obtained from the above methods on a generic flow sheet model to theoretically "upscale" and determine the effective commercialisation of *E. coli* Nissle biofuel production

# 1.2 Literature review

## 1.2.1 Biofuels

The majority of the global energy demand is fulfilled by fossil fuels. This can be seen by the fact that 88% of total energy production globally is derived from fossil fuels. It's largely understandable that the demands for energy will only increase (Konstantinovic et al., 2022). The driving factors for these include increasing industrialisation and population numbers. Not only are human numbers increasing, so are our energy needs. This increased demand has led to many existential threats: fossil fuel depletion, pollution of environments and electricity supply shortages. It is expected that because of the non-renewable nature of fossil fuels, we're expecting depletion of these sources within the next decade. Burning of fossil fuels has also led to an exponential increase in greenhouse gas emissions, which has led to detrimental effects of environments through phenomena like global warming. The quantify the level of the problem, in 2017, the levels of carbon dioxide emissions worldwide from fossil fuels went up by 1.6%. This is expected to continue to increase (Konstantinovic et al., 2022).

Because of the dire nature of the problem surrounding greenhouse gas emissions, environmentally friendly approaches need to be implemented as soon as possible to fulfil the increasing energy demands while ensuring that our environments are protected (Kasinath et al., 2021). One big way to solve for these issues is to capitalize on existing renewable energy sources that remain in abundance. This includes sources like biomass. Biomass can be used to produce fuels that are used in the transportation sector. Fuels derived from biomass are generally referred to as biofuels and are made through biological fermentation of feedstocks such as fermentable sugars, carbohydrates and lipids. Thus, these biological feedstocks can be converted into other types of energy through processes such as anaerobic digestion to produce product like biofuel and biogas (e.g., bio-oil, bioethanol, biodiesel, methanol, methane and hydrogen) (Kasinath et al., 2021) It also includes other forms of energy such

as electricity and heat. An added advantage of using biofuels in addition to being sources for renewable sources, is that they release less harmful byproducts into the environment while combusting to produce energy (Kasinath et al., 2021).

Biofuels can be broken down by the type of biological feedstocks used. There are 4 major categories of these are simply termed: first generation, second generation, third generation and fourth generation (Mahapatra et al.,2021).

First generation biofuel production is done using edible feedstocks (things like potato, rice, wheat, vegetable oils and sugarcane. Bioethanol and biodiesel are produced by oils derived from biomass processes called fermentation and transesterification (Correa et al., 2017). However, there is much debate about the use of these crops that provide sustenance for many humans to produce fuel. In addition to the crops being edible themselves, the re-direction of other important resources such as water, land, and fertilisers to produce these fuels has raised many concerns. Not only does this make the process of first-generation biofuels costly and inefficient, it also is a poor allocation of other already limited resources (like water) (Mahapatra et al.,2021). This perspective has directed many biofuel producers to look for non-food alternatives that can be used as biofuel feedstock (Correa et al., 2017)

The re-direction of other important resources, motivated by ethical questions regarding using food to produce fuel while food security is also another problem humanity faces, has led to experimentation with second generation biofuels. This generation of biofuels are produced using feedstocks not used for consumption like woody biomasses (Fontes et al.,2021). In fact, these feedstocks are mostly waste (like husks), making it a more economical and environmentally approach as this feedstock used is waste not generally used by society. Benefits to this approach are the fact it does not add to food insecurity, reduction of the need for water, land and other important resources needed for cultivation, less greenhouse gas emissions from their combustion, and encouraging the use of waste/by-products to make the whole process greener (Li et al., 2022). Despite these great benefits, the challenge with the type of biofuel is that the cost of production is higher (despite the feedstocks being a cheap by-product) since the process is more complicated, requiring the use of expensive equipment to produce second generation biofuels. An example of where the process can be complicated is the need for enzymes to break down the cellulose of the plant cells. This kind of additional process increases the cost and the time taken to produce the biofuel. The limitation of the above two types led to the exploration in a third type of biofuel (Fontes et al.,2021).

Microalgae biomass can be extracted to produce third generation biofuels. Microalgae are incredibly small in size, don't need land for cultivation (and other important resources that go with cultivation of crops), and have higher growth rates (Zewdie and Ali, 2020). This means that third generation biofuels could be cheaper, faster and more environmentally friendly to produce. As things stand currently, these microalgae reduce the worldwide $CO_2$ content by almost 40% (Zewdie and Ali, 2020). The size and growth rate of microalgae make it the ideal candidate for use in biofuel production. The limitations arise from the large amounts of freshwater and nutrients needed to maintain microalgae cultivation, which leads to increased costs. However, microalgae-derived biofuel is seen as less stable (and so highly volatile) compared to the first and second generations (Zewdie and Ali, 2020). The additional requirement of energy needed for the drying of biomass and extraction of lipids encouraged the research to evolve into understanding fourth generation biofuels.

This last classification of biofuels, the fourth generation, revolves around modifying microalgae genetically. These microalgae were engineered to have higher biofuel production rates as well as capturing an even larger amount of $CO_2$ from the environment compared to the original strains (Shokravi et al., 2022). Because of the improvement $CO_2$ capture amount, the modified microalgae strains as classified as carbon negative. This is because the amount of $CO_2$ released is lower than the amount of $CO_2$ take-up. The limitation of this approach is that not enough biological data is available across a variety of microalgal species to test many assumptions (Shokravi et al., 2022).

The following study is centred around the formation of a type of biofuel, i.e., biodiesel, and explore the different co-products involved in the production of biodiesel.

### 1.2.2 Biodiesel

Biodiesel fuel (BDF), also recognised as fatty acid alkyl esters (FAAEs), is a type of fuel produced from various types of fats and oils (animal and vegetable), which is both biodegradable and renewable (Fukuda *et al.*, 2008). Because of its positive effects, it is being strongly considered as a more sustainable alternative to conventional petrochemicals, especially in diesel engines (Fukuda *et al*., 2008).

With that in mind, the global energy demand is still being fulfilled by conventional measures like natural gas, coal and petroleum (Demirbas, 2007). Although hydroelectricity and nuclear energy are also used worldwide, their depletion is not imminent, like the finite sources of conventional ways

(Demirbas, 2007). This depletion rate is only faster due to increased energy demands. Pursuing alternative fuels like biodiesel is the most efficient way to meet rising energy demands as these do not use current fossil fuel reserves and are able to utilize waste material (Meher *et al.*, 2006). The numerous advantages of biodiesel include greenhouse gas emission reduction, increased security of energy sources, economic and social improvements due to this new sector providing more opportunities as well as political stability. All these benefits, that can solve for the current energy problems, are the reason why there is a big movement currently toward alternative cleaner fuels. The improved attention into biofuels has meant that fast strides are being made in this field, allowing these greener fuels to become cost-competitive with conventional fuels. (Demirbas, 2007). The trends of alternative fuels is only expected to grow. The motivating factors for this are due to fossil fuels resulting in: increased pollution by emissions, rising costs and increased scarcity. The current conventional reserves are expected to deplete in the coming decades, making a strong case for biological based fuels like bioethanol and biodiesel (Kirsch, 2020). Within the category of biofuels, biodiesel is one of the more promising products.

### 1.2.3 Biodiesel production

Biodiesel is a type of biodegradable and renewable alternative fuel that is produced either through the esterification of fatty acids or trans-esterification of plant and/or animal oils and fats (i.e. triglycerides) with short-chain alcohols (Encinar, Nogales-Delgado and Sánchez, 2021). The first process involves the reaction of an acid with an alcohol. The result is the production of water and an ester. The second process involves the displacement of one alcohol by another from an ester (Ranganathan *et al.*, 2008; Tan *et al.*, 2010).

Other processes that allow for biodiesel production include pyrolysis or micro- emulsification. (Ranganathan *et al.*, 2008). Pyrolysis involves the decomposition of a compound through the use of high temperatures.

### 1.2.4 Trans-esterification

Transesterification is the process that involves the displacement of a certain alcohol moiety from an ester, by another alcohol (Encinar, Nogales-Delgado and Sánchez, 2021). The alcohol moiety behaves as an acyl acceptor as presented in Figure 1. This process can be catalysed in several ways. Firstly, it can be

done chemically where liquid acids or bases are used. Secondly, it can be done with homogenous or heterogeneous solid catalysts. The third option involves enzymatically using an esterase/lipase. Lastly, it can also occur by using the alcohol and substrate in supercritical conditions (such as high pressures and temperatires) (Ranganathan *et al.*, 2008). The preferred method, especially in industrial largescale production of biodiesel, is chemical catalysis. The reasons for this is because this method has many advantages over the other processes. These advantages include larger rates of conversion, additive cost reduction, reduction in reaction times and lowered operating temperatures needed (meaning that the total energy input needed to make the product is less) (Fukuda *et al*. 2008; Sivasamy *et al*. 2009). The industry, however, still deals with issues with acid/base biodiesel production methods. The issues include the formation of soap (caused by the increase in the content of moisture), the need for complex pre-treatment steps (like the reduction of water and fatty acids) and treating the wastewater which contains the catalyst (recovering of this catalyst is large energy necessities for glycerol recovery (Fukuda *et al*. 2008; Fjerbaek *et al*. 2009).

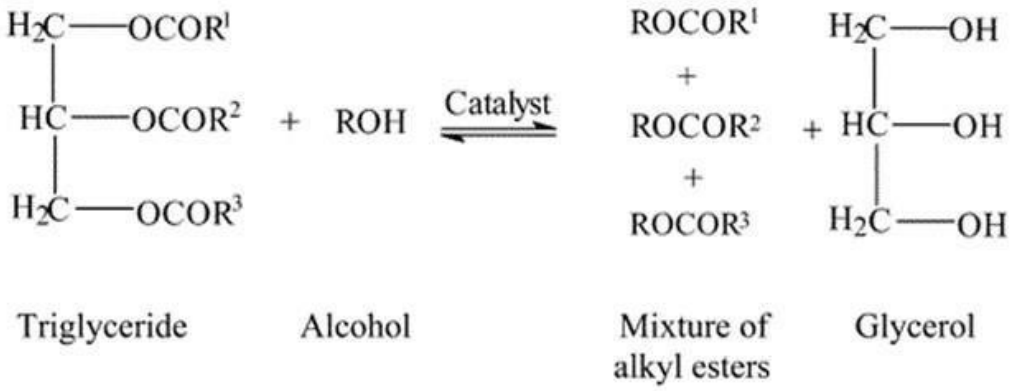



***Figure 1.1. Reaction mechanism of triglyceride transesterification.*** *The image shows one triglyceride molecule reacting with three moles of alcohol to produce one mole of glycerol and three moles of FAEE with the help of a catalyst. The above has been adopt (Pereira* et.al.*,2014)*

## 1.2.5 Enzymatic biodiesel production

The production of biodiesel through enzymatic means, which is the addition of biological catalyst to accelerate the rate of the chemical reaction, is an alternative to the commonly used chemically catalysed reaction, which is more reliant on the purity of the reactant oil used (Sebastian, Muraleedharan and Santhiagu, 2016). This process is advantageous when compared to FAAE

generations since enzymes are more tolerant to variations in feedstocks (Sivasamy *et al*., 2009). The additional fact that the use of enzymes result in a bigger yield in crude glycerol with no adverse reactions makes it more desirable for use when compared to more conventional catalysts (Lam *et al*., 2010). Additional benefits include this process being single-step (resulting in less energy use) and the reduction of polluting by-products (Fjerbaek *et al*., 2009; Fukuda *et al*., 2008; Tan *et al*., 2010). Even though the benefits of this process are numerous (as highlighted above), mass scale use is minimal. The main reason attributed to this is cost. When compared to enzymes, chemical catalysts have larger reactions rates, are lower in cost and have longer re-usability. A key point to note is that enzyme usage is known to be limited to 100 days of operation only (Lam *et al.*, 2010). Studies have been done to assess whether these bottlenecks can be alleviated. Strategies used included assessing whether the immobilization of enzymes improved the stability, which in turn might improve reusability (Tan *et al*., 2010). What was discovered was that these enzymes could be immobilized by 3 core ways: adsorption onto a carrier (supports such as synthetic polymers, glass and even polysaccharide derivatives), encapsulation within a carrier (utilising polymers like cellulose, collagen, k-carrageenan, or liposomes and microcapsules) or via cross-linkage (carrier-free; utilising reagents such as bisdiazobenzidine, hexamethylene diisocyanate and glutaraldehyde) (Tan *et al*., 2010; Datta *et al*., 2012; Sheldon and Van Pelt, 2013). It is important to note that lipases are the main enzymes that enable biodiesel production through enzymatic means.

The overall persisting problem associated with biofuel/biodiesel production (in either chemical or enzymatic means) involves the overproduction of by-products such as crude glycerol (Sebastian, Muraleedharan and Santhiagu, 2016).

## 1.2.6 Glycerol

Glycerol is a hygroscopic clear compound that has the following properties: high viscosity, sweet taste, non-toxicity, water solubility and biodegradability. This compound is an important structural element of fat and oil substances (Ayoub and Abdullah, 2012). The main use of pure glycerol in industry is its use in the manufacture of food and beverages, where it is an additive with minute structural modification. Other industries that also utilize this compound involves pharmaceuticals, the textile industry and the tobacco industry (where it is used as humectant) (Kenar, 2007; Paulo *et al.*, 2009). Crude glycerol (with about 80% purity) happens to be an inevitable waste reaction by-product of the trans-esterification process that enables biodiesel manufacture. Despite this inefficiency,

massive tax support programmes (from different governments like in the US and in Europe), compulsory mandated use and mixing into conventional transportation fuels have increased biodiesel manufacture. This scenario has led to the inevitable flooding of the refined glycerol markets (Johnson and Taconi, 2007).

Average crude glycerol prices were conventionally reasonable before the production of biodiesel started to become more popular as an avenue to reduce the use of fossil fuels (Johnson and Taconi, 2007). However, because of the over-production of glycerol (leading to a bigger supply when compared to the demand) have resulted in a steep decline of prices (IncBio, 2016). As a consequence, manufactures of refined glycerol (which were obtained from petro-chemical sources) have closed their manufacture (Da Silva *et al.*, 2009; Dharmadi, 2006; Johnson and Taconi, 2007). The over saturation in the crude glycerol market resulted in massive market volatility in the price of glycerol that was refined.

### 1.2.7 Various value uses for glycerol

Crude glycerol being produced excessively as a by-product from the conventional process of biodiesel production is major obstacle for manufacturers of biodiesel (Johnson and Taconi, 2007). Two main reasons that make it an impediment is the fact that this excess production is a potential and environmental liability. The glycerol by-product is of no value to the biodiesel producer and hence they might sell this crude version of glycerol to a refinery. This could potentially be a strategy to recoup some costs in the otherwise costly production of biodiesel. Considering that up to 90% of the cost of the production of biodiesel is made of raw material cost, this might seem like a useful strategy for the biodiesel manufacturer. However, this is not entirely an economical option either (Yazdani and Gonzalez, 2007; Paulo *et al.*, 2009). Crude glycerol is contaminated with many other compounds such as alcohol, heavy metals and salts and refinement methods needed include subsequent fractional distillation and vacuum distillation (Katryniok *et al.*, 2011). This is a necessity before glycerol can be used in other applications (Ayoub and Abdullah, 2012). Reducing the economic viability of biodiesel production even further is the fact that the producer is responsible for transportation of this crude glycerol to the refinery. In totality, all these expenses can exceed the actual value of the crude glycerol by-product (Johnson and Taconi, 2007). To remain in the growing industry and as an attempt to reduce their own internal manufacturing expenses, people in this industry and exploring ways to convert this crude glycerol by-product they have into other value-added products. This will increase the product range (and by default the revenue streams) that a biodiesel manufacturer can offer,

hence helping reduce the cost of the overall production of biodiesel (Johnson and Taconi, 2007; Trinh and Srienc, 2009; Yazdani and Gonzalez, 2007). With this approach, biodiesel manufacture may become a more investment-worthy pursuit for people in this industry.

The pursuit of the strategy to convert the crude glycerol by-product to value added products is enabled by the fact that glycerol is a product that is commercially relevant (Johnson and Taconi, 2007). It's currently produced through many types of reactions. These include bond-breaking, biochemical, oxidisation, chemical, reduction, and polymerization reactions (Kenar, 2007; Pagliaro *e. al.*, 2007). This shows that glycerol production and its refinement is a quite a mature space, allowing biodiesel manufacturers to pivot into this space. In addition, the off shoot derivates of these different reaction types also have practical value and monetary value in industry. There are many examples of this use-case. These include etherification of glycerol to produce fuel oxygenates. These can be incorporated into standard aromatic diesel fuel, which results in reduced release of unregulated polluting matter like carbon monoxide, hydrocarbons, and aldehydes
(Pagliaro *et al.*, 2007). Another example is using hydrogenolysis to produce propylene glycol (1,2propanediol). A couple of other examples include using dehydration to produce acrolein, reforming to produce syngas, and microbial fermentation to produce 1,2- and 1,3-propanediol (Pagliaro *et al.*, 2007).

The significant prices for crude oil and the volatility of these markets have encouraged the acceptance and use of glycerol as a cost-prudent alternative for numerous petroleum-derived chemicals (Transparency Market Research, 2013; Miller-Klein Associates, 2006). Glycerol substitutes that make up this market and their uses include: epichlorohydrin (used as an intermediate in items like composites, paints, coatings and epoxy resin manufacture) and polytrimethylene terephthalate (a polymer fibre that has similar properties to (Kenar, 2007; Miller-Klein Associates, 2006; Ragauskas *et al.*, 2006; Transparency Market Research, 2013). This excess availability of glycerol and the need for diversification have encouraged major chemical industry players like ADM (Archer Daniel Midland), Dow Chemical and Cargill to use glycerol as one of the main feedstocks for industrial microbial fermentation processes (Kenar, 2007; Miller-Klein Associates, 2006; Transparency Market Research, 2013).

### 1.2.8 Glycerol bioconversion

Even with opportunities for diversification in the glycerol market, the excess glycerol that biodiesel producers bring into market as it is currently, is so large that the supply far exceeds the demand,

creating market volatility (Chilakamarry *et al*., 2021). This demands the need for new applications and markets for crude glycerol in order to reduce overall market volatility back to generally accepted fluctuations and has indicated that microbial fermentation could be a viable use case scenario for this excess glycerol (Zularisam and Pandey, 2021). Microbes are naturally able to use this glycerol as a sole energy and carbon source (Yazdani and Gonzalez, 2007). This information has encouraged the biodiesel industry to explore the use of glycerol made through the biodiesel production process to produce chemicals like succinate, 1, 3-propanediol, ethanol, xylitol through bioconversion (Chilakamarry *et al*., 2021). Further motivation to pursue this strategy has come about also since using feedstock made of glycerol instead of sugars has resulted in bigger yields in biofuel production (Zularisam and Pandey, 2021). In this scenario, the problem that needs to be solved is on the refinement and purification of crude glycerol before use with microorganisms. These contaminants could inhibit microbial growth otherwise and result in an inefficient process (Johnson and Taconi, 2007).

