Microbial enhanced oil recovery (MEOR): kinetics of biodemulsification of simulated oil-water emulsion.

By

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DECLARATION

I, **Mantsha Johanna Maphoto**, declare that the contents of this thesis represents my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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ABSTRACT

Biodemulsification has been recently receiving a lot of attention due to the environmental friendliness of the resultant microbial products commonly known as biosurfactants. It has the potential to address emulsion issues that challenge the petroleum industry which have been reported. However, it is not yet fully established due to high capital costs which continue to inhibit the full industrial application of this technology, more especially with the lagging literature. Thus, more studies are required that will positively contribute to the implementation of this technology despite current challenges.

While many studies have been done, the kinetics of biodemulsification are yet to be fully documented in the literature. The aim of this study was to investigate the suitable carbon source and the effect of carbon source on the production of a *B.licheniformis* STK 01 biodemulsifier. Furthermore, to investigate the biodemulsification kinetics including the effect of temperature.

Biodemulsification experiments were conducted by initially cultivating biodemulsifiers in conical flasks containing the growth media and the various carbon sources, in an incubating shaker operating at 37 °C and 160 rpm over a 48 hr period. The produced biodemulsifiers were then used for the various demulsification studies at 37 °C, over a 24 hr period. The simulated emulsions were produced in conical centrifuge tubes with the aid of Span 60 and Tween 60 surfactants.

The study showed that all the produced *B.licheniformis* STK 01 biodemulsifiers possessed biodemulsifying capabilities but at different efficiencies. Motor oil proved to be the most suitable carbon source, resulting in a *B.licheniformis* STK 01 biodemulsifier that achieved 82,9% demulsification within 8 hrs. This is followed by diesel, paraffin, glucose, fructose and sucrose-cultivated biodemulsifiers with demulsification values of 73,7%, 61,9%, 52,9%, 45,1% and 44,7% respectively, thus, indicating the positive and significant contribution of insoluble carbon sources to the production of biodemulsifiers.

The kinetics investigations revealed that *B.licheniformis* STK 01 biodemulsifiers cultivated on soluble carbon sources adhered to third order kinetics while insoluble carbon sources followed a first order. The biodemulsification rate constants, k, for soluble substrate glucose (k_g), sucrose (k_s) and fructose (k_f) were determined to be $10 \times 10^{-5}\ dm^6/mol^2 s$, $5{,}029 \times 10^{-5}\ dm^6/mol^2 s$, and $9 \times 10^{-5}\ dm^6/mol^2 s$ respectively. The insoluble substrates motor oil (k_o), diesel (k_d) and paraffin (k_p) gave the rate constants of $11{,}561 \times 10^{-5}\ s^{-1}$, $2{,}447 \times 10^{-5}\ s^{-1}$, and $2{,}245 \times 10^{-5}\ s^{-1}$ respectively.

Finally, the relationship between the rate of biodemulsification of *B.licheniformis* STK 01 and temperature $(37 - 67\ ℃)$ was also investigated, assuming that the effect could be

independently studied. The rate of biodemulsification was found to increase with the increase in temperature; this trend was depicted using the Arrhenius equation (R^2 value of 96,3%), with the corresponding Arrhenius parameters, namely activation energy and frequency factor as $70{,}88\ KJ/mol$ and $14 \times 10^6\ s^{-1}$ respectively.

This study found that the carbon source used for the production of a biodemulsifier significantly contributes to its biodemulsification capability. It also found that insoluble carbon sources were the better carbon source option compared to soluble carbon sources and that the more complex the carbon source, the better the biodemulsifier produced. Furthermore, it found that the suitable biodemulsifier followed first order kinetics and the kinetic parameters thereof.

Keywords: Kinetics; Biodemulsification; *Bacillus licheniformis*; Biodemulsifier; Demulsification; Oilfield emulsions; MEOR

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Mantsha Johanna Maphoto
July 2019

DEDICATION

I dedicate this thesis to my Father in Heaven,
My Creator and My Redeemer. All glory unto Yahweh.

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Symbol	Description	Unit
A	is the optical constant	$dimensionless$
b	is a constant	$dimensionless$
C	Regression factor	$dimensionless$
C_A	Reactant concentration	mol/L
C_{A_0}	Reactant concentration	mol/L
C_P	Lipopeptide concentration	g/L
C_x	Dry cell weight	g/L
D	Dispersed phase diffusion coefficient in the continuous phase	m^2/s
g	Gravitational acceleration	m/s
k	Boltzmann constant	$m^2.kg/s^2.K$
k_d	Diesel biodemulsification rate constant	s^{-1}
k_f	Fructose biodemulsication rate constant	dm^6/mol^2s
k_g	Glucose biodemulsification rate constant	dm^6/mol^2s
k_o	Motor oil biodemulsification rate constant	s^{-1}
k_p	Paraffin biodemulsification rate constant	s^{-1}
k_s	Sucrose biodemulsification rate constant	dm^6/mol^2s
K	Coalescence constant	$dimensionless$
K_M	Michaelis constant	m
L	the height of the container	m
L	Specific growth rate ratio	$1/s$
$MATH$	Microbial affinity to hydrophobicity	$dimensionless$
n	The number of droplets at time t	$dimensionless$
n_0	The number of droplets at time 0	$dimensionless$
n_0	The number of droplets at $t = 0$	$dimensionless$
N	The number of non-coalesced water droplets at time t	$dimensionless$
N_0	The total number of water droplets assessed	$dimensionless$
η_0	The viscosity of the continuous liquid	m^2/s
OD_{580}	Optical density at wavelength 580nm	Abs
ϑ	Rate of Ostwald ripening	
ϕ	Volume fraction	$dimensionless$
ρ	Density	kg/m^3

r_c	The non-varying radius also known as critical radius.	m
r	Sphere/particle radius	m
R	Gas constant	$J/mol.K$
$S(\infty)$	Particle infinite radius	m
t	Time	s
t_0	The initial rupture time of the droplets assessed	s
$t_{1/2}$	The droplet half-life (half the time that it takes for the droplets to disappear)	s
T_{abs}	Absolute temperature	K
τ	is the rate of flocculation	
μ	Specific growth rate constant	$1/s$
v	Speed (revolutions or distance per second)	$rev/s\ or\ m/s$
v_c	Rate of sedimentation/creaming	m/s
V	Volume of oil separated	L
V_1	Volume of the droplets	L
$V_{t=0}^{CS}$	The cell suspension added to emulsion	L
V_t^E	The emulsion volume remaining at time t	L
$V_{t=0}^E$	The initial emulsion volume	L
V_m	The dispersed phase molar volume	L/mol
$V(\infty)$	The extrapolated volume at infinite time	L
x	The mean distance of the centrifuge tube from the axis of rotation	m
X	Microbial biomass	g/L
X	The biomass concentration	g/L
X_0	Initial biomass	g/L
X_m	Maximum possible biomass	g/L

ASP:	Alkaline surfactant polymer
CSH:	Cell surface hydrophobicity
%DE:	Biodemulsification capability
EOR:	Enhanced oil recovery
HHB:	Hydrophobic-Hydrophilic balance
MATH:	Microbial affinity to hydrocarbons
MEOR:	Microbial enhanced oil recovery
OIP:	Oil in place
OPW:	Oily produced water
O-W:	Oil in water
W-O:	Water in oil

CHAPTER 1

1. Introduction

1.1. Background

Petroleum is an important commodity to the global community, a fuel source and also a raw material for products such as plastic, chemicals, etc. It is rarely recovered without water, which is even more abundant during the declining years of the petroleum well (Speight, 2007). In some instances, the water content is due to water/steam flooding into the oil reservoir during production with the aim of enhancing oil recovery (Peña et al., 2004; Pillon, 2007). The recovered fluid at the wellhead is usually an emulsion comprising petroleum and water. Emulsions contain the drops of one liquid surrounded by a film of an emulsifying agent preventing the coalescence of the drops (Speight, 2007).

Oilfield emulsions are produced during various stages within the petroleum industry namely the recovery, storage, transportation, and refinery process (Long et al., 2013; Speight, 2007; Fang et al., 2016; Hu et al., 2015). The emulsion can be highly stable due to components such as asphaltenes, resins, naphthenic acids, etc (Long et al., 2013; Peña et al., 2004; Zaki et al., 2003).

While water content may possibly be 90% of the emulsion, oilfield emulsions are generally water-in-oil type, but the acceptable range is 0.5-2% (Parsia et al., 2016; Nadarajah et al., 2002; Huang et al., 2010a). Furthermore, many countries consider emulsions to be hazardous waste, which cannot be disposed-off ordinarily (Hu et al., 2015; Fang et al., 2016). Therefore, the water has to be separated from the petroleum before transportation (Parsia et al., 2016; Speight, 2007).