### 1.2.9 *Escherichia coli*-based glycerol fermentation

The general lack of a natural 1, 3-propanediol pathway in *E. coli* was initially identified as the main reason for the lack of innate glycerol fermentation activity in this microbe (Gonzalez *et al*, 2008; Trinh and Srienc, 2009). *In vitro* experiments of membrane-bound dehydrogenases confirmed the basis of this hypothesis. The experiments included dehydrogenases such as glycerol-3-phosphate dehydrogenase which is *glpD* encoded and is known, in the presence of oxygen, to be coupled to glycerol metabolism and the GlpABC operon. These dehydrogenases allow for glycerol-3-phosphate (G3P) conversion and is then immediately converted into pyruvate through the process of glycolysis (Dharmadi, 2006; Gonzalex *et al*. 2008). Glycerol-3-phosphate is a product that occurs from the phosphorylation of glycerol, using glycerol kinase, to dihydroxyacetone-phosphate (DHAP) (Dharmadi, 2006).

Thus, glycerol metabolism using *E. coli* was determined on the addition of various other electron acceptors via external sources to the specific medium such as nitrite, fumerate, or even trimethylalamine-*N*-oxide (Trinh and Srienc, 2009). This is the section where aerobic metabolism was vital. Metabolic engineering events that include the introduction of the 1, 3-propanediol pathway from the *Enterobacteriaceae* family was employed, if anaerobic metabolism was crucial (Dharmadi, 2006; Gonzalex *et al*. 2008). These balanced the redox potential.

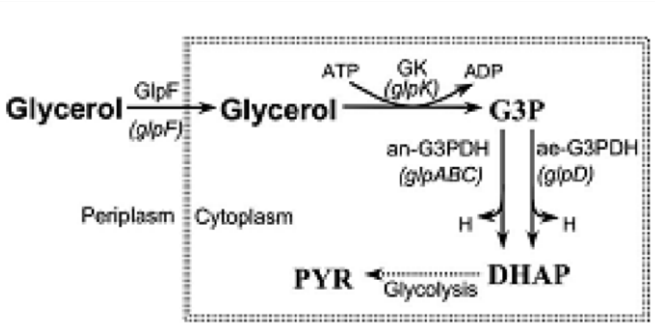



***Figure 1.2: Aerobic glycerol metabolism in E. coli****. Glycerol is metabolised by an ATP- dependant glycerol kinase (GK), as well as aerobic G3PDH (ae- G3PDH) and the electron (an-G3PDH) which are two respiratory enzymes. This is all completed in the presence of electron acceptors. H is the reducing equivalent (NADPH/NADH/FADH2), PYR is referred to as pyruvate and DHAP is dihydroxyacetone phosphate (Murarka et at. 2008).*

*E. coli*, in tightly regulated conditions was used to prove that with the absence of any kind of external electron acceptors, anaerobic metabolism of glycerol was possible (Dharmadi, 2006). The final product 1, 2-PDO had been formed with various other key fermentation end products that used glycerol as the sole carbon source (Dharmadi, 2006; Gonzalex *et al*, 2008; Murarka *et al,* 2008; Trinh and Srienc, 2009). Another report showed the pathways needed for the utilisation of glycerol in *E. coli* within an anaerobic environment were identified and characterised, with a new model for this process being suggested. Research hypothesised that the fermentation of glycerol would result in the production of 1, 2-PDO which occurs through the use of a dormant and natural pathway as an alternative to the common pathway, which results in the production of 1, 3-PDO, which is found with other enteric bacteria (Murarka *et al,* 2008). The following process is further defined in Figure 3:

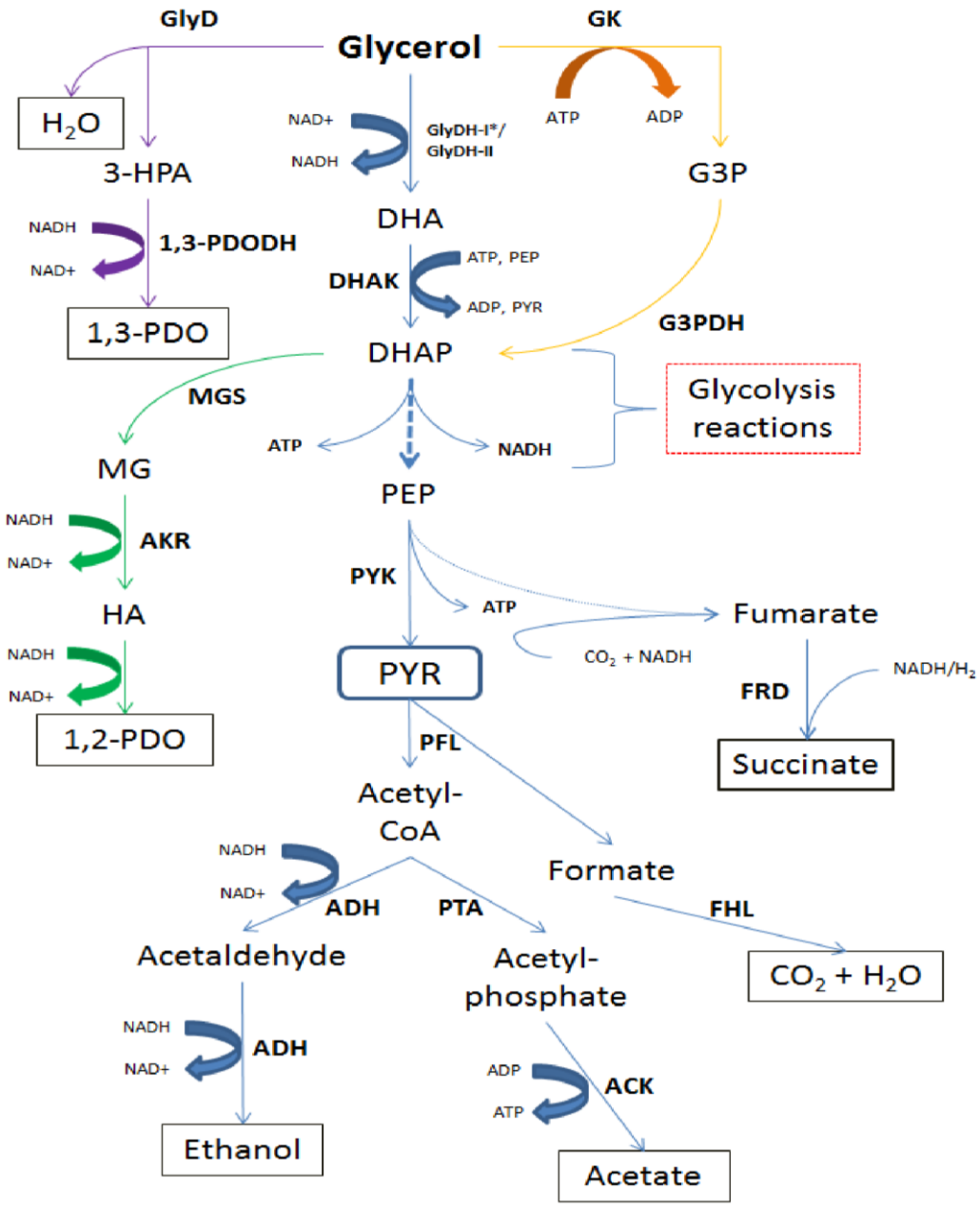



***Figure 1.3: Diagram which displays glycerol metabolism in E. coli as well as other members of Enterobacteriaceae family.*** *This indicates fermentative key products which are shown with black boxes. The various pathways are all colour coded as such: (A). The purple and orange colours show the reductive and oxidative pathways of the members of the Enterobacteriaceae family respectively. (B). As described in the text, E. coli use the blue and green pathways. Various enzymes are responsible for the reactions which are shown alongside their respective reaction (Gonzalex et al, 2008).*

In the end, the glycerol would be converted from glyDH-II to dihydroxyacetone (DHA) through dehydrogenisation. This product would then undergo phosphorylation in order to generate DHAP through DHA kinase (DHAK) use. Ultimately, these products will be reduced by methylglyoxal synthase

action to form methylglyoxal. This product is then reduced by three enzymes referred to as aldo-keto reductases, encoded by the genes: yafB, yeaE, yghZ and which generate $NAD^+$ through the reduction process, to form hydroxyacetone. This goes through another reduction process by NADHlinked glycerol dehydrogenase to 1, 2-PDO and which generates another $NAD^+$ molecule in the process (Gonzalez *et al*, 2008).

The selection of a low-cost feedstock is crucial to develop an economically feasible and sustainable biodiesel production process (Pasha et al., 2021).

## 1.2.10 *Escherichia coli*

*Escherichia coli* are a diverse and extensive group of bacteria that can form biofilms which are generally formed as a strategy for protection (Beloin *et al*, 2008). Biofilms made by *E. coli* are made from different components and is dependent on the type of strain and environmental condition. Stress resistance has been identified as an ability given to strains that are able to produce biofilms (Conway T and Cohen PS, 2015; Limoli DH *et al*., 2015). A particularly interesting *E. coli* that exemplifies this is *Escherichia coli Nissle 1917* or simply known as EcN. Research proved that this strain had anti-microbial properties (Sonnenborn, 2016) and since then, this strain has been branded as 'Mutaflor' and sold in the probiotic market as a medication for intestinal issues for almost a century! Because of its importance in probiotics, this strain has been explored in clinical trials as a potential treatment for ulcerative colitis (Chen *et al*., 2017). Further research has shown that this strain has a high potential to form biofilms. This has been attributed to the fact that this strain contains Type 1 fimbriae. The presence of this structure enormously encourages the formation of biofilms, especially on the mannoside presenting surfaces (Chen *et al*., 2017). However, this use of this strain in industrial microbiology sectors like the biofuel industry has never been explored. The focus of this study is to fill the knowledge gap that exists in the literature and hence identify if this EcN strain that has a high biofilm forming potential, can be used as a whole cell biocatalyst for improved production of biofuels, mainly biodiesel.

## 1.2.11 Whole cell biocatalyst system

In the industrial biotransformation space, the use of whole-cell biocatalysts have entrenched themselves as a reliable way to do this biotransformation work, as well reducing the typical costs of production processes when comparing to those that are enzyme based (Straathof *et al*, 2002). A variety of industries like food, pharma and agriculture apply this technique to their great benefit,

particularly, in the fine chemicals sectors where enantiomerically uncontaminated chemicals are vital for success of experiments (Ban *et al*, 2001; Narita *et al*, 2006, Straathof *et al*, 2002; Xiao *et al*, 2011). Whole cell is preferred when compared to (partly) isolated enzymes when it comes to redox and nonredox biotransformations as well. In this scenario, 2 enzymes are imperative for the biotransformation to be active (Straathof *et al*, 2002).

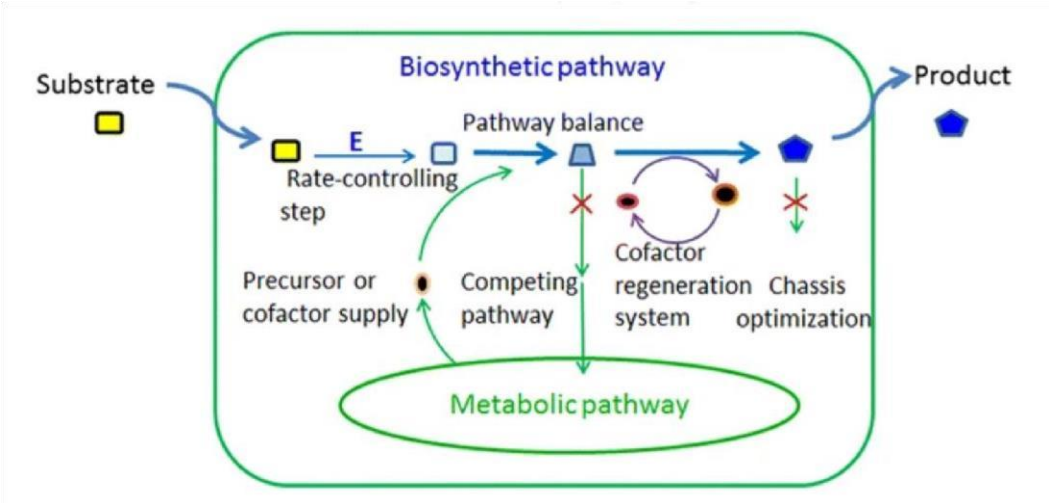



***Figure 1. 4: An example of how a whole cell biocatalyst system would function and overall ways for optimization.*** *This displays the conversion of a substate to a product using a cells internal pathway s (Straathof et al, 2002).*

Enzyme-based biotransformations generally have a few obstacles when compared to Whole-cell biocatalysis. Disadvantages of using the former techniques includes things like re-usability and recovery issues and increasing costs because of the multiple purification steps (Fukuda *et al*, 2008). However, with whole-cell biocatalysis, the ideal environment for the functioning of the enzyme is present. These include the presence of things like co-factors, regeneration networks and an extracellular environment (Fujita *et al*, 2002; Jose *et al*, 2012).

Latest research has already indicated the use of microbes like bacteria and yeast (as well as filamentous fungi) to convert triglyceride to biodiesel was successful through a whole-cell biocatalysis system (Patel et al., 2020). These kinds of research show strong signals that this type of conversion might be viable, both in the lab and potentially in industry.

Firstly, Narita *et al* (2006) had established an *E. coli* cell surface display system through the use of a motif to specifically anchor PgsA. This system was able to demonstrate both the *Candida antarctica*

lipase B (CALB) activity and the *Streptococcus bovis* 148 α-amylase activity. The *E. coli* cells that displayed CALB effectively catalyzed trans-esterification enantio-selectively. Next, Xiao *et al* (2011) obtained an *Aspergillus niger* strain and immbolized the strain within biomass support particles (BSPs) containing polyurethane. This ensured the conversion of palm oil, in a packed-bed reactor (PBR), to biodiesel using enzymatic lipase transesterification.

A high yield of FAME, Fatty Acid Methyl Esters, of no less than 90% in the PBR with a flow rate of about 15 L per hour after a 72hour reaction time and a water content of 15% had been attained following glutaraldehyde solution addition in order to stabilize any kind of lipase activity. An increased conversion rate of greater than 85% was further sustained after four palm oil batch conversion cycles in the PBR.

Whole-cell biocatalysts can benefit from lipase production in a cost-reduced, simpler and more efficient system that shows non-conventional media stability. However, disadvantages in this approach include rates of reaction that are lower, mass-transfer effects and lower yields (Fukuda *et al*, 2008). Researching the intersection between biofilm technology and whole cell biocatalysis is where there is room for more experimentation.

### 1.2.12 Whole cell biocatalysis compared to chemical catalysis

Table 1.1 A comparison of advantages and disadvantages of whole cell biocatalysis to chemical catalysis, showing pros and cons of each method (Fukuda et al, 2008).

| Advantages | Disadvantages |
|---|---|
| • Selectivity is high | • The biocatalyst stability can be affected when either the product or substrate is inhibited<br>• Extreme conditions like high temperature and pH could result in inactivation |
| • Catalytic activity is high | • The cell membrane can become a barrier when mass transport is needed in the process |
| • There is the option for recycling | • Toxicity to cells is possible due to the increase in potential for waste by-products that are unwanted. These by-products can |

| | |
|---|---|
| • Conditions needed to operate is milder, meaning less energy use, resulting in a more environmentally and cost-effective process | also be difficult to isolate from the original mixture |

## 1.2.13 Biofilm and carriers

*E. coli* have the ability to switch between a biofilm form and planktonic form due to environmental stress such as low resources or current water flow. The biofilm form of *E. coli* is about 1000 times more resistant to outside stress compared to that of their planktonic form. The formation of biofilms in *E. coli* is produced in four major steps: (1) reversible initial adhesion or attachment; (2) irreversible early development of biofilm structure; (3) Maturation of the developed biofilm and (4) Dispersion of cells from the biofilm to return to platonic state (Beloin, Roux and Ghigo, 2008).

## 1.2.14 Initial adhesion

There are many conditions in a liquid environment that affect biofilm formation: these include environmental conditions such as ionic force of medium, pH, temperature, repulsive electrostatic forces, and hydrodynamic forces (Beloin, Roux and Ghigo, 2008).

Motility is an essential factor for bacterial adhesion; this is shown with peritrichous flagella which acts as a mechanism for *E. coli* to overcome these mentioned forces and increases *E. coli* surface interaction, therefore providing the first cell-to-surface contact for adhesion. Non-motile bacteria express robust adhesion factors which also allow surface attachment without flagella (Jiang et al., 2021).

## 1.2.15 Early development of biofilm

In this stage, the synthesis of flagella is repressed when the *E. coli* cells attach to the surface. This effectively makes the cells immobile. Molecules known as cyclic-diguanylic acid (c-di-GMP) are essential for the platonic to immobile state shift. The concentration of this molecule is low in the motility state and increases during biofilm formation. Adhesive organelles which include curli fimbriae and type 1 fimbriae play critical roles in the permanent attachment of *E. coli* to the surface. The type 1 fimbriae are vital for the initial adherence to abiotic surfaces and their expression is initiated by adhesion and initial development of the biofilm (Beloin, Roux and Ghigo, 2008). The Curli

fimbriae are extra cellular structures that attach to the proteins of the extracellular matrix and allow for abiotic surface adhesion through both the enhancement of the cell-to-surface interaction and cell-to-cell communication (Jiang et al., 2021).

## 1.2.16 Maturation

Once cell-surface adhesion is complete, aggregation initiates through various cell-cell interactions. The bacteria in this phase produce the extracellular matrix needed to provide the three-dimensional structure of biofilm. There are four critical elements for biofilm formation and stabilisation (Donlan, 2001).:

*Autotransporters:*

These promote cell-to-cell adhesion which facilitates auto-aggregation and three-dimensional development and ultimately stimulates biofilm formation (Donlan, 2001).

*The extracellular polymeric Substances (EPS):*

This is the defining property of a biofilm that differentiates it from planktonic bacteria. The EPS matrix is the avenue which bacterial cells use to get attached to the surface, enable cell-to-surface interactions as well as cell-to-cell interactions. The EPS matric provides the support needed to cells forming biofilms and provides the biofilm with a three-dimensional structure. This structure plays structural and protective roles (Donlan, 2001).