The excess water weakens the market value of oil and increases the cost of transportation while; also having the potential to cause corrosion and scaling to pipelines and processing equipment (Nadarajah et al., 2002; Wen et al., 2010; Peña et al., 2004). Unfortunately, the water content within the emulsion brings forth numerous challenges to the industry and affects the commercial value of petroleum (Kim et al., 1995; Long et al., 2013).

The industry combines physical and chemical methods (Kim et al., 1995; Fang et al., 2016) for the recovery of oil from oilfield emulsions. Even though chemical demulsifiers enhance the coalescence of oil droplets by disturbing interfacial properties (Hou et al., 2014b), chemical surfactants used are:

- Refractory organic polymers (Huang et al., 2010a),
- Non-environmentally friendly (Fang et al., 2016; Wen et al., 2010),
- Synthesized from crude oil; thus production costs fluctuate with international crude oil prices (Bodour and Miller-Maier, 1998), and
- Soluble in water; thus inexorably polluting the environment when discharged (Huang et al., 2016a).

Although chemical surfactants do separate the oil from the water, it has been shown however that microorganisms and/or their products have demulsifying capabilities (Nadarajah et al., 2002; Parsia et al., 2016; Bodour and Miller-Maier, 1998), and may provide an environment-friendly method of separating oil from water.

Microorganisms have the ability to produce various products such as gases and surfactants, as shown in Table 1.1. Microbial products are therefore applied for different uses that are referred to as application in MEOR. Microbes such as *Arthrobacter*, *Pseudonomas* and *Bacillus* have been reported to have demulsifying capabilities (Coutinho et al., 2013; Das, 2001).

Table 1.1: Application of MEOR

Microbial product	**Example microbes**	**Application in MEOR**
Acids	*Clostridium, Enterobacter & Mixed acidogens*	Permeability increase, emulsification
Biomass	*Bacillus, Leuconostoc & Xanthomonas*	Selective plugging and wettability
Gases	*Clostridium & Enterobacter methanobacterium*	Increased pressure, oil swelling, interfacial tension and viscosity reduction
Polymers	*Bacillus, Brevibacterium, Leuconostoc & Xanthomonas*	Injectivity profile and viscosity modification, selective plugging
Solvents	*Clostridium & Zymomonas klebsiella*	Rock dissolution for better permeability, oil viscosity reduction
Surfactants	*Acinetobacter, Arthrobacter, Bacillus & Pseudomonas*	Emulsification and demulsification through reduction of interfacial tension

Source: Sen (2008)

Microorganisms are capable of separating oil from water; this technique is referred to as biological demulsification, also known as biodemulsification. Studies have been conducted on the isolation, identification and characterization of biodemulsifying microorganisms and various microorganisms have been found to possess biodemulsifying capabilities (Huang et al., 2010a; Huang et al., 2009; Li et al., 2012; Mohebali et al., 2012; Parsia et al., 2016).

One of the studies isolated twenty biodemulsifying strains from different locations and further characterised the strains. The strains were found to belong to eleven different genera; and that strains belonging to the same genera achieved significantly different biodemulsification ratios (Huang et al., 2010a).

Huang and co-workers investigated screening methods for biodemulsifying microbes. The study compared surface tension measurement, oil-spreading test and blood-plate hemolysis test. The results showed that microorganisms that had the highest surface activity, thus exhibiting surface tension of less than 40 mN/m, achieved the highest demulsification ratio,

thus concluding that surface tension was the most efficient screening method (Huang et al., 2009).

A different study went further and investigated the factors that contribute to the biodemulsification capability of the produced biodemulsifier. The study found a correlation between culture age, cell surface hydrophobicity and the biodemulsification capability (Coutinho et al., 2013), whilst various other studies optimized the growth conditions which included carbon source, nitrogen source, pH, and/or temperature (Huang et al., 2013).

Li and co-workers optimized the biodemulsifier preparation conditions and investigated the kinetics of the production process. The study found that the *Candida tropicalis* strain JM-1 produced a lipopeptide biodemulsifier. The optimized conditions failed to significantly affect the strain growth or amount of lipopeptide produced but increased the rate at which the biodemulsifier was produced, furthermore the biodemulsifier production followed the Logistic growth model (Li, 2011).

Numerous studies have been performed on this topic for decades, although it is still not widely used due to limited research information. However, there is hardly any published information on the kinetics of the process. This has largely restricted the application of the technology.

1.2. Research questions

- What is the suitable carbon source for biodemulsification of simulated oil/water emulsion using *B.licheniformis* STK 01?
- What is the rate of the biodemulsification of simulated emulsion by *B.licheniformis* STK 01?
- What is the activation energy of the biodemulsification process by *B.licheniformis STK 01?*

1.3. General objectives

Objective 1: Find a suitable carbon source for the production of the biodemulsifier.

Objective 2: Investigate the kinetics of the biodemulsification of simulated oil/water emulsion at oilfield conditions.

Objective 3: Determine the activation energy

1.4. Significance of study

There are numerous reported studies on biodemulsification, although there are limited studies on biodemulsification kinetics. This project will investigate the kinetic models describing the biodemulsification of a simulated oilfield emulsion with a view to providing an understanding of the effects of certain parameters such as temperature affecting the kinetics of biodemulsification. In addition, the activation energy of the biodemulsification process will

be determined. The results of this study could shed some light on the biodemulsification of oilfield emulsions, and thus provide the basis for the optimization and design of the process.

1.5. Delineation of study

The scope of this research will be limited to the investigation of biodemulsification kinetics of a *B.licheniformis* STK 01 strain grown within the specified conditions. Therefore, the following will not be considered:

- Optimization of microbial growth conditions and
- Demulsification capabilities of various strains,
- Effect of physical treatments (freezing, heating, etc.),
- Interfacial properties,
- Phylogenic analysis.

1.6. Thesis outline

The research presented in this thesis was conducted at the Cape Peninsula University of Technology (CPUT), Cape Town, South Africa. The thesis comprises seven chapters, which include this chapter.

Chapter 1 presents the introduction which highlights the background of the proposed study, including the problem statement, research objectives and the significance of the study.

Chapter 2 presents the literature review on biodemulsification of oilfield emulsions. It covers the production, treatment and challenges of oilfield emulsions as well as a review of biodemulsifying microorganisms, production of biodemulsifiers, breakdown of emulsions and biodemulsifier production kinetics.

Chapter 3 presents the materials and methodology used in this work. It elaborates on the experimental methods of the study including the production of the simulated emulsions and *B.licheniformis* STK 01 biodemulsifier used in the study, as well as the fundamental techniques of the demulsification and kinetics investigation.

Chapter 4 considers the effect of carbon sources on the biodemulsifying capability of the produced biodemulsifier. It discusses the results of the effect of both insoluble and soluble carbon sources on the biodemulsification capability of *B.licheniformis* STK 01 whilst correlating the results with the reviewed literature; and concludes regarding the most suitable carbon source for the production of *B.licheniformis* STK 01 biodemulsifier.

Chapter 5 deliberates on the investigation of the biodemulsification kinetics of *B.licheniformis* STK 01 biodemulsifier, presenting the kinetic parameters of the *B.licheniformis* STK 01 biodemulsifier and simulated emulsion reaction from the results of this study.

Chapter 6 summarizes the various conclusions that were drawn from the results presented in this thesis and recommends a perspective for future research work.

2. Biodemulsification of oilfield emulsions

2.1. Introduction

Various methods are used to recover petroleum, namely primary, secondary and tertiary/enhanced oil recovery methods. The primary recovery method depends on the natural reservoir energy to drive the petroleum to the production well. Secondary recovery methods are applied in order to recover petroleum in a reservoir with depleted or low pressure with the aid of surface or submerged pumps and/or gas or water injection (Sen, 2008; Speight, 2007). When both primary and secondary recovery methods cannot be implemented in order to recover the remaining 55-60% oil in place (OIP), enhanced oil recovery (EOR) methods are used, which is advanced secondary recovery. EOR is done in order to improve the sweep efficiency of the oil, which has to be produced (Speight, 2007).

Enhanced oil recovery methods are categorized as follows (Sen, 2008; Feng et al., 2017; Haghighat et al., 2008; Hu et al., 2015):

- Thermal (steam flooding, in-situ combustion, etc.),
- Chemical (polymer, surfactant and alkali injection),
- Gas injection (carbon dioxide, nitrogen and flue gas injection),
- Biotechnology (Microbial enhanced oil recovery (MEOR) and
- Novel (sonic stimulations, solvent extraction, freeze-thaw, etc.).

Substances such as surfactants, steam, and carbon dioxide are introduced into the oil well during EOR. However, when MEOR is applied, microorganisms and/or metabolic products are added to the oil well. All these various additions are used with the purpose of enhancing oil recovery (Sen, 2008).

Alkaline Surfactant Polymer (ASP) flooding is a common chemical flooding method that entails the use of an aqueous solution containing alkali, surfactant, and polymer. The aqueous solution is injected into an oil reserve with the intention of driving the OIP out of the oil reserve. ASP flooding is used in various oilfields such as Daqing and Shengli located in China. This technology has increased oil recovery by more than 20%. However, the emulsion produced at the oil head is somewhat more stable and complex (Li, 2011).

The use of advanced methods to recover OIP within oil reserves consequently results in emulsions with different parameters compared to those recovered during the primary and secondary stages. Altered emulsion parameters therefore require more specialized treatment of wet petroleum (Li, 2011).

In addition, recovered petroleum is treated in order remove the excess water and solids, but this also results in oily produced water (OPW) in the form of an emulsion. The OPW cannot be discharged since it does not meet discharge regulations and therefore has to be treated further (Fang et al., 2016). Although demulsification has been identified as an effective way

to enhance the separation of oil from water, more stable emulsions require increased amounts of chemical demulsifiers (Li, 2011; Liu et al., 2011b), thus, potentially increasing treatment costs. Emulsion stability has been found to range from minutes to years depending on the complexity of the petroleum composition (Mosayebi and Abedini, 2013). Therefore, unconventional recovery methods combined with complex petroleum composition present complex and stable emulsions.

Many have studied oilfield emulsions and found that they can be classified as either water-in-oil (W-O) or oil-in-water (O-W) and the majority is W-O type (Huang et al., 2010a; Ali and Alqam, 2000). The more complex emulsions are classified as either oil-in-water-in-oil or water-in-oil-in-water (Atta et al., 2014). When examining the physical stability of an emulsion, it is important to measure rheological parameters as a function of temperature. Various studies have shown that the following parameters significantly affect the emulsion stability (Li, 2011):

- Zeta potential
- Interfacial tension
- Interfacial rheology
- Temperature
- Oil/water ratio
- Water droplet size
- Alkaline surfactant polymer components.

Chemical, physical and biological methods are currently, widely used in the industry, with chemical demulsifiers being the most common (Huang et al., 2016a; Zaki et al., 2003; Peng et al., 2016b). Various methods studied for demulsification of emulsions from different stages of petroleum (e.g. production, transportation, and processing) include the following (Hu et al., 2015; Zaki et al., 2003; Huang et al., 2016a):

- Solvent extraction and freeze thaw
- Flotation
- Carbon dioxide
- Centrifugation
- Electrochemical techniques
- Membrane separation
- Magnetic nanoparticles
- Ultrasonic wave treatment
- Biodemulsification

Despite the various options available operational feasibility and other important factors generally dictate the options chosen by various industries.

2.2. Biodemulsification

Numerous microorganisms (see Table 2.1) and/or their products have been reported to have demulsifying capabilities and capacity to separate oilfield emulsions. The biodemulsifying

performance of the various microorganisms can be anything from non-existent to excellent. Pure biodemulsifying microorganisms such as *Nocardia*, *Corynebaterium*, *Rhodococcus* and *Torulopsis* cultures have been found to perform. These pure strains separated emulsions whether used alone or combined with common chemical demulsifiers (e.g. tretolite e 3453, F 46 and/or polynucleolyte). These are therefore, capable of matching or outperforming the demulsifying capabilities of chemical demulsifiers (Das, 2001).

Table 2.1: Various microorganisms with biodemulsification capabilities

Microorganisms with biodemulsification capabilities		
Achromobacter sp.	*Bacillus* sp.	*Nocardia amarae*
Acinetobacter sp.	*Bacillus subtili*	*Ochrobactrum anthropi*
Acinetobacter calcoaceticus	*Brevibacillus* sp.	*Ochrobactrum* sp.
Aeromonas	*Corynebacterium petrophilum*	*Pseudonomas aeruginosa*
Aeromonas sp.	*Corynebacterium* sp.	*Pseudonomas* sp.
Alcaligenes sp.	*Dietzia* sp.	*Pusillimonas* sp.
Alcaligenes sp. S-XJ-1	*Micrococcus* sp.	*Rhodococcus*
Alteromonas	Mycobacterium sp.	*Rhodococcus aurantiacus*
Alteromonas sp.	*Nocardia sp.*	*Rhodococcus* sp.
Arthrobacter sp.	*Nocardioform Actinomycetes*	*Sphingopyxis* sp.
Bacillus mojavensis		*Streptomyces* sp.
		Torulopsis bombicola

Sources: (Huang et al., 2016a; Huang et al., 2010a; Parsia et al., 2016; Wen et al., 2010; Peng et al., 2016a)

Microorganisms since ancient times have been used to produce useful products (e.g. biosurfactants) for various industries (Doran, 2013). Biosurfactants, known as amphiphilic compounds, are capable of reducing both surface and interfacial tensions (Haghighat et al., 2008; Bodour and Miller-Maier, 1998). Biosurfactants drastically affect the physiological behaviour of microbes, which includes cell mobility (Amodu et al., 2014), cell communication, nutrient accession and cell-cell competition (Van Hamme et al., 2006). Biosurfactants also seem to play a role when microbes come across an interface. Some biosurfactants such as acetoin, polysaccharide, glycolipid, glycoproteins, phospholipid and rhamnolipid have demulsifying capabilities (Wen et al., 2010).

Research shows that there are various factors that affect the performance of microorganisms and the type and amount of biosurfactant produced. These relevant factors include carbon and nitrogen sources, growth conditions (temperature, pH, oxygen, nutrient limitations etc.), cell surface properties, and cell concentration to name a few (Peng et al., 2016a). Furthermore, the appropriate combination of these factors improves the biodemulsification capability of some microorganisms (Huang et al., 2014; Liu et al., 2011b).

Culture medium is a fundamental part of the biodemulsifier production and is estimated to contribute to nearly half the cost of production of which carbon source costs the most. The production of biodemulsifiers seems to require hydrophobic materials for the synthesis of the

appropriate biosurfactant that includes, decane, crude oil, kerosene, etc. (Liu et al., 2010; Liu et al., 2011b).

The advantages and disadvantages of biodemulsifiers are as follows (Bodour and Miller-Maier, 1998; Coutinho et al., 2013; Haghighat et al., 2008; Hou et al., 2014b; Huang et al., 2010a; Li et al., 2012; Liu et al., 2010; Liu et al., 2011b; Long et al., 2013; Peng et al., 2016a; Wen et al., 2010):

Advantages:

- Low toxicity,
- Biodegradable,
- Efficient under harsh conditions,
- Environmentally friendly,
- Higher interfacial activity,
- Structural diversity, and
- Microbial cells might be reusable.

Disadvantages:

- Cost ineffective due to low product yield, and
- High instability of product at transportation
- Capital costs.

Despite the efficiency and environmental friendliness, biodemulsifier technology seems to be hindered by high capital and operating costs equipment even more for the use of pure bacteria (Nadarajah et al., 2002). Furthermore, the fragility of products contributes to the hindrance of biodemulsification technology advancement.

2.2.1. Biodemulsifying microorganisms

Only a number of microorganisms have the ability to effectively demulsify oilfield emulsions. It seems that the location where the microbe is retrieved is a contributing factor to its biodemulsification capabilities, extraction and purification and production costs. Strains in the same genera may have a large difference in biodemulsification capability, surface properties, and biochemical and physiological characteristics (Huang et al., 2010a). Furthermore, studies have shown that bacteria are more adaptable to the environment (Khoshdel et al., 2016)

Biodemulsifying microorganisms were mostly found in petroleum-contaminated or undisturbed environments (Huang et al., 2010a). Additionally, the reported microorganisms isolated from petroleum-contaminated soil mostly had a biodemulsifying capability of more than 70% compared to those isolated from domestic wastewater and landfill leachate treatment plants (see Table 2.2). However, not all strains isolated from petroleum contaminated environment were efficient biodemulsifiers.

Table 2.2 shows different strains of microorganisms obtained from various locations. The various *Alcaligenes* sp. strains listed in Table 2.2 show that although the microorganisms

may be of the same genera, they may possess different characteristics which resulted in different biodemulsification capabilities. However, this may also be a result of exposure to different growth conditions or the hydrophobic-hydrophilic balance (HHB) of the cell wall which has been found to significantly affect the biodemulsifier performance (Hou et al., 2014b). This reveals the intricacy of the biodemulsifier composition and biosynthesis (Liu et al., 2011b).