*Quorum sensing (QS):*

This is a chemical signalling system that is reliant on the density of cells. In this phenomena, singular cells release signal molecules called quormons or autoinducers, which are very small, to the surrounding to promotes intraspecies communication. Therefore, this is the 'messaging system' utilized by bacteria to co-ordinate and communicate their functions and behaviour so they are in sync like a multicellular organism. This QS system and it also controls gene expression during both biofilm formation and maturation (TALSMA, 2007).

*Stress resistance genes:*

During the formation of a biofilm, the induction of stress resistant genes assists in protection the biofilm from aspects such as harsh environment.

## 1.2.17 Dispersion

The final stage of biofilm formation includes the detachment of bacteria from a mature biofilm and their initial dispersal. This results in the transmission of the dispersed bacteria to a platonic state, lodging at distant sites and the formation of separate biofilms. This detachment occurs as a result of enzymatic degradation of both the QS and the biofilm matrix due to various environmental changes such as oxygen depletion and nutrient levels as a result of external forces (Sharma *et al*., 2016). Understanding cell surface and use of elements such as carriers are vital in understanding attachment, the initial phase in the formation of biofilms (TALSMA, 2007).Surfaces can be made up of a variety of types, from: extremely hydrophobic ones like silicone to highly charged hydrophilic ones like glass. These surfaces can be smooth like glass or textured like in the environment (Donlan, 2001).

Microbial attachment can be influenced by the properties of a substance like a 'carrier (Donlan, 2001). Properties that are influenced include the level and rate of attachment. The tendency of biofilms to grow faster on hydrophobic and rough surfaces has been established. The surface of the microbial cell is also an important consideration when it comes to biofilm formation. Appendages like flagella can influence the rate of microbial attachment since the cell needs to overcome repulsive forces in materials and structures like flagella can adhere the microbial cell to the surface until a more robust mechanism for attachment takes over (Korber *et al*. 1989). Cells that are permanently attachment to a given surface start reproducing, resulting in micro-colonies and extracellular polymers. These are defining elements of a biofilm (TALSMA, 2007).

Biofilm technology and knowledge is already implemented in sectors like the wastewater treatment industry. There are certain characteristics of biofilms that might make them more conducive to building a more robust biofuel production process. These include the ability to increase the conversion process efficiency by increased rate of reactions when a significant concentration of cell-associated hydrolytic enzymes at the biofilm-substrate interface occurs. As an added benefit not seen in suspended cultures, a contained biofilm microenvironment can 'reward' cells with superior phenotypes conferred from either signal exchange or intercellular gene transfer. This creates a more robust colony of microbes which might contain more desirable traits. Considering all information cited above, a critical element of biofilms that might make it an important strategy in biofuel production overall is immobilization. Immobilization can ensure that the producer of the biofuel (the microbial

biomass) remains adhered to the fermenter. This means that the biofuel can be separated easily from the biomass as well as allow for continuous reactions (Wang and Chen, 2009).

Research developments has shown that production of biodiesel offers clear environmental benefits however its economic competitiveness is crucially dependent on the type of feedstock used, production capacity and technological choice (Pasha et al., 2021). Therefore, evaluating biofilm technology utilizing waste glycerol as a feedstock for the means of biodiesel production could allow for increased economic competitiveness.

# Chapter 2

# 2.1 Methods

## 2.1.1 Preparation of *Escherichia coli* Nissle bacterial strain

An *E. coli* Nissle strain (EcN) which was procured from Austria was prepared by suspending one Mutaflor capsule containing the bacterial strain in 50 mL Lysogeny broth. This was incubated at 37°C overnight under controlled static conditions. The resulting bacterial suspension had been diluted with fresh LB at the ratio of 1:100, and re-incubated overnight at 37°C without shaking. The resultant bacterial suspension was plated on Luria Bertani agar and Mckonkey Agar after serial dilutions have been done. The plates were placed in a 37°C incubator for 24 hours. A single colony of EcN from the plate was inoculated into the fresh LB broth (50 mL) and incubated overnight with 200 rpm orbital shaking at 37°C. The concentration of the bacterial suspension was adjusted to $[OD]_{600} = 0.25$, $10^8$ CFU $mL^1$ for further use.

## 2.1.2 *Escherichia coli* Nissle Growth Curve with LB Media

A single colony from the LB agar plates was taken and inoculated in 5 ml of LB broth within a 15 ml falcon tube. Both tubes were incubated overnight at 37°C under static conditions. The next day, 1 ml of the resultant bacterial suspension was taken from the falcon tube and inoculated in 100 ml of LB Broth in a shake flask and incubated on a 200 rpm orbital shaking at 37°C for 16 hours. The OD reading of the suspension was done every hour of the incubation (Hall *et al*, 2013).

## 2.1.3 Production of competent cells and transformation of *Escherichia coli* Nissle

## 2.1.4 Competent cells

A falcon tube containing 1 ml of LB was inoculated with a single colony of ECN. The tube was placed on an orbital shaker at 200 rpm and in a 37°C incubator for 12 – 16 hours (Sciences *et a*l., 2022).

From the overnight culture, 1 ml was combined with 99 ml of fresh LB in a 5L flask (1:100 dilution, no antibiotics). The flask was placed on an orbital shaker at 200 rpm and in a 37°C incubator for 3-4 hours or until OD reached about 0.4 (Sciences *et al*., 2022).

Once an OD of around 0.4 was reached, the culture was separated into four 250 ml ice-cold centrifuge bottles. They were placed on ice for 20 minutes first and then centrifuged at 4°C at 4000 rpm for a total of 10 minutes. The resultant supernatant was discarded by tipping the bottles over a discard bin and any remaining media was aspirated. The remaining pellet in each bottle was suspended with

20 ml ice-cold 0.1 M $CaCl_2$ and incubated on ice for 30 minutes. The bottles were then centrifuged at 4°C at 4000 rpm for 10 minutes and the resultant supernatant was discarded by tipping the bottles over a discard bin and the remaining pellets were combined by suspending in 5 ml ice-cold 0.1 M $CaCl_2$ with 15% glycerol. A few 50 µl samples were taken out and stored in tubes at -80°C (Sciences *et al*., 2022).

## 2.1.5 Transformation of *Escherichia coli* Nissle

Transformation of the previously produced competent cells were done with the vector pIRES2-EGFP. The following vector information map is as follows:

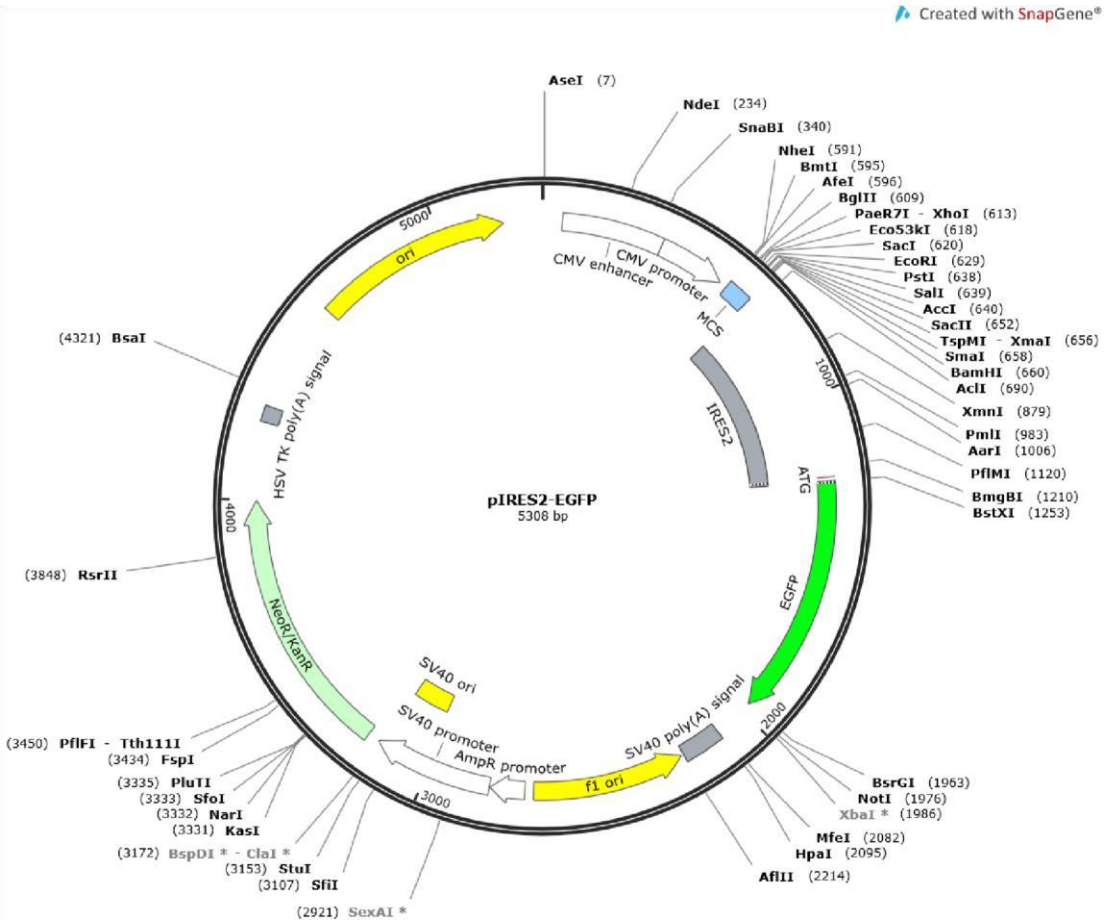



***Figure 2.1. The plasmid pIRES2-EGFP****. The main points of interest in this figure are the light green arrow between the 3000 – 4000 indicators which refers to the kanamycin resistance gene and the other one is the neon green arrow between the 1000– 2000 indicator which refers to the EGFP (pIRES2-EGFP Sequence and Map, 2022).*

The competent cells were thawed on ice and 1-5 µl (10 pg-100 ng) of the plasmid, pIRES2-EGFP, was added to the thawed competent cells. The mixture was incubated in ice for 30 minutes. The mixture was then heat-shocked by placing it in a 42°C water bath for exactly 30 seconds and placed on ice for 2 minutes. The mixture was removed from the ice and 1 ml of pre-warmed SOC medium was added and the mixture incubated at 37°C, at 200 rpm and for 1 hour.

Spread plates of 1:10 and 1:100 dilutions were done on selective media (LB media containing kanamycin) and incubated at 37°C for 12-16 hours.

## 2.1.7 Plasmid extraction from the transformed EcN

The plasmid extraction protocol was completed using the Zyppy™ Plasmid Miniprep Kit.

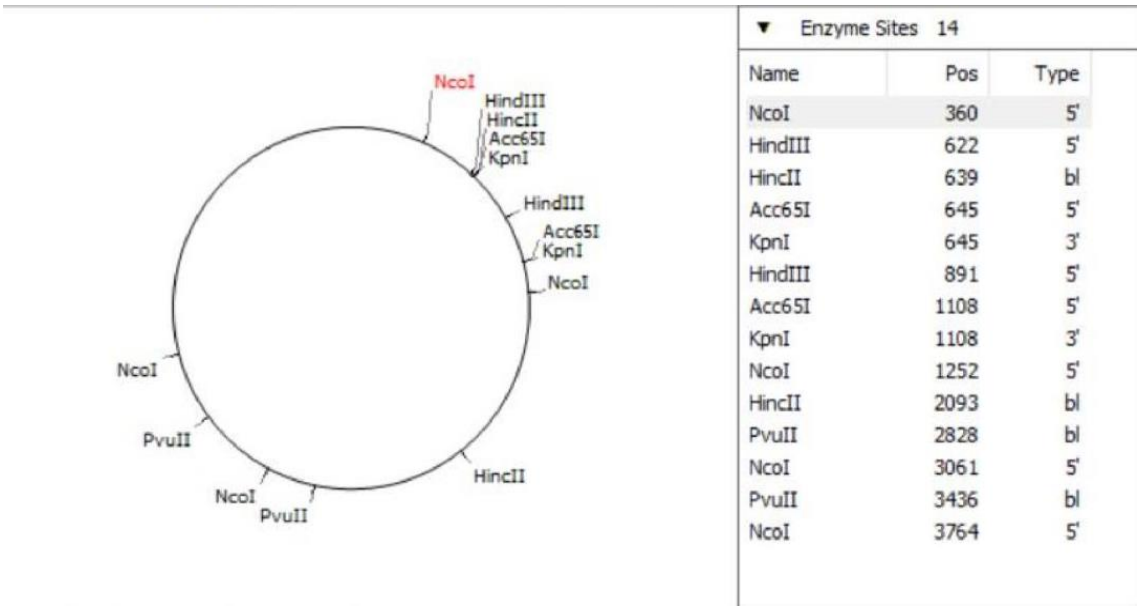
The following procedure was performed at room temperature (15-30°C).

A colony, taken from the transformed *E. coli* Nissle plates, was streaked on new kanamycin plates to obtain a more isolated colony sample. This colony was taken and added to LB media and allowed to grow overnight at 30°C and at 200 rpm. A 600 ul sample of the overnight bacterial culture was added to an empty 1.5 ml microcentrifuge tube. A 100 ul of 7x lysis buffer (blue) was added to the tube and mixed by inverting the tube 4-6 times and then incubated at room temperature for 1-2 minutes. After the mixture turned clear blue (indicating complete lysis), 350 ul of cold Neutralization buffer (yellow) was added and mixed again via inversion. The mixture turned yellow and resulted in a precipitate. The mixture was then centrifuged for 2-4 minutes at 2988 rpm.

A Zymo-Spin™ column was placed in a collection tube and the supernatant obtained after the centrifugation step was transferred into the column. The column was centrifuged for 15 seconds at 2988 rpm with the flow-through being discarded. A 200 ul sample of Endo-wash buffer was added to the column and then centrifuged for 30 seconds at 2988 rpm and then 400ul of Zyppy™ wash buffer was added to the same column and recentrifuged for 1 minute at 2988 rpm. The column was then transferred into a clean 1.5 microcentrifuge tube and 30ul of Zyppy™ Elution buffer was added directly to the column matrix and incubated for 1 minute at room temperature. The solution was centrifuged for 30 seconds at 2988 rpm to elute the plasmid DNA. The eluted DNA was then stored at -20℃ for further use.

## 2.1.8 Restriction enzyme digestion of extracted plasmid

Suitable restriction enzymes were chosen to digest the plasmid to determine whether transformation was successful and whether the plasmid was active. The following figure indicates the selected enzymes and the areas which they affect:




Enzyme Sites 14

| Name | Pos | Type |
|---|---|---|
| NcoI | 360 | 5' |
| HindIII | 622 | 5' |
| HincII | 639 | bl |
| Acc65I | 645 | 5' |
| KpnI | 645 | 3' |
| HindIII | 891 | 5' |
| Acc65I | 1108 | 5' |
| KpnI | 1108 | 3' |
| NcoI | 1252 | 5' |
| HincII | 2093 | bl |
| PvuII | 2828 | bl |
| NcoI | 3061 | 5' |
| PvuII | 3436 | bl |
| NcoI | 3764 | 5' |

***Figure 2.2 Restriction enzyme sites on the plasmid pIRES2-EGFP map.*** *The main sites of interest are the HindIII and HincII sites*

1. *HindIII*

The plasmid DNA obtained was mixed with a master mix (as shown in Appendix A), incubated at 37°C for 1 hour and then placed in an 80°C water bath for 20 minutes to allow for heat inactivation to complete digestion. The reaction was done as follows:

2. HincII

The plasmid DNA obtained was mixed with a master mix (as shown in Appendix A), incubated at 37°C for 5-15 minutes and then placed in a 65°C water bath for 20 minutes to allow for heat inactivation to complete digestion.

## 2.1.9 Agarose gel electrophoresis

A 1% agarose gel was produced according to standard procedures. The agarose was allowed to cool to a temperature of 50°C and 2.5 µl of Ethidium Bromide (EtBr) was added to the gel; EtBr binds to the DNA and allows for the visualization of the DNA under ultraviolet (UV) light. After mixing in the EtBr, the agarose was poured into a gel tray with a well comb in place and allowed to sit at room temperature for 45 minutes, until it completely solidified (Lee *et al*, 2012). The solidified gel was placed in an electrophoresis unit and filled with

1x TAE until the gel was covered and the comb was then safely removed. Loading dye, 2 µl, was added to all the DNA samples: the uncut plasmid, the HincII cut plasmid and the HindIII cut plasmid. The 1kb DNA ladder (molecular weight ladder) was loaded into the first lane of the gel and the other samples (mixed with the loading dye) was added into the additional wells of the gel in the order of: uncut plasmid, HincII plasmid and HindIII plasmid. The gel was run at 100V until the dye line was approximately 75-80% of the way down the gel, which took a time of 1 hour and 10 minutes. Once completed the power was turned off, the gel was removed and the DNA fragments were analysed (Lee *et al*, 2012)

### 2.1.10 EcN growth curve with LB media and M9 media

Two single colonies from the LB agar plates were taken and inoculated in 5 ml of LB broth and 5 ml M9 media respectively within a 15 ml falcon tube with a negative control following the same procedure. All the tubes were incubated overnight at 37°C under static conditions and followed the standard procedure for bacteria growth curves. (Hall *et al*, 2013).

### 2.1.11 Preparation of carriers

#### 2.1.11.1 Preparation of the calcium alginate carrier

Alginate gel was made through the combination of both a calcium chloride solution and sodium alginate solution. The sodium alginate solution was made at a 3% solution and various concentrations were made for the calcium chloride solution (0.4 M, 0.6 M, 0.8 M, 1.0 M, 1.2 M and 1.4 M). The sodium alginate solution was poured into a container filled with the calcium chloride solutions and left for one hour to allow for solidification (Lee, Ravindra and Chan, 2013). The solid pieces of alginate were removed and stored in a sealed bag for 48 hours to allow for more solidification and drying to occur. Once dried the alginate gel was then cut into 2 x 2 $cm^2$ cubes. These 2 x 2 $cm^2$ cubes were used in the biofilm growth step.

#### 2.1.11.2 Preparation of water pebble carriers

Home Décor water pebbles were used in this step. They were used as they were bought, with no alterations made.

### 2.1.11.3 Preparation of the glass balls carriers

Glass balls were used as carriers. They were used as they were obtained, with no alterations made.