Studied biodemulsifying microorganisms, some with exceptional biodemulsification capabilities were found (Huang et al., 2009; Li et al., 2012) to be either cell-bound and/or extracellular biodemulsifier producers. Cell-bound biodemulsifiers possess the effective biodemulsifying components on the cell walls and generally produce the biodemulsifier as the cells adapt to the surrounding conditions, which means that without the cells, there will not be effective biodemulsification (Huang et al., 2013). Extracellular biodemulsifiers are biological metabolites/products that cells produce when exposed to certain conditions and these generally surround the cells, and thus separate. These metabolites/products generally referred to as biosurfactants can be separated from the cells, and the biosurfactants possessing effective biodemulsifying components will effectively demulsify an emulsion (Das, 2001; Hou et al., 2014b; Li et al., 2012; Long et al., 2013). However, a different study found that cell bound biodemulsifiers were more efficient at the separation of complex emulsions compared to both demulsifiers and biosurfactants (Huang et al., 2016a).

Molecular biology analysis was used to identify the microbial phylogenic evolution of some biodemulsifiers and it indicated that the microorganisms belonged to three divisions of *Eubacteria* phylum and two divisions of *Archaea* phylum (Huang et al., 2010a). However, various other potential biodemulsifiers isolated from different locations suggests that further studies should be done before the exact scientific classification can be realized.

Table 2.2: Various biodemulsifiers, isolated from different locations and their biodemulsification capabilities.

Microorganisms	**Isolation location**	**%DE**
Alcaligenes strain S-XJ-1	Petroleum contaminated soil	*81*
Dietzia natronolimnaea strain LL 51		*72*
Pseudonomas sp. strain BFXJ-8		65
Rhodococcus sp. strain F12		*88*
Rhodococcus sp. strain E33		67
Alcaligenes sp. strain mp-2		*91*
Dietzia sp. strain ES18		*100*
Bacillus cereus strain LH-6		*95*
Mixed bacteria		*96*

Pseudonomas aeruginosa strain MSJ		*97*
Bacillus mojavensis strain XH1		*70*
Ochrobactrum anthropic strain RIPI5		*72*
Paenibacillus alvei strain ARN63		*77*
Micrococcus	Unknown	100
Gordonia sp. strain D2	Refinery wastewater treatment plant	*70*
Brevibacillus agri strain NCHU1002	Landfill leachate treatment plant	65
Alcaligenes sp. strain Ic4		68
Brevibacillus borstelensis strain MH301	Produced water treatment station in Shengli oilfield	*99*
Bacillus badius strain NBRC 15713		58
Brevibacillus borstelensis strain T2–1		67
Sphingopyxis granuli strain Kw07	Domestic wastewater treatment plant	59
Alcaligenes sp. strain ESPY2		62
Dietzia sp. strain ES18		69
Ochrobactrum intermedium clone kl-2		50
Pusillimonas terrae strain BN9		*98*
Gordonia sp. strain D2	Oilfield produced water	50
Achromobacter sp. strain EP177		56
Bacillus sp. strain BSi20511		68

Sources: (Coutinho et al., 2013; Das, 2001; Hou et al., 2014a; Huang et al., 2010a; Li et al., 2012; Wen et al., 2010)

2.2.2. Biosurfactants

Biosurfactants refer to a collection of surface-active biological products that are amphiphatic such as lipopeptides, glycolipids, polysaccharides, etc. (Amodu et al., 2014; Huang et al., 2010a; Wen et al., 2010). Microorganisms produce biosurfactants so that they are able to access the nutrients despite harsh environments. Biosurfactants produced by microorganisms are not all biodemulsifiers; some will emulsify, degrade certain elements (biodegradation), while others are used for other industrial applications (e.g. cosmetics, antibiotics, etc.) (Li et al., 2012; Liu et al., 2011b).

Studies have shown that carbon source and other cultivation conditions dictate the production and type of biosurfactant produced (Huang et al., 2010a). Furthermore, the exposure to sufficient or insufficient nutrients affects the quality and amount of the biosurfactant produced (Li et al., 2012; Liu et al., 2011b). Rhamnolipid is a commercially available glycolipid biosurfactant with a high surface activity, known for its potential industrial

application of waste crude oil demulsification (Doran, 2013). Waste crude oil could not be demulsified by common chemical surfactants that included sodium dodecyl, poly-oxy-ethylene-octyl-phenol-ether and various other several polyether-type surfactants. However, It has been shown that rhamnolipid was able to enhance the recovery of more than 98% of the crude oil (Long et al., 2013).

B.licheniformis has been known to produce lipopeptide biosurfactants such as lichenysin (Doran, 2013). Lipopeptides were found to have amino-acid chain that is the hydrophilic aspect of the biodemulsifier while a hydrocarbon chain is the hydrophobic aspect (Huang et al., 2009; Huang et al., 2010a). The hydrophilic aspect enhances the biodemulsification ability of the lipopeptide.

2.2.3. Interfacial tension

According to literature, interfacial film strength reflects the degree of demolition of the film and this is evaluated according to the below mentioned equation (see Equation 2.1).

$$\ln\left({}^{N}/_{N_0}\right) = -K(t_{1/2} - t_0)^n$$

Equation 2.1: Interfacial film strength

Where: N is the number of non-coalesced water droplets at time t, N_0 is the total number of water droplets assessed, t_0 is the initial rupture time of the droplets assessed, K is the coalescence constant and $t_{1/2}$ is the stabilization half time (Wen et al., 2010).

Biodemulsifiers are capable of reducing the interfacial tension despite the presence of emulsifiers in emulsions and this is due to their high surface activity (Hou et al., 2014b; Long et al., 2013; Wen et al., 2010). The relationship between interfacial tension and biodemulsification efficiency is owed to situations in which the interfacial wave amplitude is determined by the interfacial tension (Long et al., 2013).

Studies found that the coalescence constant (K) increased while t_0 and $t_{1/2}$ decreased, with an increase in biodemulsifier cell concentration, which implied that the interfacial film was in an unstable state at high biodemulsifier cell concentrations. Therefore, a biodemulsifier can efficiently reduce the interfacial film strength and shorten the time till rupture of water droplets, even more when biodemulsifier cell concentration was increased (Wen et al., 2010).

Interfacial tension was found to decrease with an increase of biodemulsifier concentration (Hou et al., 2014b; Long et al., 2013; Wen et al., 2010). The interfacial tension decrease was still visible when the emulsifier concentration was increased (Wen et al., 2010). This implies a higher surface activity since the interfacial activity of a biodemulsifier has to be large

enough to overwhelm the interfacial tension gradient thus enhancing film drainage and coalescence (Long et al., 2013).

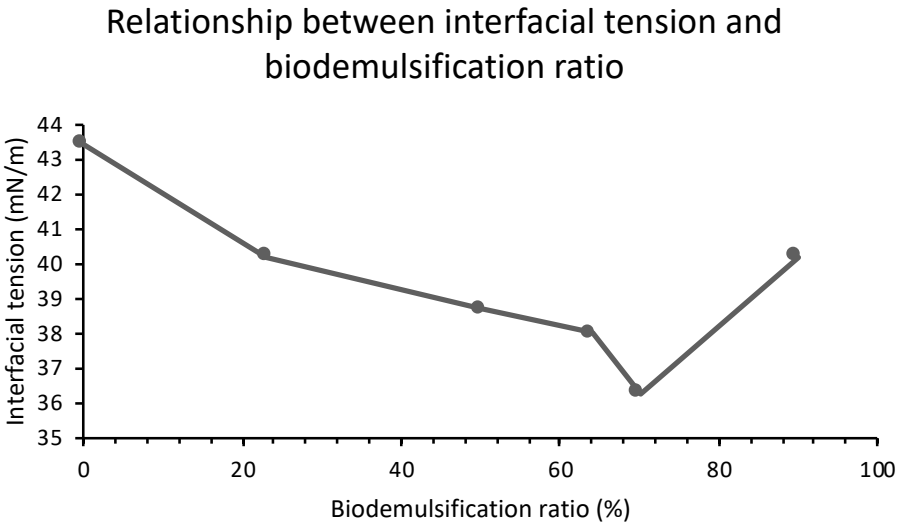

Figure 2.1: Relationship between surface tension and biodemulsifier efficiency (Hou et al., 2014b)

In another study, an increase in biodemulsifier concentration resulted in an interfacial tension decrease (Hou et al., 2014b). However, the lowest interfacial tension value was not at the optimum biodemulsifier concentration (see Figure 2.1), thus there was no consistent pattern relating biodemulsifier efficiency and concentration to the interfacial tension. This endorses the statement that “Demulsification only occurs when the interfacial activity of the demulsifying bacteria is high enough to suppress the interfacial tension gradient” (Wen et al., 2010).

2.2.4. Zeta potential

Zeta potential is referred to as the measure of surface charge which is a physiochemical property of interfacial surfaces. Natural and synthetic surfactants are known to affect the physiochemical properties of interfacial surfaces such as cell surface charge (essentially zeta potential), interfacial tension and interfacial rheology. The physiochemical properties are relevant to the overall stability of emulsions or function of cells (Huang et al., 2016a; Liu et al., 2011; Nikkhah et al., 2015).

Emulsion stability generally occurs at the droplet interface; surfactants will adsorb to oil-water interface, causing significant changes to the droplet surface, thus enhancing the formation or stability of emulsions (Wang et al., 2011). A study found that when a surfactant adsorbed to the oil-water interface and caused emulsion stability, the zeta potential increased while the interfacial tension decreased. Furthermore, the addition of NaOH contributed to a further zeta potential increase and interfacial tension decrease, though too much NaOH did the opposite (Wang et al., 2011).

It has been suggested that there is some relationship between the cell surface hydrophobicity and zeta potential of a demulsifying strain. This study found that the zeta potential of a demulsifying strain increased (higher negative charge) with the decrease of the cell surface hydrophobicity. However, the relationship is yet to be mathematically outlined. Another study found that a higher cell surface hydrophobicity and lower zeta potential enhanced the production a biodemulsifier (Liu et al., 2011).

2.2.5. Cell surface hydrophobicity

The biodemulsifying capability of a biodemulsifier is based on its ability to adsorb to the water-oil interface and can be affected by the cell surface hydrophobicity (CSH) and hydrophobic-hydrophilic balance (HHB) of the cell wall (Coutinho et al., 2013). This is only applicable if the demulsifying activity is related to the cell surface when the biodemulsifier is the actual cells or intracellular biosurfactant. Cells with high hydrophobicity destabilized O-W emulsions. The high affinity of the cells to oil is described by Huang et al. (2010), in Equation 2.2:

$$MATH = \left(1 - \frac{OD_{580}(final)}{OD_{580}(initial)}\right) \times 100\%$$

Equation 2.2: Affinity of cells to oil

Where: $OD_{580}(initial)$ is the initial optical density of the emulsion, and $OD_{580}(final)$ is the emulsion optical density after a period. A high MATH value indicates high affinity of the cells to oils.

Research has been done on *Alcaligenes* sp. S-XJ-1 as a biodemulsifier and the biosurfactant on the cell walls was identified as a lipopeptide. The presence of a lipopeptide caused a high affinity value of 85%, which added to the demulsification capacity of the *Alcaligenes* strain (Wen et al., 2010). The study correlated with other studies that showed that a high CSH increased the cell affinity to kerosene, thus increasing the biodemulsification ability of the cell (Liu et al., 2011a; Huang et al., 2013). This owed to the CSH of the cell-enhancing contact with the oil-water interface and amphiphilic trait of the lipopeptide promoting the adsorption of the cell to the oil-water interface (Liu et al., 2011a; Nadarajah et al., 2002; Wen et al., 2010).

In another study, researchers found that the initial MATH value of original cells was 37.6% more than that of the remaining cells after the demulsifying compound was extracted. Therefore, the biodemulsifying capability of a cell can be determined using its CSH value (Huang et al., 2013).

According to a different study, the CSH of a biodemulsifier is extensively affected by the initial culture pH. *Alcaligenes* sp. S-XJ-1 showed an increase in CSH when the cultivation pH

was increased. However, there was a sudden decrease of CSH when the cultivation pH was increased to 11 (see Figure 2.2) (Liu et al., 2010).

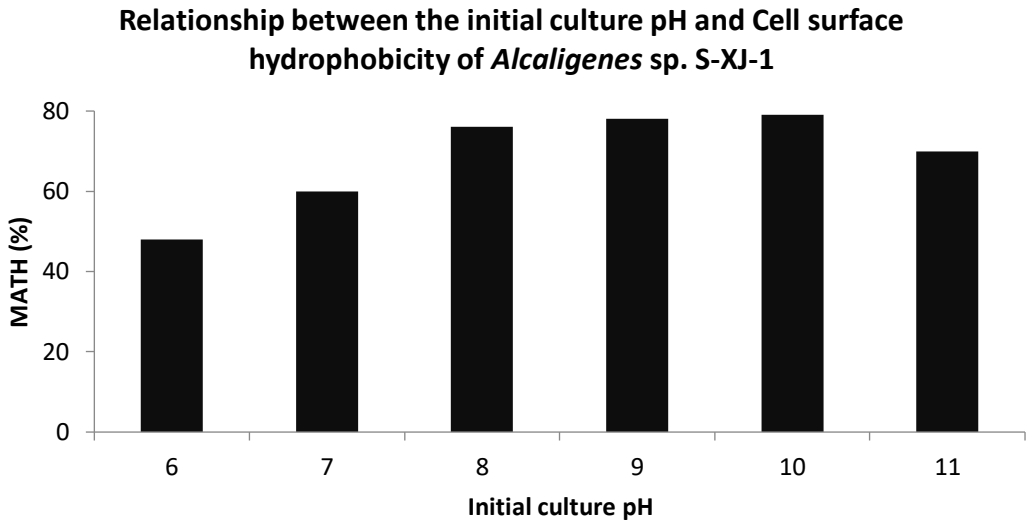

Figure 2.2: Cell surface hydrophobicity (MATH%) of Alcaligenes sp. S-XJ-1 (Liu et al., 2010)

Liu and co-workers investigated the effect of different carbon studies on the CSH, and the results obtained showed that hydrophilic carbon sources decreased the CSH (MATH value below 20%). Furthermore, the hydrophobic carbon sources resulted in CSH values well above 50%. Liu et al., (2010) proposed that the CSH "surely correlated" with the biodemulsifier performance when the biodemulsifier was cultivated with different hydrophobic carbon sources (Liu et al., 2010).

An *Ochrobactrum anthropic* strain RIPI5-1 was investigated as a biodemulsifier and during various growth stages of the microbe, the demulsifying activity and CSH exhibited a similar trend (Mohebali et al., 2012), further suggesting that biodemulsification could be a phenomenon mediated at the cell surface. This correlated with a suggestion by Coutinho et al. (2013) who investigated *Pseudomonas aeruginosa* MSJ as a biodemulsifier.

2.3. Biodemulsifier efficiency

A good biodemulsifier has to be miscible in emulsions, highly diffusive, and surface active (Huang et al., 2010a). The amphipathic nature of a biodemulsifier qualifies its adsorption to the oil/water interface (Das, 2001) thus leading to surface property changes (Huang et al., 2010a). The biodemulsification capability (%DE) is expressed by Equation 2.3, which compares the initial volume of the emulsion with the remaining emulsion after biodemulsification (Wen et al., 2010).

$$Biodemulsification\ capability = \left(1 - \frac{V_t^E}{V_{t=0}^E + V_{t=0}^{CS}}\right) \times 100\%$$

Equation 2.3: Biodemulsification capability

Where: V_t^E is the emulsion volume remaining at time t, $V_{t=0}^E$ is the initial emulsion volume, and $V_{t=0}^{CS}$is the cell suspension added to emulsion.

There are various aspects that affect the biodemulsification efficiency of microorganisms and thus inferior microorganisms in this case can sometimes be improved by altering nutrient and cultivation conditions (Huang et al., 2010a). Studies have shown that microorganisms may be capable of demulsifying both O-W and W-O emulsions (Das, 2001); however; a microorganism may excel in demulsifying one type of emulsion. *Micrococcus* sp. demulsified O-W at a faster rate than its demulsification of W-O emulsion (Das, 2001). A study on *Alcaligenes* as a biodemulsifier showed that an increase in cell concentration resulted in enhanced biodemulsification performance. At low (<50 mg/L) cell concentrations, only oil separation took place. However, at cell concentrations above 100 mg/L, both oil and water separation took place (Wen et al., 2010).

2.3.1. Cell concentration

An increase in cell concentration was found to advance the biodemulsifier performance. During a study, 2.5 mg/L of cell concentration resulted in an oil separation ratio of 29.2% within 24hrs; whereas 500 mg/L of cell concentration resulted in an oil separation ratio of 83.3% within the same period (Wen et al., 2010).