### 2.1.11.4 Preparation of the agarose gel carriers

A 1% Agarose gel was prepared by the addition of 1 g of agarose to 100 ml of 1x TAE buffer. The flask was microwaved in 30 second pulses with occasional swirling for a total time of 3 minutes to avoid over boiling and the subsequent decrease in concentration (Lee *et al*, 2012). The agarose was cooled in a gel tray and allowed to sit at room temperature for 45 minutes, until it has completely solidified. Once dried, the alginate gel was then cut into 2 x 2 $cm^2$ cubes. These 2 x 2 $cm^2$ cubes were used in the biofilm growth step.

### 2.1.12 *Escherichia coli* Nissle biofilm growth

The carriers were divided as follows:

1. The calcium alginate substrates were divided into 1x1 $cm^2$ pieces per container
2. The 1% Agarose substrates were divided into 1x1 $cm^2$ pieces per container
3. The water pebble substrates were divided into 8 pebbles per container
4. The glass bead substrates were divided into 8 beads per container
5. Each of the above samples were placed in 15 ml tubes and inoculated with M9 Media. The substrates were inoculated at 37°C under controlled conditions for 8 days consecutively. This resulted in EcN biofilm formation. The culture was changed daily during this period of growth. Once the biofilm growth was completed, the substrates were rinsed three times with PBS solution in order to remove any EcN that were planktonic. This was done prior to microscopic imaging.

### 2.1.13 EcN biofilm imaging

All images of the biofilm structure were obtained using the Olympus BX63 OFM microscope. Each carrier with an attached biofilm were attained via the reflected bright-field and green fluorescence protein options using a 40x objective. Specialised tools and software associated with the Olympus BX63 OFM microscope were used for image procurement and examination.

### 2.1.14 Biofuel production and determination

#### 2.1.14.1 Growth period of EcN biofilm

The biofilm growth process was repeated as per the standard methods. Once the biofilm had grown after the 6day period, M9 media was removed and replaced with fresh M9 media. Each sample containing the carrier, biofilm and fresh M9 media was moved to a makeshift “glove box”. This allowed for the removal of oxygen in the sample through the addition of argon gas. Since argon gas is heavier, it pushed the oxygen gas out the sample and allowed for an anaerobic environment. Care was taken not to disturb the biofilms and carriers during this process. Once the samples were effectively sealed, they were stored at 30°C and 37°C at fixed conditions for 78 hours to allow for biofuel production. After the 78-hour period, the media was removed from the tube and placed in a round bottom flask for fractional distillation.

#### 2.1.14.2 Fractional distillation of microbially produced media

The M9 media containing the produced ethanol was added to the round bottom flask and heated on a hotplate to 120°C. This temperature allowed the thermometer above the fractional column to reach 78°C for ethanol. Components of the M9 media with lower boiling points (ethanol and water) began to vaporise and rise through the fractional column. Ethanol began to boil first (ethanol’s boiling point is 78, 5°C while water’s boiling point is 100°C). The rising vapor contained some molecules of other substances, but the vapor contained more ethanol the higher it rose in the column. The heavier molecules fell back down and turned back into liquid in the round bottom flask.

The vapor that reached the top of the column was collected into a condensing column which cooled the ethanol vapor back into a liquid form. This was then collected into a beaker. The collected sample was then taken to complete a proton NMR to confirm if the sample did contain ethanol and no other substances.

### 2.1.14.3 Proton NMR of distilled ethanol

Proton NMR data was obtained on a Bruker Advance 300MHz spectrometer. A 10 mg sample was dissolved in 4.5 mL of deuterated chloroform ($CDCl_3$). Chemical shifts are reported in parts per million and were referenced against an internal standard, TMS (trimethyl silane) which occurs at zero ppm (de B. Harrington and Wang, 2017)

## 2.1.15 Biodiesel production:

### 2.1.15.1 Control biodiesel formation using standard procedure:

Biodiesel was produced using different types of oil and alcohol as a test against the biodiesel produced from the *E. coli*. The two types of oil used was grapeseed oil and olive oil; the alcohol used was ethanol. The catalyst used was Potassium hydroxide.

The 20 ml oil used was heated to 54°C using a hotplate and 0.14 g potassium hydroxide was dissolved in 4 ml of the alcohol. Once the desired temperature for the oil was obtained, the alcohol mixture was added to the oil and shaken for 5 minutes until the colour of the oil changed from yellow to bright orange (Olive oil) or milky white (Grapeseed oil). The mixture was then kept at room temperature for 24 hours.

### 2.1.15.2 Biodiesel formation using media produced from EcN as an ethanol substitute:

The 20 ml oil used was heated to 54°C using a hotplate and 0.14 g Potassium hydroxide was dissolved in 10 ml of fermented EcN mixture. Once the desired temperature for the oil was obtained, the EcN fermented mixture was added to the oil and shaken for 5 minutes until the colour of the oil changed from yellow to bright orange (olive oil) or milky white (grapeseed oil). The mixture was then kept at room temperature for 24 hours.

### 2.1.15.3 Biodiesel formation using media produced from EcN as an ethanol and catalyst substitute:

The 20 ml olive oil used was heated to 54°C using a hotplate. Once the desired temperature for the oil was obtained, the EcN fermented mixture (with no potassium hydroxide) was added to the oil and

shaken for 5 minutes until the colour of the oil changed from yellow to bright orange (olive oil) or milky white (grapeseed oil). The mixture was then kept at room temperature for 24 hours.

### 2.1.15.4 Biodiesel formation using EcN produced media as the oil, ethanol and catalyst substitute:

The 20 ml EcN fermented mixture was heated to 54°C using a hotplate. Once the desired temperature for the oil was obtained, the EcN fermented mixture (with no Potassium hydroxide) was added to the oil and shaken for 5 minutes until the colour of the oil changed from yellow. The mixture was then kept at room temperature for 24 hours.

## 2.1.16 Theoretical up-scaling: Biofuel production

The CEBER Modeller, developed by Prof. Kevin Harding, was used to perform theoretical up-scaling (Harding, K.2008). This Excel model uses functions and algorithms to simulate the upscaled production of compounds, taking into consideration the required media needs and proposed energy consumptions. The model was used by inserting data collected from the various experiments completed. This up-scaling simulation was done to anticipate the media and energy needs for large scale bioreactor industrial fermentations. The following inputs were selected:

## GENERIC FLOWSHEET

| Product Location | Aerboic/Anaerobic | Process Type | Cooling Options | | |
|---|---|---|---|---|---|
| (•) Extra cellular | ( ) Aerobic | (•) Continuous | Cooling water temp. | 18 | °C |
| ( ) Intra cellular | (•) Anaerobic | ( ) Batch | Chilled water temp. | 10 | °C |
| ( ) Microbial growth | | | Min. allowed temp. differences across streams in heat exchangers | 10 | °C |
| | | | No. of significant figures | 4 | |

Total mass of crude product required: 1000 kg

OR Total mass of product required in final stream: 650 kg

## MICROBIAL GROWTH CONDITIONS

### Product

| | |
|---|---|
| Product Category | OTHER |
| Specific Product | |
| Product Phase | Liquid |

Density ρ: 1221,3 kg/m³

Product:biomass ratio: 1 kg/kg

Product name: Biodiesel

Chemical Formula:

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| C | 17 | 17 | S | 0 | Mg | 0 | |
| H | 34 | 34 | Na | 0 | Ca | 0 | |
| O | 2 | 2 | Cl | 0 | Si | 0 | |
| N | 0 | 0 | K | 0 | Al | 0 | |
| P | | 0 | Fe | 0 | Cu | 0 | |

### Biomass

| | |
|---|---|
| Biomass | *Escherichia coli* |

Ash Content: 8,5 %

Chemical Formula:

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| C | | 1 | S | 0 | Mg | 0 | |
| H | | 1,77 | Na | 0 | Ca | 0 | |
| O | | 0,49 | Cl | 0 | Si | 0 | |
| N | | 0,24 | K | 0 | Al | 0 | |
| P | | 0 | Fe | 0 | Cu | 0 | |

### Secondary Anaerobic Product

*This is for the growth phase only

| | |
|---|---|
| Anearobic Product | Ethanol |

Anaerobic product:biomass formation ratio: 0,5 — 0,5 kg/kg

Chemical Formula:

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| C | | 2 | S | 0 | Mg | 0 | |
| H | | 6 | Na | 0 | Ca | 0 | |
| O | | 0 | Cl | 0 | Si | 0 | |
| N | | 0 | K | 0 | Al | 0 | |
| P | | 0 | Fe | 0 | Cu | 0 | |

### Carbon Source

| | |
|---|---|
| Main category | OTHER |
| Specific source | Glycerol |

Carbon excess: 1 %

Chemical Formula:

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| C | 3 | 3 | K | 0 | | | |
| H | 8 | 8 | Fe | 0 | | | |
| O | 3 | 3 | Mg | 0 | | | |
| N | | 0 | Ca | 0 | | | |
| P | | 0 | Si | 0 | | | |
| S | | 0 | Al | 0 | | | |
| Na | | 0 | Cu | 0 | | | |
| Cl | | 0 | | | | | |

☐ Add another carbon source?

### Nitrogen Source

| | |
|---|---|
| Main category | Organic |
| Specific source | OTHER |

Nitrogen excess: 5 %

Nitrogen name: *Nitrogen source

Chemical Formula:

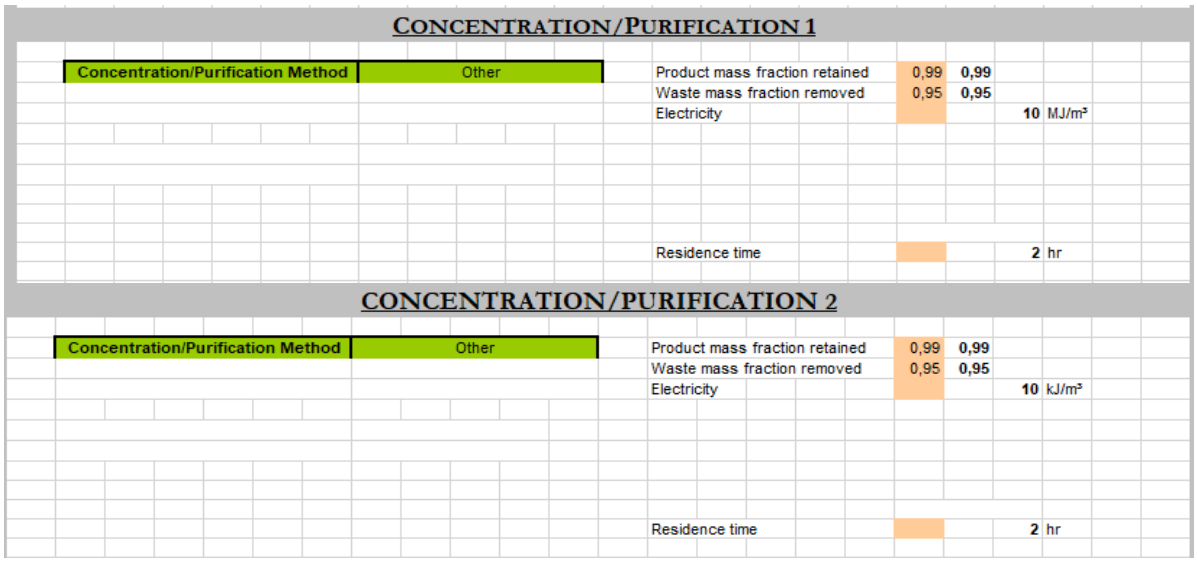
| | | | | | | | |
|---|---|---|---|---|---|---|---|
| C | 0 | 0 | K | 0 | | | |
| H | 4 | 4 | Fe | 0 | | | |
| O | 0 | 0 | Mg | 0 | | | |
| N | 1 | 1 | Ca | 0 | | | |
| P | | 0 | Si | 0 | | | |
| S | | 0 | Al | 0 | | | |
| Na | | 0 | Cu | 0 | | | |
| Cl | 1 | 1 | | | | | |

### Reactor Temperature

| | | | |
|---|---|---|---|
| Reactor temperature | $T_{rct}$ | | 37 |
| Ambient temperature | $T_a$ | | 20 |

### Growth Rate

| | | | | | |
|---|---|---|---|---|---|
| Initial biomass concentration | $C_{x,in}$ | | 1 | g/l (kg/m³) | |
| Final biomass concentration | $C_{x,final}$ | 6,2 | 6,2 | g/l (kg/m³) | |
| Max. specific growth rate | $\mu_{max}$ | 0,35 | 0,35 | hr⁻¹ | |
| Limiting nutrient conc. giving half max. growth rate* | $K_s$ | | 8,33 | mg/l | *Carbon source I used as reference |



| CONCENTRATION/PURIFICATION 1 | | | | | | |
|---|---|---|---|---|---|---|
| Concentration/Purification Method | Other | Product mass fraction retained | 0,99 | 0,99 | | |
| | | Waste mass fraction removed | 0,95 | 0,95 | | |
| | | Electricity | | | 10 | MJ/m³ |
| | | Residence time | | | 2 | hr |

| CONCENTRATION/PURIFICATION 2 | | | | | | |
|---|---|---|---|---|---|---|
| Concentration/Purification Method | Other | Product mass fraction retained | 0,99 | 0,99 | | |
| | | Waste mass fraction removed | 0,95 | 0,95 | | |
| | | Electricity | | | 10 | kJ/m³ |
| | | Residence time | | | 2 | hr |

***Figure 2.3: CEBER Modeller showing all the inputs that were selected for the CEBER Modeller excel sheet****. Each category with missing information (either coloured green or light orange) was completed to obtain a final result*

# Chapter 3

## 3.1 Results

### 3.1.1 *Escherichia coli* Nissle strain preparation

The following figures show the growth of the EcN strain on both LB media and MacConkey media. The LB media has all general nutrients in abundance for bacterial growth.

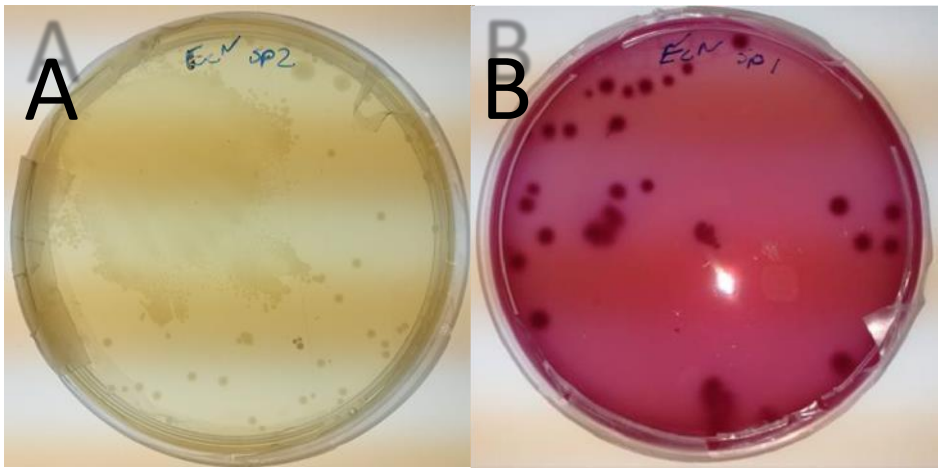


***Figure 3.1: Growth of E. coli Nissle on two different types of growth media for verification.****The bacteria were grown on first (A) LB media for preparation and then (B) MacConkey media for E. coli specific growth. A 100ul of bacterial suspension was added to each plate and incubated for 24 hours at 37°C.*

## 3.1.2 Growth curve of untransformed EcN in LB media

A single colony was taken from the MacConkey plates and inoculated to develop a growth curve. The growth curve shows that the lag phase occurred for 1 hour where it then increased into the exponential phase for the next 12 hours where it then plateaued at 4.2 absorbance. It took a total of 15 hours for the EcN strain to reach maximum growth.

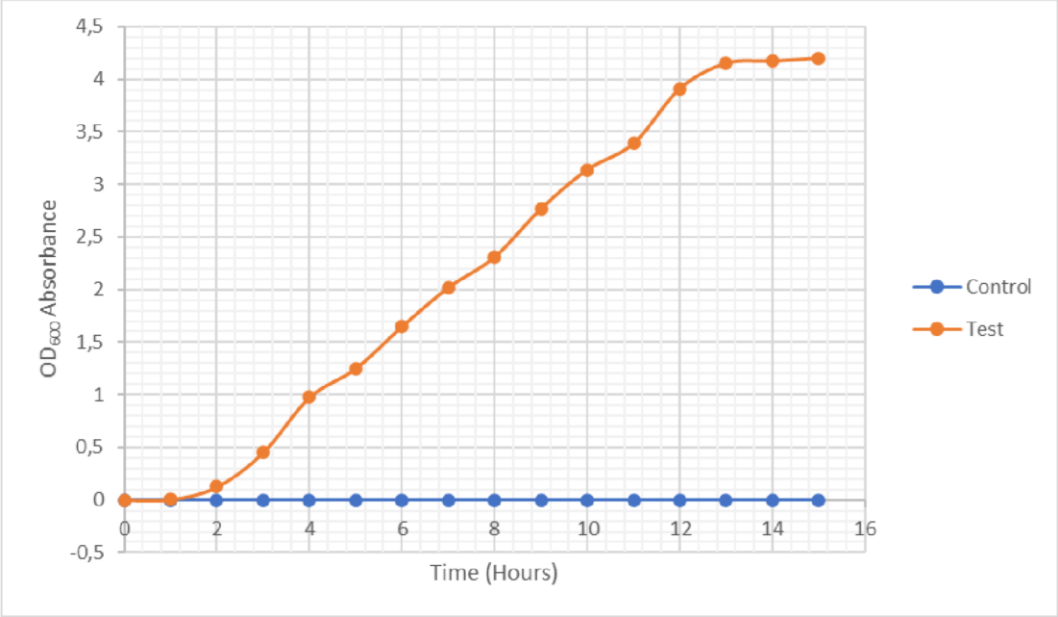



***Figure 3.2: The growth curve of E. coli Nissle in LB media showing the absorbance (OD600) over a period of time(hours).*** *The results show the growth of E. coli Nissle over a 15-hour period with the absorbance read every hour. The average of three test growth curves containing the E. coli Nissle and a control containing no E. coli Nissle was completed over the 15-hour period.*

## 3.1.3 Agarose gel electrophoresis

The EcN sample was taken and made competent through the calcium chloride method to allow for transformation with the vector: pIRES2-EGFP (which contains a kanamycin resistant gene and enhanced green florescent protein). Plasmid extraction and restriction enzyme digestion was completed on enzyme sites: HindIII and HincII. These were then run on a 1% agarose gel. As shown on the figure 3.3, the enzyme cuts at the relevant spots but the bands are incredibly light due to low plasmid DNA count after extraction.