Furthermore, the effect of cell concentration on the biodemulsifier performance was seen during the treatment study of O-W emulsions with the aid of *Micrococcus* sp. cells (Das, 2001). 4 mg of cells per mL of emulsion resulted in a complete separation of oil from water within 2.5 hrs, whereas, 0.2 mg of cells per mL of emulsion did not fully separate the same emulsion after 485.5 hrs (see Table 2.3).

Table 2.3: Effect of cell concentration on the emulsion volume

Cell concentration (mg/mL of emulsion)	Emulsion volume remaining (hr)					
	0	**0.5**	**1.5**	**2.5**	**20.5**	**485.5**
0.2	100	98	98	98	96	28
0.6	100	98	98	98	86	24
1.0	100	98	98	98	76	14
2.0	100	98	95	92	**0**	-
3.0	100	91	48	**0**	-	-
4.0	100	81	28	**0**	-	-

Source: (Das, 2001)

2.3.2. Chemical treatment

Researchers investigated the effect of cell chemical treatment on the biodemulsification capability of the cells. It was found that acetone, dichloromethane, and methyl tert-butyl ether (MTBE) treated *Alcaligenes* sp. cells had a higher demulsification efficiency compared to untreated cells (see Table 2.4). Ethyl acetate treated cells had similar demulsification efficiency to that of the untreated cells (Huang et al., 2013). These chemicals affected the cells in different ways despite the fact that they are all organic compounds.

Table 2.4: Biodemulsifier performance after exposure to chemical treatment.

Chemical treated cells	% Biodemulsification efficiency	
	24hrs	48hrs
Untreated	82.3 ± 2.1	87.3 ± 2.3
Acetone	92.0 ± 1.3	92.8 ± 1.3
Dichloromethane	90.5 ± 0.5	93.1 ± 0.6
MTBE	83.1 ± 2.4	87.3 ± 1.6
Ethyl acetate	82.0 ± 1.0	86.0 ± 1.3
Petroleum ether	80.0 ± 0.5	87.2 ± 1.3
n-Butanol	76.7 ± 1.6	83.5 ± 3.2
Distilled water	73.0 ± 0.5	82.4 ± 0.7
Methanol	32.7 ± 2.0	63.5 ± 1.3
0.1 M Sodium hydroxide	11.5 ± 2.1	19.5 ± 2.3

Source: (Huang et al., 2013)

It is common for microorganisms grown in nutrient media comprising of alkanes to incline towards accumulating the alkanes at the cell walls, which enhances the cell surface hydrophobicity. Thus, chemical treatment of cells definitely alters the biodemulsifier demulsification efficiency for the superior or the inferior demulsification capability (Huang et al., 2013).

2.3.3. Effective biodemulsifying components

Microorganisms are known to be harnessed by varying growth conditions so that they may produce certain products that can possibly be used for different applications. Hence it is possible to have the same strain produce different products for different purposes. This however does not imply that the different products will both perform excellently for various purposes (Coutinho et al., 2013; Das, 2001; Hou et al., 2014a; Huang et al., 2010a; Li et al., 2012; Wen et al., 2010).

Extreme temperature may deteriorate the stereo conformation and active sites of proteins and inhibit their enzymatic activity, simply the active components on the cell surface. Therefore, the active biodemulsifying components might be extracellular proteins. However, there was no solid pattern between the produced extracellular proteins and the biodemulsification ratio (Hou et al., 2014b).

Bacillus mojavensis XH-1 was treated with trypsin (hydrolyzes proteins), proteinase K (which tears the carboxyl group from aliphatic and aromatic amino acids) and urea. These drastically reduced biodemulsification efficiency, thus indicating the presence of extracellular proteins (Hou et al., 2014b). The *Bacillus mojavensis* XH1 bacteria strain secreted a number of different extracellular proteins such as pyruvate carboxylase, bacillopeptidase F, and extracellular serine protease (see Table 2.5). These extracellular proteins may be of great importance in the biodemulsification process (Hou et al., 2014b), however the topic is yet to be studied further.

Table 2.5: Various proteins secreted by *Bacillus mojavensis* XH1

Protein name	Peptide count	Cover percent	Theoretic MW (KD)
2',3'-Cyclic nucleotide 2'-phosphodiestrerase/3'-nucleotidase bifunctional periplasmic precursor protein6.88	7	6.98	159.705
Pyruvate carboxylase	7	6.88	127.936
Extracellular serine protease	5	7.94	85.607
Bacillopeptidase F	4	2.44	154.577
YjbG	4	8.05	69.813
Gamma-glutamyltranspeptidase	4	7.16	64.188
Hypothetical protein BSU 11540	4	7.39	70.144
Dihydrolipoamide dehydrogenase	3	8.72	49.721
Hypothetical protein BSU0I660	3	5.30	70.580
Oxalate decarboxylase	3	8.05	43.566
Cell wall-associated protein precursor (CWBP23, CWBP52)	3	3.80	96.487
Endo-1,4-beta-xylanase A precursor	3	9.39	23.474
Hypothetical protein BSU3I080	2	13.81	19.256
Hypothetical protein BSU17690	2	11.20	26.597
Glycoside hydrolase	2	4.07	70.384
OxdC	2	5.73	43.479
Glycoside hydrolase	2	6.82	39.462

OxdC	2	9.91	22.775
Triosephosphate isomerase	2	7.51	27.114
Polyribonucleotide nucleotidyltransferase	2	3.23	78.181

Source: (Hou et al., 2014b)

A different study speculated that the nitrogen substance of a produced biodemulsifier played a significant role in the biodemulsifier performance while carbonyl groups contributed negatively. Furthermore, there could be a correlation between the biodemulsifying capability of a microorganism with the functional groups produced on the cell surface (Liu et al., 2011b). However, the specific role of each compound, protein and/or combined role of proteins is yet to be determined (Liu et al., 2011b; Hou et al., 2014b).

2.4. Production of biodemulsifier

2.4.1. Cell incubation time

It has been observed that cell age has a great effect on the biodemulsification capability of microbial cells. Microbial cells seem to require a period before biodemulsification in order to adapt to the conditions that they are exposed to, thus triggering their ability to demulsify (Hou et al., 2014a; Li et al., 2012). A study found that the cells achieved maximum biodemulsification after about 21 hrs of cultivation (see Figure 2.3), which was around the time that it achieved the lowest surface tension, while glucose had already been totally consumed (Li et al., 2012).

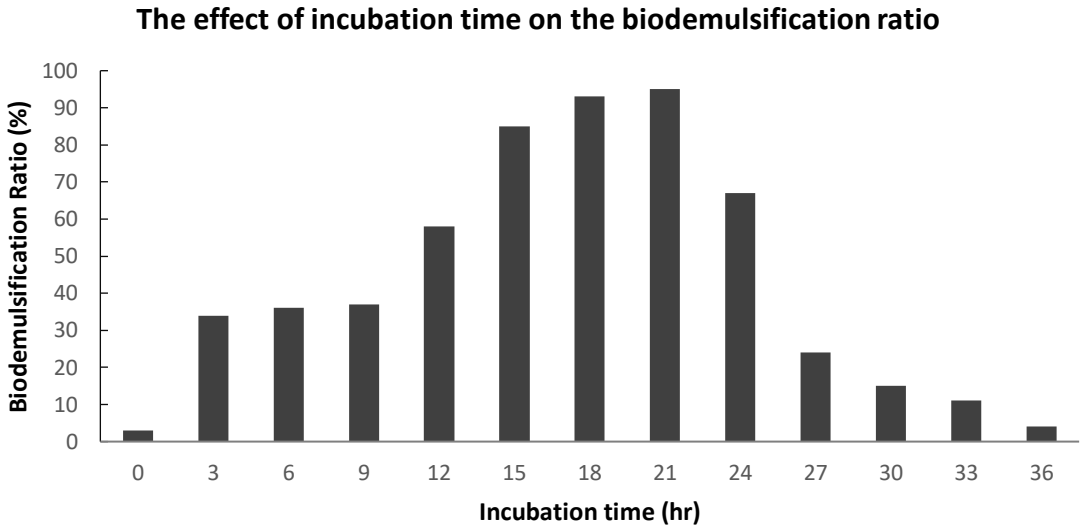

Figure 2.3: The effect of incubation time on biodemulsification capability (Li et al., 2012)

However, once the biodemulsifier reached its maximum biodemulsification efficiency, an extended period resulted in the reduction of the biodemulsification efficacy (Li et al., 2012). This was not the case for *Bacillus cereus*; its biodemulsification efficiency reached a peak

and then decreased, but only to about 60-70% where it remained constant for the remaining period (Hou et al., 2014b). Therefore, the incubation period that resulted in maximum biodemulsification efficiency differed for some microorganisms.

2.4.2. Nutrients

Microorganisms seem to have preferences in nutrients (Huang et al., 2010a). The biodemulsification efficiency is considerably affected by the growth medium composition of the biodemulsifier (Coutinho et al., 2013). For example, *Micrococcus* sp. cells grown on n-tetradecane were discovered to be capable of demulsifying both O-W and W-O emulsions (Das, 2001). While another study found that NaCl affected the production of lipopeptide by *Bacillus subtilis* and *licheniformis* strains (Haghighat et al., 2008). Therefore the nutrients (e.g. carbon source) exposed to the microorganisms during growth extensively affect the biosurfactant produced (Liu et al., 2011b).

2.4.2.1. Carbon source

Various studies have shown that microorganisms with biodemulsification properties tend to require hydrocarbon substrates as the carbon source (Liu et al., 2010; Mohebali et al., 2012). On the contrary, there is information that some microorganisms have biodemulsifier potential despite being grown in conditions comprising non-petroleum hydrocarbon substrates (Mohebali, 2015). Other studies suggested that hydrophilic carbon sources inhibited the production of biodemulsifiers (Liu et al., 2010).

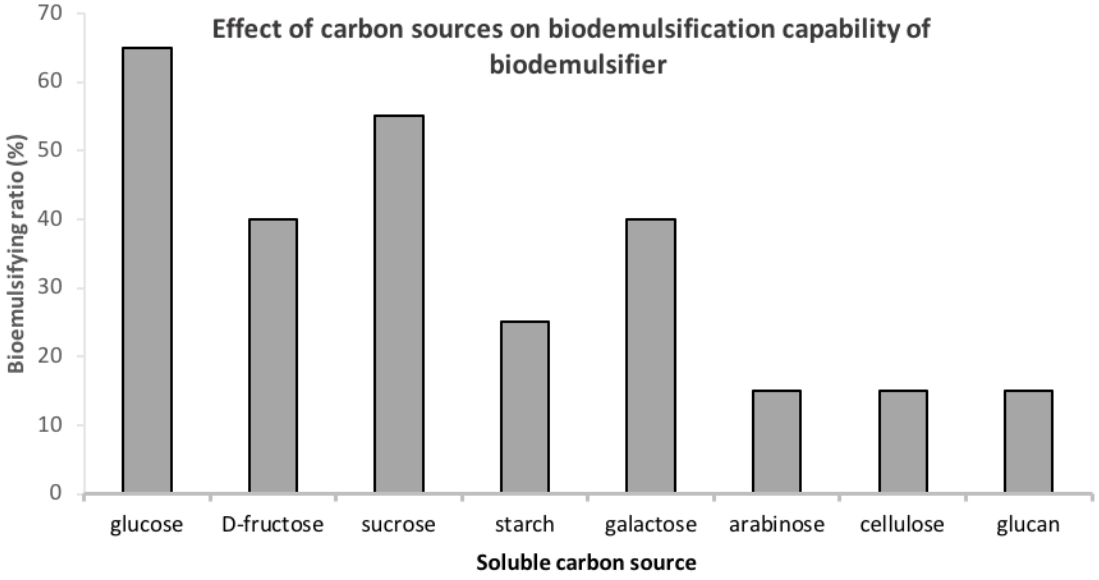

Figure 2.4: Biodemulsification capabilities of Bacillus mojavensis sp. grown on various soluble carbon sources (Li et al., 2012)

Research on *Bacillus mojavensis* showed that glucose-grown *Bacillus mojavensis* had the greatest demulsifying capability, implying that glucose may be the best soluble carbon

source for the biodemulsifying strain (Li et al., 2012). The glucose performance could be due to monosaccharide properties, which enable the cell growth energy and play a role in the synthesis of the lipopeptide produced (Li et al., 2012). This is associated with the common industrial use of glucose as a growth substrate.

The combination of glucose and liquid paraffin outperformed the biodemulsification capabilities of *Bacillus mojavensis* sp. grown on liquid paraffin or glucose only (see Figure 2.4 and Figure 2.5) (Li et al., 2012). This correlates with studies that have shown that microbial growth on multicarbon growth media resulted positively compared to single carbon growth media (Kovárová-Kovar et al., 1998). On the contrary, this did not give similar results with a *Bacillus cereus* sp. that performed the least when glucose and liquid paraffin were the carbon source; glucose was more of an inhibitor (Hou et al., 2014a). However, liquid paraffin seems to be the most suitable carbon source for biodemulsifier for both W-O and O-W emulsions (Hou et al., 2014a; Li et al., 2012; Liu et al., 2010).

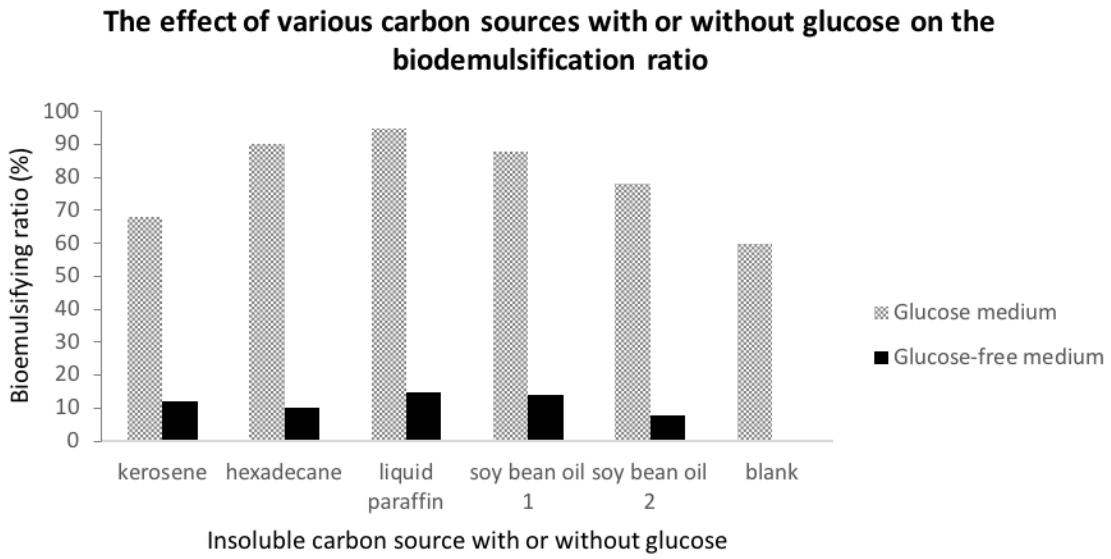

Figure 2.5: Biodemulsification capabilities of *Bacillus mojavensis* sp. grown on various insoluble carbon sources with or without glucose (Li et al., 2012)

Furthermore, there is some correlation with the biomass-produced biodemulsifier efficiency and carbon source. There was less biomass produced when the biodemulsifier efficiency was low compared to the high biodemulsification efficiency coupled with more biomass production (Li et al., 2012). There is therefore a need for further studies to determine the correlation of biodemulsifiers and carbon source.

A study revealed that some bacterial isolates grown in growth media that contained a hydrocarbon (n-hexadecane) as the sole carbon source did not produce biosurfactants. On the contrary, various other bacterial isolates in different studies produced biosurfactants, despite being grown on n-hexadecane as the sole carbon source (Amin, 2012).

Furthermore, *Bacilli Licheniformis* and *subtilis* were able to consume different types of carbon sources for the production of biosurfactant (Haghighat et al., 2008). A different study proposed that crude oil was the best carbon source for biosurfactant production (Haghighat et al., 2008). This demonstrates the complexity of applying the same concept on different types of microbes, highlighting even more the need for tailored growth conditions for specific microorganisms.

2.4.2.2. Nitrogen, Phosphate and trace mineral solution

Nitrogen, phosphate and trace mineral sources present in growth media have some effect on the biodemulsification efficiency of microorganisms (Li et al., 2012). Ammonium citrate was found to be the best nitrogen source for *Alcaligenes* sp. compared to ammonium nitrate, urea, potassium nitrate, sodium nitrate, ammonium molybdate, ammonium sulphate, ammonium chloride and yeast extract (Liu et al., 2010).

However, yeast extract also exhibited positive effects to the biodemulsification efficiency as a sole nitrogen source for *Alcaligenes* sp. (Liu et al., 2010). Yeast extract combined with ammonium sulphate proved to be the best nitrogen source for *Bacillus cereus* sp., even though ammonium sulphate was generally the best sole nitrogen for microorganisms (Hou et al., 2014a). The microbe seems to have a preference for complex nitrogen sources because the fast consumption of single nitrogen sources did not produce the relevant extracellular metabolites required for biodemulsification (Hou et al., 2014a).