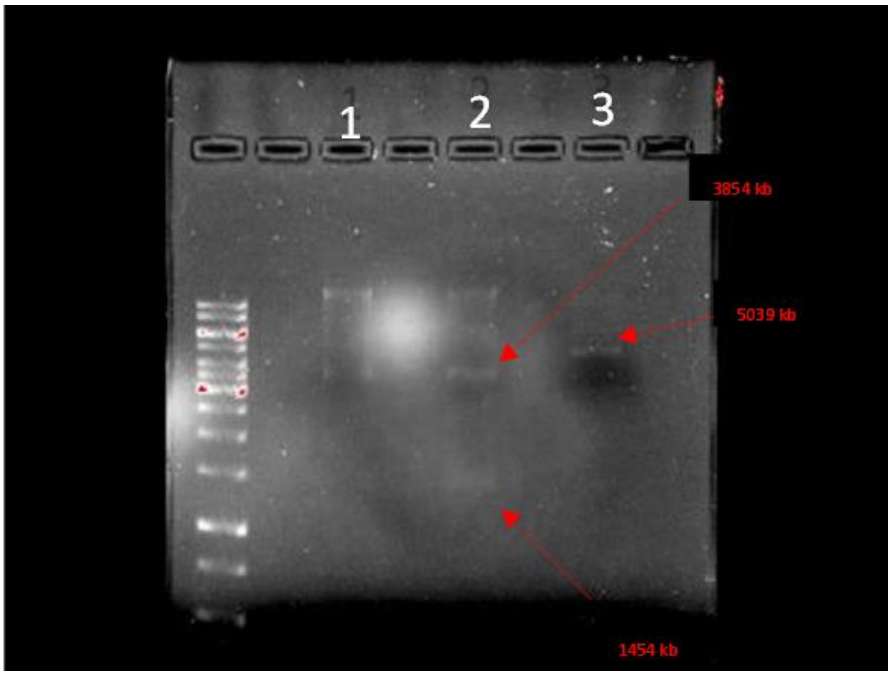



***Figure 3.3: A 1% Agarose gel of the extracted plasmid showing two sites that have gone through restriction enzyme digestion****. Lane 1 contains the uncut plasmid DNA. Lane 2 contains the HincII cut DNA fragments. Lane 3 contains the HindIII cut DNA fragments.*

## 3.1.4 Growth curve of transformed EcN in LB media

A single colony was taken from the transformed *E. coli* Nissle colonies and inoculated to produce a growth curve. The growth curve shows that the lag phase occurred for 2 hours where it then increased into the exponential phase for the next 5 hours, where it then plateaued at 3.5 absorbance as shown in figure 3.4. It took a total of 9 hours for the EcN strain to reach maximum growth as shown in figure 3.4.

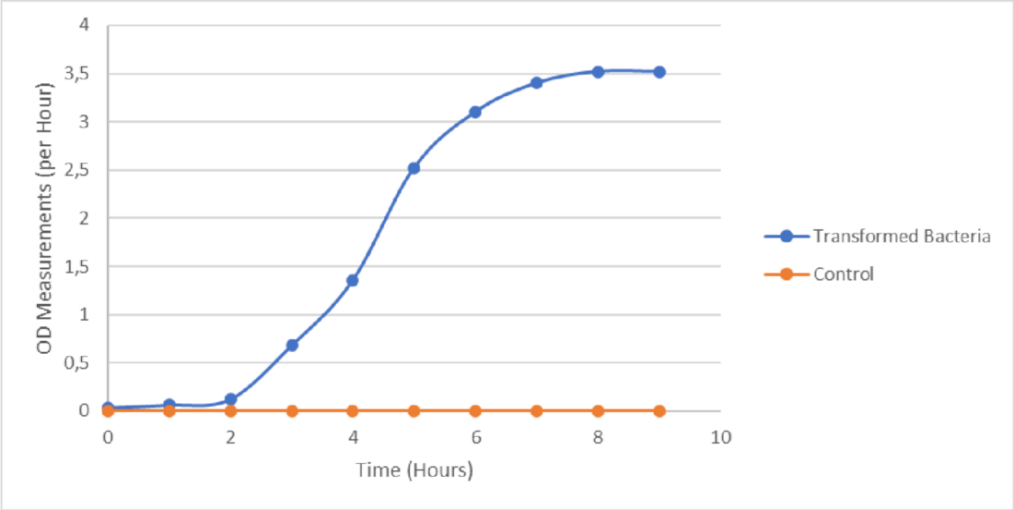



***Figure 3.4: The growth curve of Kanamycin transformed E. coli Nissle in LB media showing the absorbance (OD600) over a period of time(hours).*** *The results show the growth of E. coli Nissle over a 9-hour period with the absorbance read every hour. The average of three test growth curves containing the E. coli Nissle and a control containing no E. coli Nissle was completed over the 9-hour period.*

## 3.1.5 Growth curve of transformed *Escherichia coli* Nissle in M9 media

A single colony was taken from the MacConkey plates to produce the growth curve shown in figure 3.5. The growth curve shows that the lag phase occurred for 5 hours where it then increased into the exponential phase for the next 6 hours, where it then plateaued at 2.5 absorbance as shown in figure 3.5. It took a total of 9 hours for the EcN strain to reach maximum growth as shown in figure 3.5.

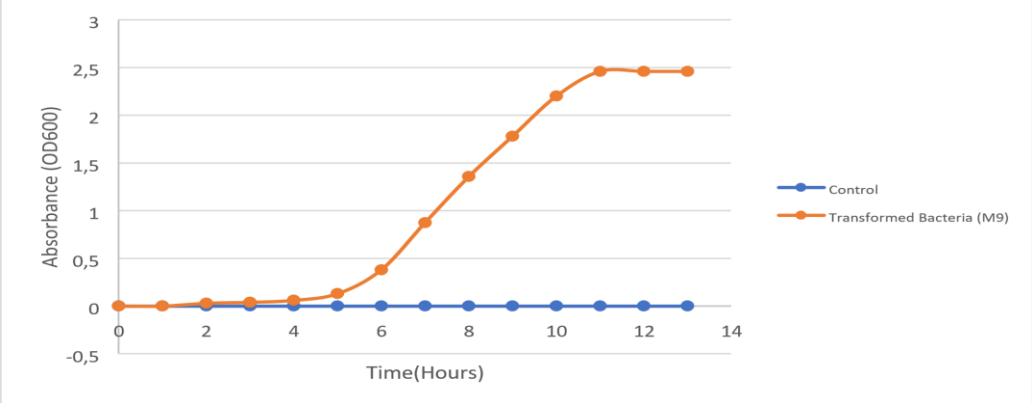



***Figure 3.5: The growth curve of Kanamycin transformed E. coli Nissle in M9 media showing the absorbance (OD600) over a period of time(hours).*** *The results show the growth of E. coli Nissle over a 13-hour period with the absorbance read every hour. The average of three test growth curves containing the E. coli Nissle and a control containing no E. coli Nissle was completed over the 13-hour period*

## 3.1.6 Preparation of carriers for EcN biofilm growth

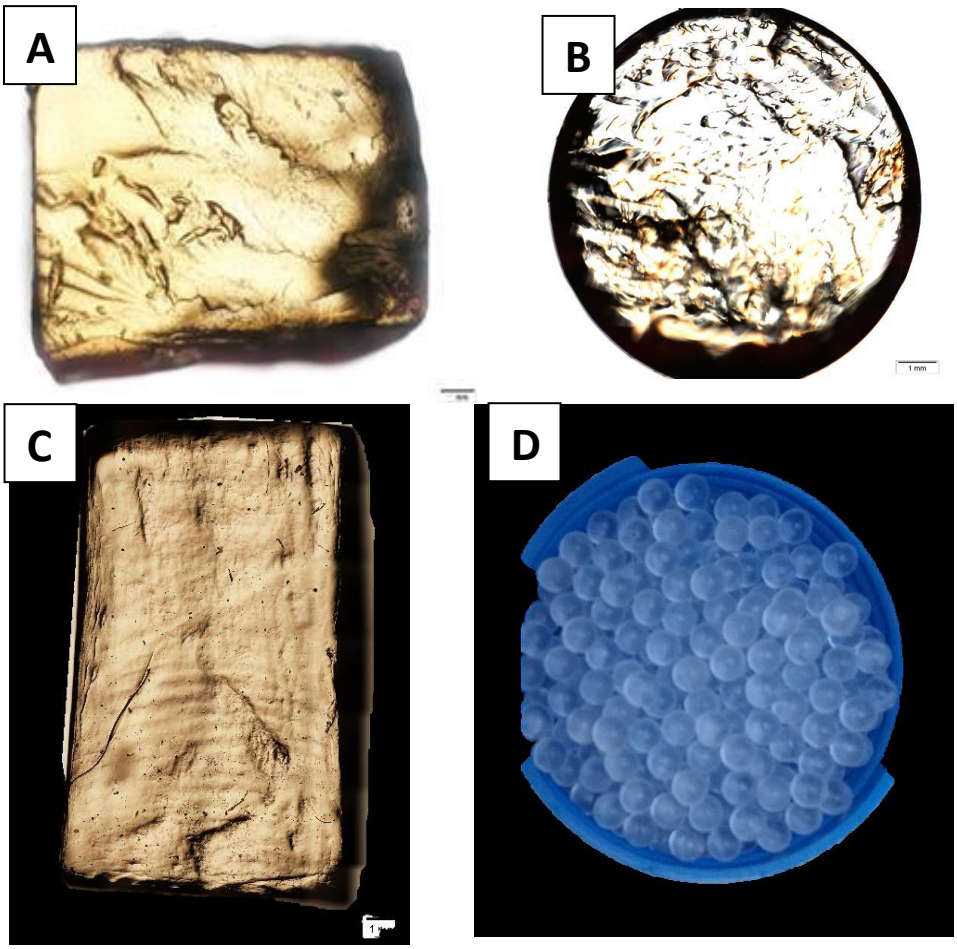



***Figure 3.6: Microscopic image of the different carriers formulated.*** *The results display the carriers after a 24-hour development period (A) Calcium Alginate carrier, (B) Water pebble, (C) 1% Agarose Gel and (D) Glass balls*

## 3.1.7 Biofilm growth

## 3.1.8 EcN biofilm imaging

Both standard bright field and *florescence* Images of the biofilm growth on the three different carriers: Calcium alginate, 1% agarose gel and water pebble were taken as shown in figures 3.7, 3.8 and 3.9 respectively.

- Calcium Alginate

Images of the biofilm growth on the calcium alginate carrier was taken. The figures contain both the bright field view of the carrier and biofilm growth at 4x magnification and the green florescence view of the biofilm and carriers at 100 ms exposure with 2x grain.

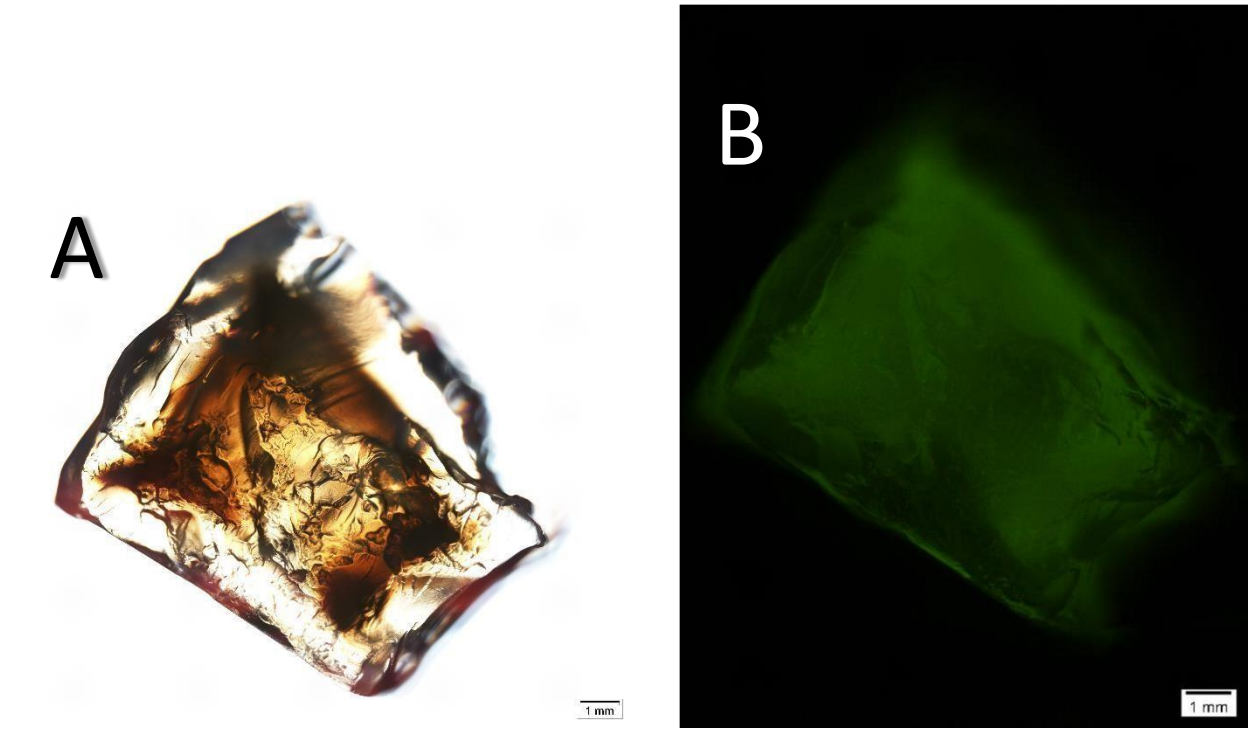



***Figure 3.7: Microscopic image of the E. coli Nissle biofilm on the calcium alginate carrier****. The image on the left (A) displays the bright field view of the biofilm on the alginate carrier at 4X magnification. The biofilm is indicated by the dark colouring in the middle of the alginate. The image on the right (B) displays the florescence view of the biofilm on the alginate carrier at 4X magnification The biofilm is indicated by the green fluorescence on the alginate.*

- <u>1% Agarose gel</u>

  Images of the biofilm growth on the 1% agarose gel was taken. The figures contain both the bright field view of the carrier and biofilm growth at 4x magnification and the green florescence view of the biofilm and carriers at 100 ms exposure with 2x grain.

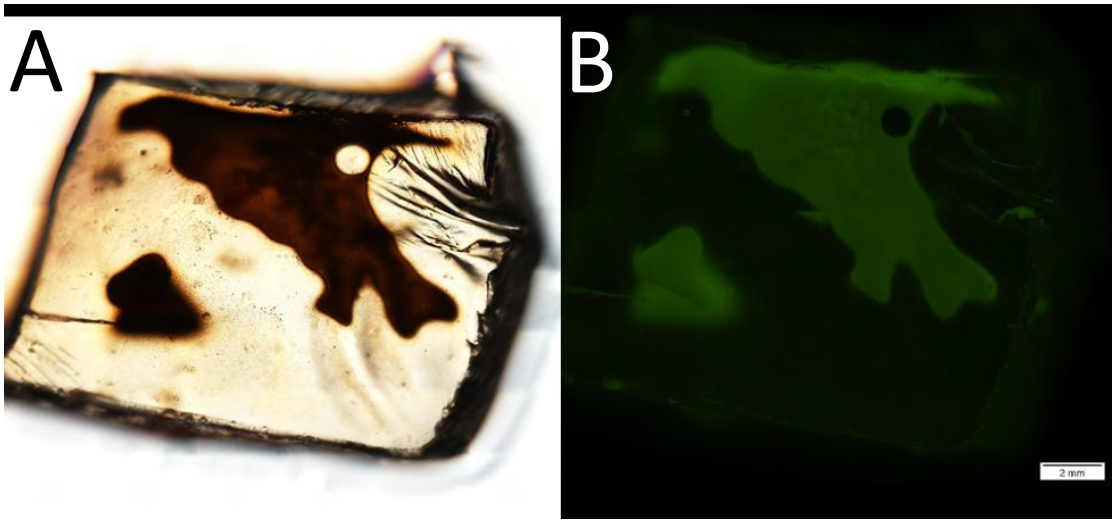



***Figure 3.8: Microscopic image of the E. coli Nissle biofilm on the 1% agarose gel carrier****. The image on the left (A) displays the bright field view of the biofilm on the agarose gel at 4X magnification. The biofilm is indicated by the dark colouring in the middle of the agarose. The image on the right (B) displays the florescence view of the biofilm on the agarose gel at 4X magnification. The biofilm is indicated by the green fluorescence on the agarose*

- Water Pebble

  Images of the biofilm growth in the water pebble was taken. The figures contain both the bright field view of the carrier and biofilm growth at 4x magnification and the green florescence view of the biofilm and carriers at 100 ms exposure with 2x grain.

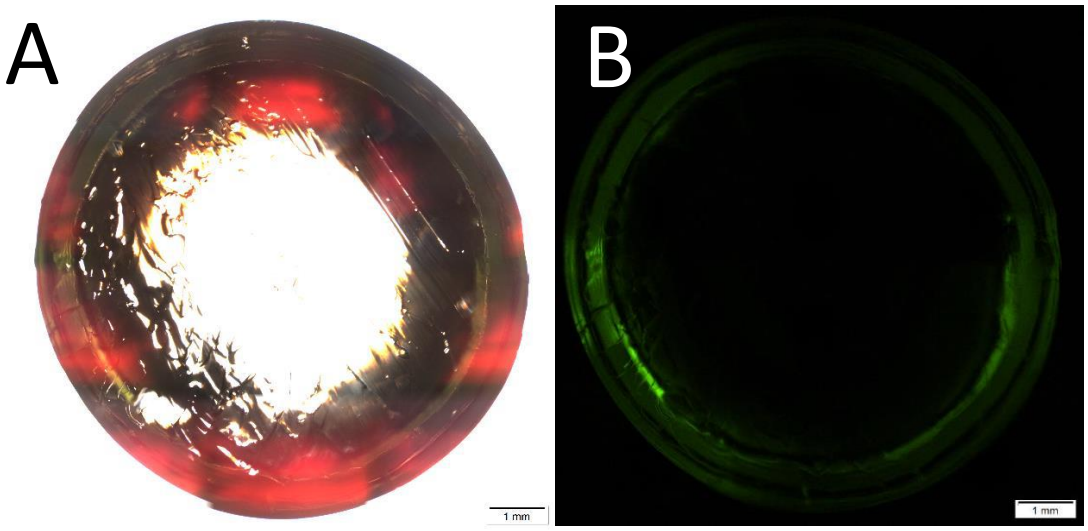



***Figure 3.9: Microscopic image of the E. coli Nissle biofilm on the water pebble carrier****. The image on the left (A) displays the bright field view of the biofilm in the water pebble at 4X magnification. The biofilm is indicated by the dark colouring around the outer edges of the water pebble. The image on the right (B) displays the florescence view of the biofilm in the water pebble at 4X magnification. The biofilm is indicated by the green fluorescence around the outer edges of the water pebble*

## 3.1.9 Biofuel production and determination

## 3.1.10 Determination of EcN ethanol production

Using fractional distillation, the data shown in the table below (table 3.1), was obtained regarding ethanol production from the EcN biofilm. The data takes into account the three types of carriers that we used: Water pebble, Calcium Alginate and Agarose gel; the incubation temperature of the samples; ideally where we expect the biofilm to grow and how long it takes the biofilm to grow in general; where of not the biofilm grown was stable on the carrier; the size of each of the carriers; the dry cell weight of the grown EcN after the selected days and the amount of ethanol after distillation.

These results show that it took longer for the EcN biofilm to show any growth with the water pebbles as these were expected to grow internally once absorbed compared to the external development of both the calcium alginate and the agarose gel. Due to the size of the water pebbles and the amount of liquid it can contain more carriers were used compared to that of the other two carriers to mitigate any issues that may results from it. Ultimately this shows that both the dry weight of the EcN biofilm as well as the amount distilled for both the calcium alginate and agarose gel were incredibly similar and far exceeded

the amount distilled from the water pebble even though the same amount of M9 media was used to supplement all samples. The main difference between the samples was that of the stability, where the water pebbles were the most stable under shaking and vortexing conditions, the calcium alginate carrier was stable but slightly less so under those conditions and the calcium alginate was not stable and the EcN was dispersed under extreme condition.

Table 3.1 showed that the average amount of distilled ethanol from each carrier differed: the water pebble produced an average of 0.3706 ml of distilled ethanol, the calcium alginate produced an average of 1.92 ml of distilled ethanol and the agarose gel produced an average of 1.94 ml of distilled ethanol. The dry cell weight also differed in the average amount per carrier: the water pebble had a dry cell weight amount of 0.0124 g: the calcium alginate had a dry cell weight amount of 0.1465 g and the agarose gel had a dry cell weight amount of 0.1380 g. This shows a linear correlation between the dry cell weight of EcN to the amount of ethanol produced as well as that the square carriers were able to hold more dry cell weight to that of the circular carriers. Both the square carriers (calcium alginate and 1% agarose) had a higher visibility of growth compared to that of the circular carrier (water pebble) which also allowed it to accumulate more dry cell weight.

Table 3.1: The production and the calculated results from the fractional distillation to determine the amount of ethanol that was distilled from the fermented sample. This table considers all the variables concerning the EcN biofilm growth

| Sample Number | Carrier Type | Area where the biofilm growth occurred | Growth of film visibility | | | | | | | | Base stability of biofilm on carrier | Number of Carriers | Carrier Size (g) | Dry Cell weight of EcN (g) | Ethanol Distillation Amount (ml) | Amount of M9 media per sample (ml) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | | | | | | |
| 1 | Water Pebble | Inside | No | No | No | No | No | Yes | Yes | Yes | Stable | 8 | 0.4343 | 0.0124 | 0.373 | 8 |
| 2 | Water Pebble | Inside | No | No | No | No | No | Yes | Yes | Yes | Stable | 8 | 0.4343 | 0.0124 | 0.370 | 8 |
| 3 | Water Pebble | Inside | No | No | No | No | No | Yes | Yes | Yes | Stable | 8 | 0.4343 | 0.0124 | 0.369 | 8 |
| 4 | Calcium Alginate | Outside | No | No | No | No | Yes | Yes | Yes | Yes | Stable | 1 | 0.7983 | 0.1496 | 2.04 | 8 |
| 5 | Calcium Alginate | Outside | No | No | No | No | Yes | Yes | Yes | Yes | Stable | 1 | 0.7980 | 0.1387 | 1.92 | 8 |
| 6 | Calcium Alginate | Outside | No | No | No | No | Yes | Yes | Yes | Yes | Stable | 1 | 0.7978 | 0.1513 | 1.80 | 8 |
| 7 | Agarose Gel | Outside | No | No | No | No | No | Yes | Yes | Yes | Not Stable | 1 | 0.6241 | 0.1324 | 2.00 | 8 |
| 8 | Agarose Gel | Outside | No | No | No | No | No | Yes | Yes | Yes | Not Stable | 1 | 0.6230 | 0.1358 | 1.87 | 8 |
| 9 | Agarose Gel | Outside | No | No | No | No | No | Yes | Yes | Yes | Not Stable | 1 | 0.6235 | 0.1460 | 1.95 | 8 |

## 3.1.11 Proton NMR

This procedure was used as a quick way to determine whether ethanol was present in the distilled EcN media obtained. This showed that both the water pebble and Calcium alginate and 1& agarose samples did contain ethanol through the distillation but also contain large amounts of water which happened to be distilled at 78.5°C. The calcium alginate and agarose sample contained a large impurity as shown in figure 3.11 whereas the water pebble sample as negligible impurity.

- Water Pebble

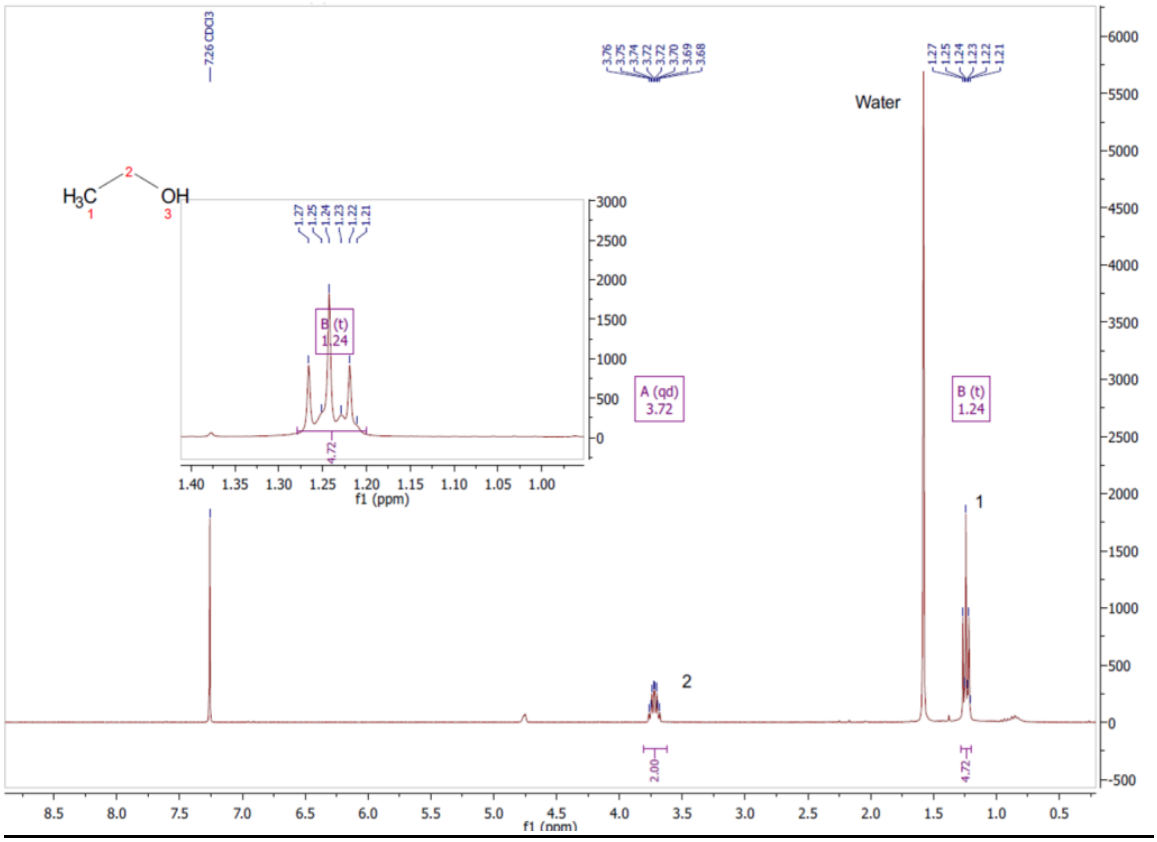



***Figure 3.10: The proton NMR of fraction distillation obtained from the distilled EcN media utilizing a water pebble as the carrier****. The peak at 1,25 ppm labelled "1" relates to the H3C group, the peak at 1,55ppm labelled "water" relates to the OH group and the peak at 3,75 ppm labelled "2" relates to the $H_2C$ group as shown on the legend on the top-left part of the figure. The peak at 7,25 relates to the deuterated chloroform that the ethanol sample was dissolved in. The square box above the deuterated chloroform is a zoomed version of peak "1". This shows the 2 distinct peaks relating to the H group.*

- Calcium Alginate & Agarose

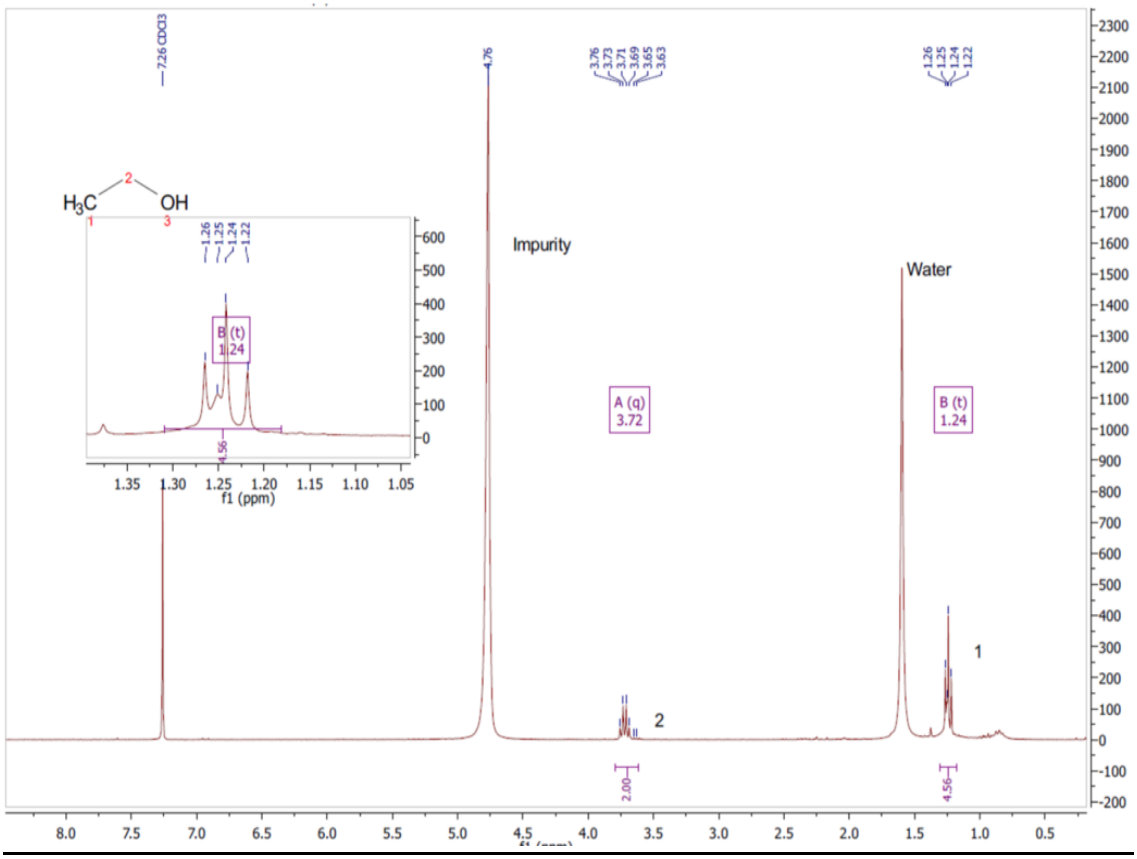



***Figure 3.11: The proton NMR of fraction distillation obtained from distilled EcN media utilizing a calcium alginate and 1% agarose as the carrier***. *The peak at 1,25 ppm labelled "1" relates to the H3C group, the peak at 1,55ppm labelled "water" relates to the OH group and the peak at 3,75 ppm labelled "2" relates to the $H_2C$ group as shown on the legend on the top-left part of the figure. The peak at 7,25 relates to the deuterated chloroform that the ethanol sample was dissolved in. The square box above the deuterated chloroform is a zoomed version of peak "1". This shows the 2 distinct peaks relating to the H group. The peak at 4,75 ppm labelled "impurity" is some form of contamination that occurred during distillation.*

## 2.1.12 Biodiesel formation test

Three different tests were done to determine biodiesel formation.

## 3.1.13 Control biodiesel formation using standard procedure:

This test was done as a control to determine biodiesel production using the classic method of mixing the three initial components (oil, alcohol and a catalyst) to produce biodiesel and glycerine waste, as shown in the table. The results shown in figure 19 indicate a successful biodiesel conversion where 20 ml of the relative oil was converted to 20 ml of biodiesel, and the 4 ml of ethanol was converted to 4 ml of glycerine for both Olive and Grapeseed oil. The only

difference between the samples was that the biodiesel colour of olive oil was light yellow whereas the grapeseed oil colour was opaque.

*Table 3.2:* ***The initial components of biodiesel production that was added together to achieve the final biodiesel and glycerine for both olive oil and grapeseed oil***

| Label | Oil Type | Initial Components | | | Final Products | |
|---|---|---|---|---|---|---|
| | | Oil (ml) | Ethanol (ml) | KOH (g) | Biodiesel (ml) | Glycerine (ml) |
| A | Olive | 20 | 4 | 0.14 | 20 | 4 |
| B | Grapeseed | 20 | 4 | 0.14 | 20 | 4 |

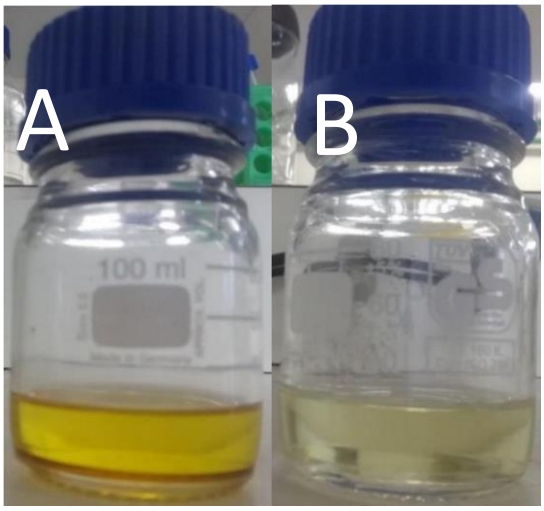



***Figure 3.12: Biodiesel control production via the classic/well known method of producing biodiesel****. Sample (A) shows the biodiesel produced from olive oil as a light-yellow liquid with a light brown line at the base, which was glycerine. Sample (B) shows the biodiesel produced from grapeseed oil as an opaque coloured liquid with an opaquer line at the bottom, which was glycerine*

## 3.1.14 Biodiesel formation using EcN produced media as an ethanol substitute:

This test was done to determine biodiesel production using the EcN produced media as an ethanol substitute to produce biodiesel, with glycerine as waste, as shown in the table. The alcohol used in this test was that of the ethanol produced via the anaerobic fermentation of the EcN biofilm in M9 media. The results shown in figure 20 indicate varying results depending on the oil used and mixture obtained from the different biofilms and their respective carriers. In all the samples, a different amount of emulsion was formed. With regards to the olive oil sample, the

water pebble fermented mixture produced a better final result compared to the other carriers. With regards to the grapeseed oil, there was no difference between the final products produced, regardless of with carrier was utilized.

Table 3.3 ***The initial components of oil, alcohol and KOH that was added together to achieve the final biodiesel, soap and glycerine.*** *This was for both olive oil and grapeseed oil. This further was split between the three types of carriers that were used*

| Label | | Oil Type | **EcN Nissle Carrier type** | **Initial Components** | | | **Final Products** | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | **Oil (ml)** | ***E. coli* fermentation mixture (ml)** | **KOH (g)** | **Biodiesel (ml)** | **Emulsion (ml)** | **Glycerine (ml)** |
| C | | Olive | Water Pebble | 20 | 10 | 0.14 | 10 | 5 | 15 |
| D | | Olive | Agarose | 20 | 10 | 0.14 | 7 | 13 | 10 |
| E | | Olive | Calcium Alginate | 20 | 10 | 0.14 | 9 | 7 | 14 |
| F | | Grapeseed | Water Pebble | 20 | 10 | 0.14 | 20 | 1 | 9 |
| G | | Grapeseed | Agarose | 20 | 10 | 0.14 | 20 | 1 | 9 |
| H | | Grapeseed | Calcium Alginate | 20 | 10 | 0.14 | 20 | 1 | 9 |

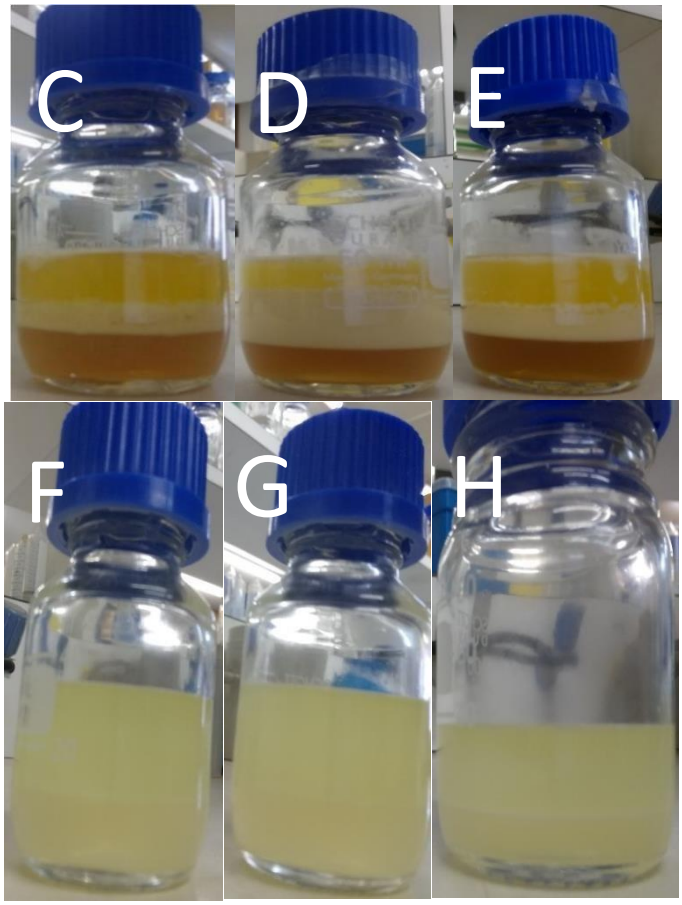



***Figure 3.13: Biodiesel production* using EcN produced media as an ethanol substitute.** *Sample (C) shows the biodiesel produced from olive oil and the water pebble carrier as a light-yellow liquid with a white segment being soap formation and a light brown line at the bottom being glycerine. Sample (D) shows the biodiesel produced from olive oil and the agarose gel carrier as a light-yellow liquid with the white segment being emulsion formation and the light brown line at the bottom being glycerine. Sample (E) shows the biodiesel produced from olive oil and the calcium alginate carrier as a light-yellow liquid with the white segment being emulsion formation and light brown line at the bottom being glycerine. Sample (F) shows the biodiesel produced from grapeseed and the water pebble carrier as a milky white liquid with the light white segment at the bottom being glycerine. Sample (G) shows the biodiesel produced from grapeseed and the agarose carrier as a milky white liquid with the light white segment at the bottom being glycerine Sample (H) shows the biodiesel produced from grapeseed and the calcium alginate carrier as a milky white liquid with the light white segment at the bottom being glycerine*

## 3.1.15 Biodiesel formation using EcN produced media as an ethanol and catalyst substitute:

This test was done to determine biodiesel production using the classic method of mixing the three initial components (oil, alcohol and a catalyst) to produce biodiesel and glycerine (as waste), as shown in table 3.4. The alcohol and catalyst used in this test was that of the fermented mixture produced via the anaerobic fermentation of the EcN biofilm in M9 media. As the colour of the olive oil was more distinct compared to that of the grapeseed oil, it was chosen for this specific test. With regards to the olive oil tests, the water pebble fermented mixture produced the best results and thus was chosen for this test as well. The results shown in figure 21 and table

4 show that 20 ml of olive oil was converted to 20 ml of biodiesel and the 10 ml of fermented mixture was converted to glycerine.

***Table 3.4: showing the initial components of oil, alcohol and KOH that was added together to achieve the final biodiesel and glycerine**. Only olive oil and the water pebble fermented mixture was used for this test with no addition of KOH.*

| **Label** | **Oil type** | **EcN Nissle Carrier type** | **Initial Components** | | | **Final Products** | | |
|---|---|---|---|---|---|---|---|---|
| | | | **Oil (ml)** | ***E. coli* fermentation mixture(ml)** | **KOH (g)** | **Biodiesel (ml)** | **emulsion (ml)** | **Glycerine (ml)** |
| I | Olive | Water<br>Pebble | 20 | 10 | 0 | 20 | | 10 |

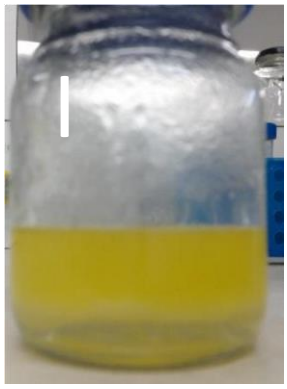


***Figure 3.14: Biodiesel production using* EcN produced media as an ethanol and catalyst substitute**. *Sample (I) shows the biodiesel produced from olive oil as a bright yellow liquid with the clear liquid at the bottom being glycerine.*

## 3.1.16 Biodiesel formation using EcN produced media as the oil, ethanol and catalyst substitute:

This test was done to determine biodiesel production using the classic method of mixing the three initial components (oil, alcohol and a catalyst) to produce biodiesel and glycerine waste as shown in table. The oil, alcohol and catalyst used in this test was that of the fermented mixture produced via the anaerobic fermentation of the EcN biofilm in M9 media. No reaction occurred over multiple tests with no colour changes as shown in figure 3.16.

***Table 3.5: The initial components of oil, alcohol and KOH that was added together to achieve the final biodiesel and glycerine****. Only olive oil and the water pebble fermented mixture was used for this test with no addition of KOH.*

| **Label** | **Oil type** | **EcN Nissle Carrier type** | **Initial Components** | | | **Final Products** | | |
|---|---|---|---|---|---|---|---|---|
| | | | **Oil (ml)** | ***E. coli* fermentation mixture(ml)** | **KOH (g)** | **Biodiesel (ml)** | **emulsion (ml)** | **Glycerine (ml)** |
| I | Olive | Water<br>Pebble | o | 20 | 0 | **0** | 0 | 0 |




***Figure 3.15: Biodiesel production using* EcN produced media as the oil, ethanol and catalyst substitute.** *Sample (J) shows the results of heating 20ml of the EcN mixture and adding 10 ml of unheated EcN mixture. No reaction occurred and the colour remained milky*

## 3.1.17 Theoretical up-scaling

The theoretical up-scaling of biodiesel production on a commercial and industrial scale was tested.

Based on the average total mass product of 1000 kg that the excel sheet provides and given the

inputs shown in the theoretical up-scaling methods section, the total product mass obtained would be 650.3 kg which is 65.03% purity and a recovery of 90.25%. The main inputs shown in figure 3.1 in product composition are the 138.6 kg of EcN and 5479 kg of glycerol feedstock to produce 650.3 kg of biodiesel and 0.1365 kg of glycerol waste.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Product Name** | Biodiesel | | | | | **Total Product Mass** | | 1000 | kg |
| | | | | | | **Product Mass** | | 650,3 | kg |
| | | | | | | **Purity** | | 65,03 | % |
| * Specific names not given | | | | | | **Recovery** | | 90,25 | % |
| **Product Composition** | | | **IN** | | | **OUT** | | | |
| Glycerol | 0,1356 | kg | Energy/Utilities | | | **Total Crude Product** | | | |
| *Nitrogen source | 0,04626 | kg | Electricity | | 1,476 GJ | | | | |
| Escherichia coli | 2,148 | kg | | | | Product | | 1000 | kg |
| Biodiesel | 650,3 | kg | Inputs | | | | | | |
| Ethanol | 0,9007 | kg | Municipal water | | 138,6 m³ | **Waste** | | | |
| Water | 346,4 | kg | Escherichia coli | | 138,6 kg | Water emissions: | | | |
| | | | Glycerol | | 5479 kg | Water | | 138,2 | m³ |
| | | | *Nitrogen source | | 388,6 kg | Escherichia coli | | 857 | kg |
| | | | | | | Ethanol | | 359,4 | kg |
| | | | | | | Glycerol | | 54,11 | kg |
| | | | | | | *Nitrogen source | | 18,46 | kg |
| | | | | | | Biodiesel | | 70,26 | kg |
| | | | | | | Air emissions: | | | |
| | | | | | | CO2 | | 3461 | kg |
| | | | | | | Cl2 | | 245,3 | kg |
| | | | | | | H2 | | 287,7 | kg |
| | | | | | | Solid waste: | | | |
| | | | | | | None | | | |
| | | | | | | Chemical Oxygen Demand: | | | |
| | | | | | | COD | | 3325 | kg |
| | | | **Total Mass** | **144,6** | **ton** | **Total Mass** | **144,6** | **ton** | |

***Figure 3.1: CEBER modeller of the theoretical product composition, input, output and waste of materials for the upscaling of ethanol and biodiesel on a commercial scale.***

# Chapter 4
# 4.1 Discussion
## 4.1.1 Growth curve (untransformed)

The growth curve for the untransformed Nissle had an unusual growth pattern where it had only 1 hour of lag before it immediately went to the exponential phase. This phase had breaks in its duration as shown in figure 3.2 and did not increase at a constant rate. The time in the exponential phase was also much longer than the average of 8 hours (Pletnev et al., 2015), where it remained at 12 hours. This was a 50% increase in the exponential phase time. Possible contamination of another organism could have caused this unusual growth pattern. Cell counts, replating, and microscopy of the EcN was completed but no contamination was found with all experimentation done under a laminar flow.

The untransformed Nissle was used in the next step, which was the biofilm growth step. During this step, the process that was followed was as prescribed, resulting in Figure 4.1. The biofilm did not grow during this step on multiple tests, resulting in "fragmented pieces" of calcium alginate which did not resemble a biofilm. The conclusion developed was that contamination was present was inhibiting the bacterial biofilm growth of Nissle. The plasmid pIRES2-EGFP was obtained to alleviate this issue as shown by figure 2.1.

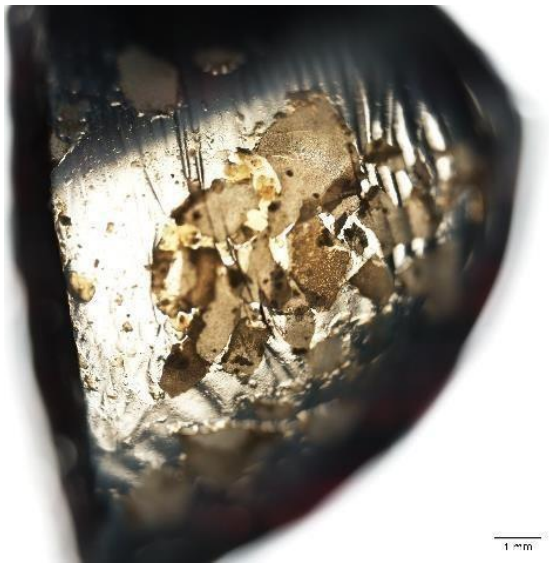


***Figure 4.1: Microscopic image of the E. coli Nissle biofilm on the calcium alginate carrier.*** *The image shows the bright field view of fragmented pieces on the alginate carrier at 4X magnification.*

## 4.1.2 Agarose gel electrophoresis

The bands shown in figure 3.3 were extremely light due to the low DNA count after transformation had occurred. Multiple tests were done but still resulted in low DNA yield. Even though the bands were light in colour, the two fragments of HincII (which are 3854kb and 1454kb), could be identified, and only 1 of the fragments of HindIII (5039kb), could be identified. This corresponds to the HindIII cut sites of 622 and 891 and the HincII cut sites of 639 and 2093 as shown in the Add gene sequence analyser site for plasmid pIRES2-EGFP (pIRES2-EGFP Sequence and Map, 2022). This was because the electrophoresis procedure did not run until the end of the gel as shown by figure 3.3. Multiple tests were done and either the procedure ran off the gel and did not produce a result at the other HindIII fragment (which is 269kb), or it stopped just before the fragment point appeared. Better optimization of the agarose gel electrophoresis procedure would need to be done to obtain a better result such as the agarose percentage of the gel, adjusting the time spent along with the volts used. Regardless, figure 10 showed the required outcome, which was that the transformation was successful, and the plasmid was taken up by the *E. coli* Nissle strain.

## 4.1.3 Growth curve differences between the transformed EcN and the untransformed EcN

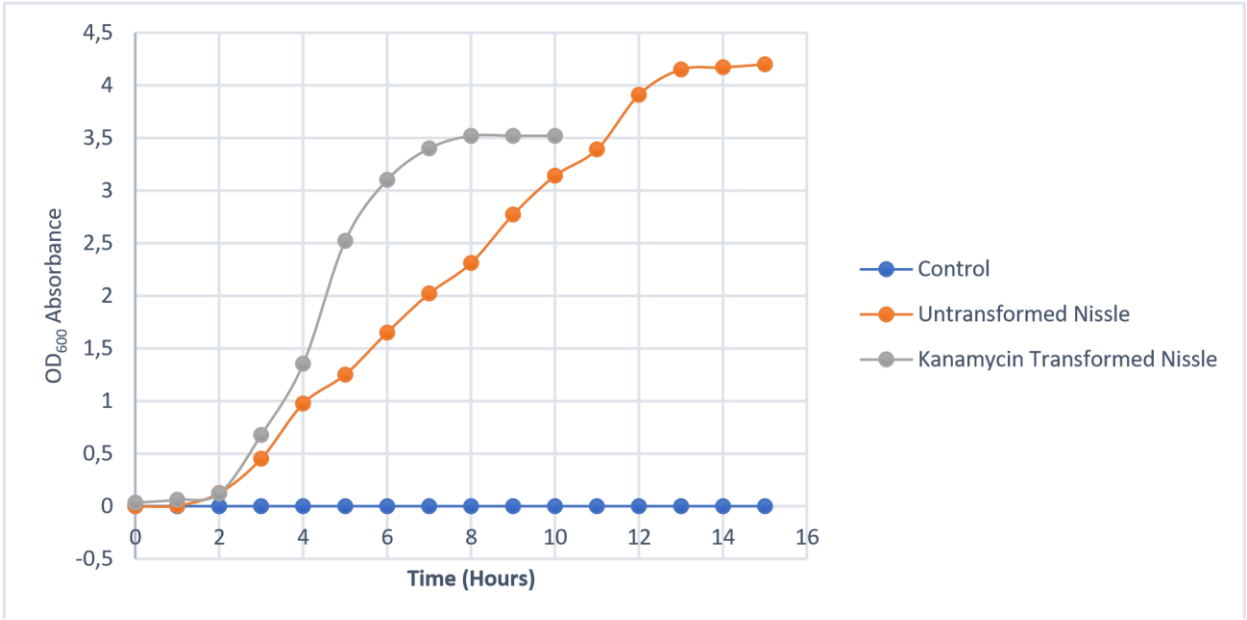



***Figure 4.2: The growth curve of transformed vs untransformed E. coli Nissle in LB media showing the absorbance (OD600) over a period of time(hours).*** *The results show a major difference between the two growth curves and that the transformed Nissle develops at a much more consistent rate with no interference compared to that of the untransformed Nissle*

Comparing the untransformed *E. coli* Nissle to the kanamycin transformed *E. coli* Nissle shows a substantial difference as seen in figure 4.2 between the two growth curves. The transformed growth curve takes 5 hours less time to reach the stationary phase as well as having no continual increases and decreases in the bacterial concentration as seen in the untransformed curve shown in figure 4.1.3. The media and conditions used for the growth curves remain constant, with only the kanamycin being the difference. The maximum specific growth rate for the untransformed EcN is calculated at 0.216 while for the transformed EcN is calculated at 0.4. This is a 1.8 times faster growth rate for the transformed kanamycin clearing indicating that there were enough nutrients available for maximum division and growth. This highlights the fact that there was some unknown microbial contamination that occurred, which the kanamycin was able to mitigate thus giving a more accurate curve for the *E. coli* species. A secondary use of the plasmid was the fact that it contained an EGFP which allowed for easier identification when using imaging tools. Due to the above reason and positive outcome of the transformed EcN, it was selected to be used in the rest of the procedures.

## 4.1.4 Growth curve differences M9 media and LB media for the transformed EcN

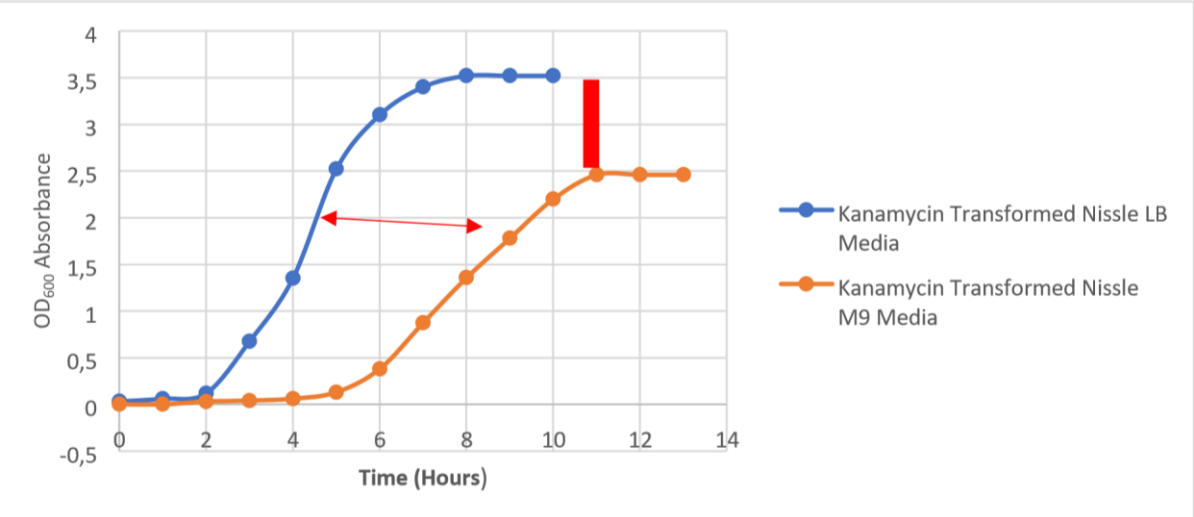



***Figure** 4 .3*: ***The growth curve of transformed E. coli Nissle in LB media and M9 media showing the absorbance (OD600) over a period of time(hours).*** *The results show a major difference between the two growth curves and that the EcN still takes longer to adjust to the M9 media due to the bare minimum growth salts present in the media*

M9 media is known as a minimal growth medium used for bacteria cultures (Azatian, Kaur and Latham, 2019). The three main advantages of this media are that it has a low autofluorescence (at 488nm), low absorbance and is a cheap media to produce. Due to these qualities, this media was selected for *E. coli* Nissle biofilm growth. There are differences between LB media and M9 media as shown in figure 4.3. The main difference is due to the minimal salts necessary for bacterial

growth which LB media has a high quantity (Sezonov, Joseleau-Petit and D'Ari, 2007). This results in a much more extended lag phase while the *E. coli* acclimatised to the environment. There was a very similar growth pattern in the exponential phase when comparing the two media. The maximum specific growth rate of the kanamycin transformed EcN in M9 media is calculated at 0.35 while for the kanamycin transformed EcN in LB media is calculated at 0.66. This is a 1.8 times slower growth rate due to the bare minimum salts present. Ultimately this was taken into consideration when using the M9 media to grow the EcN biofilms. The M9 media was also selected due to the lower autofluorescence of the media which would allow easier identification of the bacteria without any interference from the LB media autofluorescence.

### 4.1.5 Differences between the carriers and biofilm formation

The four carriers that were used produced varying results as discussed below when the EcN biofilm was formed either internally or externally. Firstly, the glass beads carrier did not have any biofilm attachment over the growth period. The biofilm rather formed in suspension and was easily dispersed through shaking and vortexing. Due to this, the glass beads carrier was not taken further in the procedures. The other three carriers (agarose gel, calcium alginate and the water pebble) each allowed for biofilm attachment in different ways. The calcium alginate and agarose carrier had external biofilm development while the water pebble had internal development. The attachment for both the calcium alginate and agarose carrier had occurred due to the surface being dried out before use. This developed a rough and textured surface, making it ideal for attachment. The water pebble carrier started at a fraction of the size and over 8 -10 hours, absorbed the EcN media which it was submerged in, to increase in size. When cut open, the inside of the water pebble was also rough and textured, but more hydrophilic compared to that of the other two carriers. This allowed for the bacteria to attach internally, on the outer most layer, while still having a flow of media through the water pebble as shown in figure 4.4.

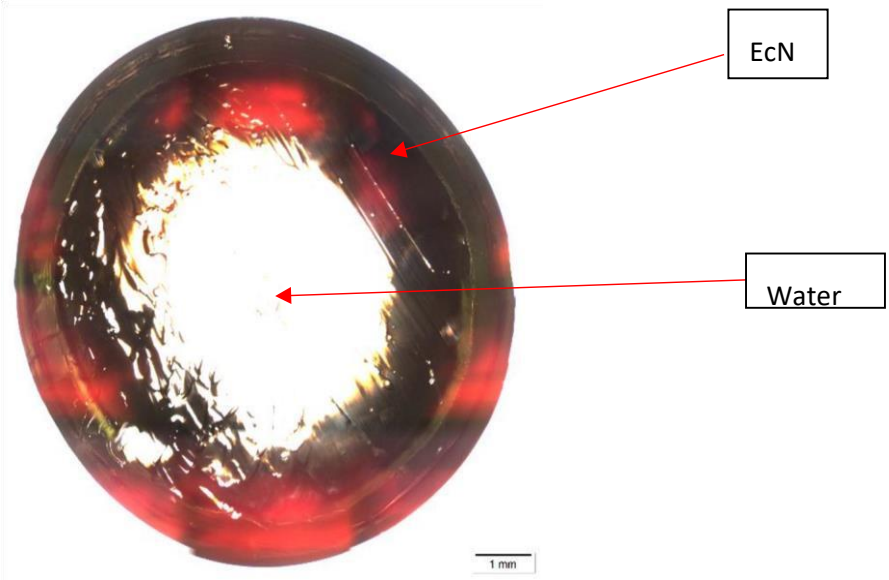



***Figure 4 .4: Microscopic image of the E. coli Nissle biofilm on the water pebble carrier.*** *The image indicates that the EcN growth occurs on the outer most layer of the water pebble while water and other aspect of the M9 media permeate further into the centre of the water pebble.*

The common quality between the carriers that allowed biofilm growth is that of a rough and textured surface which is a common aspect needed for biofilm growth.

The internal aspect of the water pebble was considered for a variety of reasons. Firstly, the stability of the biofilm of each carrier differed. The agarose gel biofilm had very little stability where it was only able to remain attached with light shaking around 120 rpm, but become more unstable as the speed increased to 150 rpm on an orbital shaker. The calcium alginate carrier had better stability compared to that of the agarose gel as even at 150 rpm, majority of the biofilm remained stable and situated on the carrier, with very little movement occurred when testing with low level movement. The water pebble had the best stability due to the internal growth of the EcN as both the 150-rpm speed and low level vortexing did not cause any carrier damage or biofilm detachment. The price difference between the carrier was also a factor as both the calcium alginate and agarose gel required the purchase of a variety of substances such as sodium alginate, calcium chloride and agarose. These can be bought in bulk which could be quite costly. If using Merck has a base point, the calcium chloride would cost R615.44 for 100 g, the sodium alginate would cost R638.96 for 100g and the agarose would be R889.04 for 10 g. These gel carriers ultimately would need time to be developed whereas the water pebbles are inexpensive compared to the other carriers, can be obtained easily through any local store, such as Crazy store which would cost R20 for 80 water pebbles (as it is commonly used for plant growth) and requires no preparation time (it can be used instantly). As the 8 – 10hour absorption period is part of the EcN growth period.

Two major differences in the biofilm development between the carriers was the dry cell weight and the distillation amount. Both the calcium alginate and agarose gel carriers had an average dry cell weight of 0.1 – 0.2 g for one carrier while the water pebble was 0.01 g per pebble and 0.09 g when all 8 were considered. The other difference was that the calcium alginate and agarose gel carriers had a higher distillation amount near 2 ml per 8 ml sample while the water pebbles had only 0.37 per 8 ml sample. The proton NMR did show that there were high water quantities in all samples, but the water pebble had higher ethanol quantities and no impurities based on figure 3.10. This figure has a high peak at 3,75 ppm labelled "2", combined with the fact that there was a lower ethanol distillation amount based on table 3 indicating that most of the distilled liquid from the water pebble carrier was ethanol. Both calcium alginate and agarose carriers had a much higher distilled amount shown in table 3 and a lower alcohol peak and contamination on figure 3.11. This shows that the water pebbles were a much better carrier for EcN compared to the other due to the cheap cost as stated in the costs, as well as lower impurities when taking ethanol into account. The impurities that were present were most likely due to substances from the EcN biofilm being distilled with the M9 media. Multiple vortexing procedures will need to be done to assist in mitigating the large number of impurities present.

### 4.1.6 Difference between biodiesel produced from the carriers

Three major tests were done with regards to biodiesel production. The first test was done to determine if there was enough ethanol production from the EcN fermentation to cause the reaction needed for biodiesel formation (using both olive oil and grapeseed oil). Out of the Olive oil tests, the water pebble EcN fermentation mixture produced the most biodiesel with the least amount of emulsion which correlates with the potential higher ethanol quantities shown in the proton NMR.

The emulsion formation occurred due to the combination of water, biodiesel, glycerine, and unreacted oil. Both the calcium alginate and agarose gel mixtures contained lower biodiesel formation with a higher emulsion content. This occurs as the M9 fermented media has a highwater quantity even after the fermentation process. Using the produced glycerine is an easy and quick ways to remove the emulsion that occurs by dissolving the emulsion and precipitating the waste back down for removal. Water present in the media can reduces it efficiency thus any water presence has a negative effect on biodiesel formation which correlates with work shown by Fevzi Yaşar, (Yaşar, 2020) when comparing the biodiesel formation of various feedstocks and how water can effect biodiesel formation. Overall, the glycerine content between the three tests were at similar levels.

Out of the Grapeseed oil tests, all the carrier fermented mixtures produced the exact same amount of biodiesel, emulsion, and glycerine formation. This showed a better yield of biodiesel from EcN compared to that of Olive oil. When comparing the oil types, olive oil comprises primarily of monosaturated fats while grapeseed oil comprises of polysaturated fat. However, monosaturated fatty acids have been reported to be better for biodiesel usage due to oxidative stability and low temperature fluidity while a high level of polysaturated fats could hamper the practical applications of biodiesel (Cao et al., 2014).

The second test was done to determine if there was enough ethanol and hydroxide catalyst production from the EcN fermentation to cause the reaction needed for biodiesel formation using Olive oil. Olive oil was chosen, despite having a lower yield compared to that of Grapeseed oil, due both to the yellow colour of the oil which would allow for easy identification and large quantities of monosaturated fats. Out of the test, the 20ml of Olive oil was converted into 20ml of biodiesel with 10 ml of clear glycerine and other waste. The glycerine produced had the same viscosity of pure glycerine but with small amounts of contamination unlike the dark crude glycerine present in the normal biodiesel production shown in the control and test 1 which contain higher levels of waste contamination. This could signify easier purification of the crude glycerine compared to that of the darker glycerine and could be easily used as feedstock for *E. coli* Nissle for the fermentation processes which already is expensive and highly wasteful. The colour of the biodiesel also differed from the control and test 1. It was more neon yellow compared to that of the usual golden colour. The control and test 1 biodiesel had the same viscosity and thickness of the test 2 biodiesel. The difference is colour of the two tests can be due to high waste content still contain in the biodiesel liquid. Slight water-washing would assist in identifying if this was due to waste.

Water washing was done on both the samples shown in figure 4.5, (A) using EcN produced media as an ethanol substitute and (B) the biodiesel formation using EcN produced media as an ethanol and catalyst substitute, to determine if contaminants in the biodiesel caused the colour difference. After double water washing, both the test 1 and test 2 samples showed very similar colours as shown in figure 27 further solidifying that it was waste contamination.

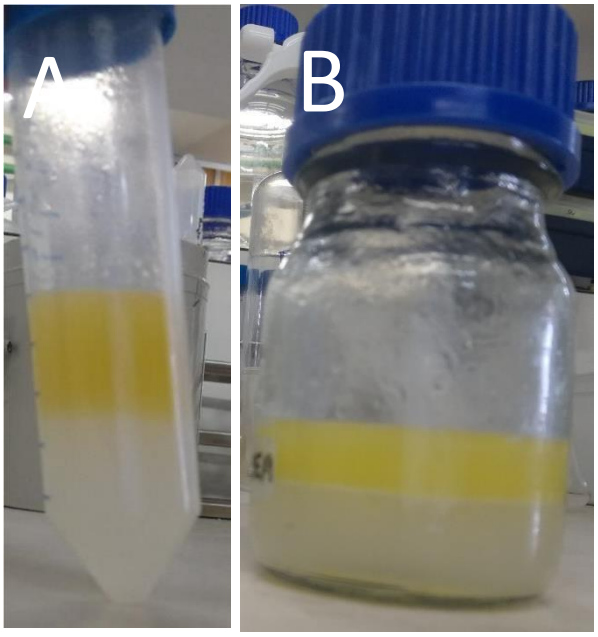



***Figure 4.5. The water-washed results of test 1 and 2 biodiesel production.*** *(A) This is the water washed sample from the biodiesel formation using EcN produced media as an ethanol substitute with clear biodiesel on upper level and contamination in the lower layer. (B) This is the water-washed sample from the biodiesel formation using EcN produced media as an ethanol and catalyst substitute with clear biodiesel on upper level and contamination in the lower layer.*

Lastly, the third test (Biodiesel formation using EcN produced media as the oil, ethanol, and catalyst substitute) was done to determine if there was enough fatty acid, ethanol and hydroxide catalyst production from the EcN fermentation to cause the reaction needed for biodiesel formation. This reaction was completed under similar conditions to the other tests. No reaction occurred in the sample as there was no colour change, no biodiesel or glycerine formation as was shown in figure 4.5. Ultimately this is due to no fatty acid production during the process. All the other tests performed confirmed that the initial compounds necessary for biodiesel conversion were readily available. Thin layer chromatography and/or Gas chromatography–mass spectrometry would need to be performed to determine how much, if any, fatty acid is produced during the process.

### 4.1.7 Theoretical up-scaling

Through up-scaling, the removal of the pre-biodiesel production stages is one of the main outcomes required which is the refining of the oil before use in biodiesel production. The pre-treatment and refining of various oils used is not necessary and the produced crude glycerol can

immediately recycle back as feedstock for *E. coli* Nissle. Unfortunately, at the current state of ethanol production in the sample of EcN as well as the lack of fatty acid production of the microbe, large scale production of these crude products would be impure and at very low levels. The biodiesel production shown in figure 3.13 and figure 3.14 show that large-scale production of biodiesel is a possibility if *E. coli* Nissle was to be engineered for increased ethanol and fatty acid production. The CEBER modeller theoretically calculates the inputs and outputs required for large scale production of biodiesel using EcN as the selected biomass. The general inputs needed would be 138.6 kg of EcN, 5479 kg of glycerol, 1.476 GJ of electricity and 138.6 $m^3$ of water. The general outputs are divided between two main sections: Water and air. The main outputs that are focused on are the: EcN being at 857 kg, glycerol being at 54.11 kg, biodiesel being at 70.26 kg and ethanol at 359.4 kg. When comparing the product composition to the waste shown in figure 3.1, it seems that at a larger theoretical scale there will be around 10% waste biodiesel loss and 7% crude glycerol loss (which is like the 10% glycerol production from the standard biodiesel production) (Monteiro et al., 2018). There is 359.4 kg of waste bioethanol being produced from the EcN switch can be processed and cleaned to be used as an alternative fuel source on its own outside biodiesel used. At the larger scale, production of biodiesel from this method would produce a purity of 65.03% when considering the tow purification steps shown in figure 2.3. Both purification steps act as a decantation-like step like immiscible liquid separation, separating the produced biodiesel from the waste product. Two purification steps were done to increase the purity of the product to 65% and the ensure that there is a 90.25% recovery of the produced biodiesel with only 9.75% being lost to waste during the purification steps. The purity of the biodiesel produced is essential as the 90% allows for less water washing to occur and therefore keep the water waste lower than that of normally produced biodiesel, which needs to wash away both crude glycerol and the solvent (generally methanol due to the fast reaction rates it provides) which is incredibly toxic to the environment (Chozhavendhan et al., 2020). As shown by research highlighted by Chozhavendhan et al (2020), sunflower oil reacting with potassium hydroxide and using methanol as the alcohol produces a yield of 91.67% like that of the theoretical up-scale model. Ultimately, the CEBER model indicated by figure 3.1 shows that under conditions that EcN can produce enough free fatty acids to in turn produce biodiesel, it can produce biodiesel at a proportion to that of the standard production shown by Chozhavendhan et al (2020) but in a way that produces less harmful/toxic contaminants by using waste glycerol as the feedstock.

### 4.1.8 Conclusion

Ultimately EcN test shown in figure 3.14 indicates that *E. coli* Nissle 1917 can produce both an alcohol, bioethanol, and a hydroxide catalyst through fermentation using glycerol as a carbon source. The various tests show that EcN can produce enough alcohol and catalyst naturally to

convert oil at the same rate and amount to that of ethanol and potassium hydroxide. It also produces a purer version of glycerine compared to that of the cruder glycerol produced when using potassium hydroxide as shown by the lighter and clearer colour compared to the dark and murkier colour of the cruder glycerol which itself could assist in making the purification step more effective and efficient.

Unfortunately, the EcN cannot produce enough fatty acids to initiate a reaction to produce biodiesel without modifying the fatty acid pathway in *E. coli* Nissle. This is an avenue that can be explored for EcN to be considered as a cost-efficient strain that has a high potential for commercial production of biofuels like bioethanol, higher chain alcohols, and biodiesel.

### 4.1.9 Future work

Firstly, more tests such as gas chromatography would need to be completed to accurately identify the amount of fatty acid production, if any, that occurs with *E. coli* Nissle naturally without any modification in the pathway. After that has been identified then modifying the pathway can be considered. There already has been work done by Wu, Karanjikar and San (2014) with regards to fatty acid production from glycerol in *E. coli* where the overexpression of PntAB, NADK and FabZ was combined with fabR deletion was able to enhance the production of fatty acids thus able to reach 83,21% of the maximum theoretical pathway yield and shown to have only slight less effacing when crude glycerol is used as a carbon source. I believe the above can be used by combining the already shown ethanol and catalyst production of the EcN biofilm, it can be beneficial into developing the EcN into an organism that can produce biodiesel without external inputs such as oil. Secondly, would be to identify the growth and fermentation rate of *E. coli* Nissle when using crude glycerol as all tests were performed using pure glycerol to determine the best efficiency of the processes. *E. coli* is an industrial organism which can easily be considered the workhorse of modern biotechnology and can use glycerol to produce a variety of products and fuels during the fermentative process and can be engineered to produce fuels such as n-butanol, isobutanol, n-propanol, isopronanol and biodiesel as shown by Koppolu and Vasigala (2016) and is ultimately an amazing replacement for diesel.

# Chapter 5

## 5.1 References

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# Appendix A

**Solutions and buffer list**

Lysogeny broth (500ml)

- 5g tryptone
- 2.5g Yeast Extract
- 2.5g NaCl
- Filled with 400ml dH2O
- Adjusted to pH 7.0
- Filled to 500ml dH20
- Autoclaved

Luria Bertani Agar (1L)

- 10g Tryptone
- 5g Yeast Extract
- 5g NaCl
- Filled with 995 ml dH20
- Addition of 1.25 ml NaOH ( 10% w/v)
- Addition of 15g Agar
- Filled to 1L dH20(if needed)

- Autoclaved

Mcconkey Agar (250ml)

- Add 12.5g of powdered mcconkey agar to 250ml of dH2O
- Mix until completely dissolved
- autoclave

**Preparation of carrier 1 (alginate)**

Calcium Chloride Solutions (100ml)

0.4M: 5.88g CaCl in 100ml dH20

0.6M: 8.82g

0.8M: 11.76g

1.0M: 14.7g

1.2M: 17.64g

1.4M: 20.58g

Sodium Alginate solution (500ml)

3%: 15g in 500 ml dH20

**Production of competent cells and transformation of EcN**

CaCl2 Buffers Preparation

1M CaCl2 (stock solution, 10x working concentration)

- 11.1g of anhydrous CaCl2
- Add to 80mL of dH2O
- Mix solution until CaCl2 is fully dissolved

- Top up to 100mL
- Filter sterilize through a 0.22µm pore
- Autoclave
- Stored at 4C

0.1M CaCl2 (working solution)

- Add 10mL of 1M CaCl2 to 90mL of dH2O for a 1:10 dilution
- Filter sterilize through a 0.22µm pore
- Autoclave
- Stored at 4C

0.1M CaCl2 + 15% glycerol (working solution)

- Mix 6mL 1M CaCl2 with 9mL sterile glycerol and 45mL dH2O
- Autoclave
- Stored at 4C

Restriction Enzyme Digestion

- HindIII

| **Component** | **50ul Reaction** |
|---|---|
| DNA | 1ug |
| 10x NEBuffer 2.1 | 5ul (1x) |
| HindIII | 1ul |
| Nuclease-free Water | To 50ul |

- HincII

| **Component** | **50ul Reaction** |
|---|---|

| | |
|---|---|
| DNA | 1ug |
| 10x NEBuffer 3.1 | 5ul (1x) |
| HindIII | 1ul |
| Nuclease-free Water | To 50ul |

One-liter 50X stock of TAE

- Tris-base: 242 g Acetate
- (100% acetic acid): 57.1 ml
- EDTA: 100 ml 0.5M sodium EDTA
- Add dH2O up to one litre.
- To make 1x TAE from 50X TAE stock, dilute 20ml of stock into 980 ml of DI water

Agarose gel

- 1g of agaose
- 100ml of 1X TAE

M9 media

- Make M9 salts
- Measure ~700ml of distilled $H_2O$ (sterile)
- Add 200ml of M9 salts
- Add 2ml of 1M $MgSO_4$ (sterile) (mw- 120,366)
- Add 20 ml of 20% glycerol ($C_3H_8O_3$)
- Add 100ul of 1M $CaCl_2$ (sterile) (mw- 110,98)
- Adjust to 1000ml with distilled $H_2O$

M9 Salts

- To make M9 Salts aliquot 800ml $H_2O$ and add

○ 64g $Na_2HPO_4$-7$H_2O$ (mw-268,07) ○ 15g $KH_2PO_4$ (mw-136,086) ○ 2.5g NaCl (mw- 58,44) ○ 5.0g $NH_4Cl$ (mw- 53,491) ○

Stir until dissolved ○

Adjust to 1000ml with distilled $H_2O$ ○ Sterilize by autoclaving