The absence of nitrogen, phosphate, and trace minerals contributed negatively to biodemulsification efficiency. This could be due to the fact that nitrogen is known to induce the production of protein (Li et al., 2012), which could possibly be the effective biodemulsifying component (Hou et al., 2014b). However, the contribution of phosphates to biodemulsification efficiency is yet to be studied.

2.4.3. pH

Bacterial growth and collection of biological products have been found to be influenced by pH (Hou et al., 2014b). *Bacillus cereus* LH-6 demonstrated that its biodemulsification capability is dependent on the initial pH value. Biodemulsification capability increased with initial pH ranging between 5 and 7 but decreased with initial pH ranging between 8 and 11 (Hou et al., 2014b, Hou et al., 2014a).

Various studies proposed a similar relation between pH and biodemulsification capability, but the studies were on different microorganisms such as *Paenibacillus alvei* ARN63, *Pseudomonas aeruginosa*, and *Alcaligenes* sp. S-XJ-1 (Amirabadi et al., 2013; Coutinho et al., 2013; Mohebali et al., 2012). Therefore, biodemulsification relation to pH (Mohebali et al., 2012) indicates that biodemulsification is “sensitive to the degree of ionization of any

ionizable group on the bacterial surface or in compounds present in the culture supernatant" (Coutinho et al., 2013).

A study suggested *Alcaligenes* sp. S-XJ-1 as an alkaliphilic bacterium, which is a bacterium that is likely to have a specific alkaliphilic enzyme to promote the survival and biodemulsifying trait during alkali conditions. Researchers proposed that the initial pH culture had the potential to alter the cell surface properties and develop the functions of alkaliphiles (Liu et al., 2010). Another study showed that *Bacilli licheniformis* and *subtilis* were found not to produce a substantial amount of lipopetides when grown under acidic conditions, implying that their cell growth was more favourable under alkali-approaching or alkali conditions (Haghighat et al., 2008).

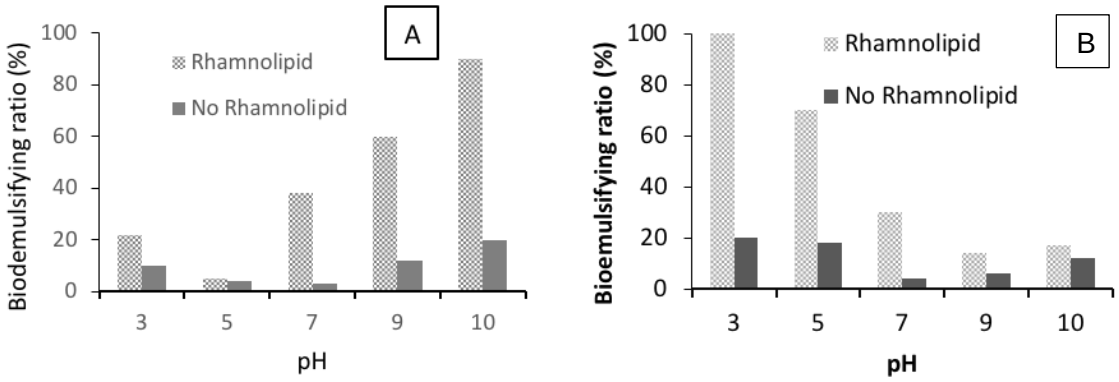

Figure 2.6: pH effect on the biodemulsification capability of rhamnolipid used to treat waste oil emulsions; A: W-O and B: O-W (Long et al., 2013)

Rhamnolipid, a high surface active biosurfactant was used to demulsify problematic waste crude emulsions. Under alkaline conditions, the rhamnolipid performed best for water-in-oil emulsion, whereas its demulsification of O-W emulsions was best achieved under acidic conditions (see Figure 2.6) (Long et al., 2013). Furthermore, Figure 2.6 shows that a pH adjustment enhances demulsification to some extent even without the injection of a demulsifier.

2.4.4. Cultivation temperature

Bacillus mojavensis XH1 was exposed to various temperature conditions and it continued to depict biodemulsifier capabilities of 85.5% after exposure to temperatures below 75 °C (Li et al., 2012). Due to a decline in biodemulsification capabilities when exposed to higher temperatures ranging between 75 °C and 120 °C, researchers suggested the presence of both thermally stable and thermally unstable components (Li et al., 2012). Also, the biodemulsifier can separate emulsions even under harsh conditions due to thermal stability over a wide range of temperatures (Liu et al., 2010).

Bacillus cereus LH-6 initially demonstrated an increasing biodemulsification performance, as the cultivation temperature was increased. However, the biodemulsifier later decreased as

the cultivation temperature was continually increased. Therefore extremely low or high temperatures affect the biodemulsifier capability (Hou et al., 2014a). This correlates with a study where exposure to high temperature (e.g. 121 °C) completely deteriorated the biodemulsifying capability of the microbes (Hou et al., 2014b; Nadarajah et al., 2002). The loss of biodemulsification activity indicates that the extra cellular proteins of the cells might be the active ingredients of the biodemulsifier (Hou et al., 2014b).

Some studies revealed that the emulsion half-life ($t_{1/2}$) decreased concurrently with an increase in temperature (Mohebali et al., 2012). On the contrary, a study on *Pseudomonas aeruginosa* MSJ showed that the biodemulsification ability reduced with increasing temperature during biodemulsification of W-O (Coutinho et al., 2013).

An increase in temperature commonly reduces the viscosity of the oil, thus increasing the density variance between the oil and water. This in turn causes the diminishing of the interfacial film and promotes droplet coalescence (Mohebali, 2015; Singh et al., 2012). Therefore biodemulsification rate is dependent on the temperature (Mohebali, 2015).

2.5. Demulsification kinetics

Ample studies have been conducted on the biodemulsification of various types of emulsions including oilfield emulsions. A critical review of literature revealed that information/data that dealt on kinetic studies of biodemulsification of oilfield emulsions was not available. The available studies conducted over the past two decades focused mostly on the topics such as listed in Table 2.6 below. However kinetics models are critical to designing an appropriate biodemulsification process

Table 2.6: Existing research focus

Existing research focus	References
Biochemical and physiochemical characterization	(Hou et al., 2014a)
Cell surface hydrophobicity	(Huang et al., 2010a; Wen et al., 2010)
Characterization of demulsifying microbes	(Huang et al., 2010a; Li et al., 2012)
Demulsification capabilities	(Das, 2001; Hou et al., 2014a; Li et al., 2012, Long et al., 2013; Wen et al., 2010)
Effect of physical and chemical treatments	(Coutinho et al., 2013)
Interfacial properties	(Wen et al., 2010)
Biodemulsifier growth kinetics	(Li. 2011)
Phylogenic analysis	(Huang et al., 2010a)
Optimization of biodemulsifier performance and/or production	(Hou et al., 2014b; Li et al., 2012)

2.5.1. Breakdown of emulsions

Destabilisation mechanism of emulsions refers to the various processes that occur during the separation of oil from water when exposed to a demulsifier (whether synthetic or

biological). For decades many have been interested in understanding the core factors affecting the breakdown and stability of emulsions. And thus far, various breakdown mechanisms are distinguishable for the disintegration of emulsions, namely sedimentation, creaming, flocculation, coalescence, Ostwald ripening and phase inversion (Tadros, 2004; Taylor, 1992; Tadros et al., 2004; Taylor, 1995; Schuster, 1996). Evaluation of these above-mentioned breakdown mechanisms occurs under the assumption that the spheres of the dispersed liquid are uniform, non-deformable and non-interacting (Tadros et al., 2004). Though these mechanisms were identified mostly during the study of demulsification due to synthetic demulsifiers, they will be further elaborated due to their fundamental nature.

A recent study has shown that biodemulsifiers follow different sequences, although It was proposed that the main biodemulsification mechanism to be solubilization and replacement. On the contrary, an *Alcaligenes* sp. was used to demulsify a W-O emulsion and it was observed that biodemulsification commenced with the cells rapidly dispersing in the oil phase, thus, triggering adsorption on the oil-water interface (Wen et al., 2010).

It was further observed that creaming, clarification, coalescence and flocculation and sedimentation occurred within the initial 24hrs (Wen et al., 2010).

- Creaming process: amount of water at the top reduced with steady oil separation,
- Clarification process: water droplet coalescence and settling at the bottom of the emulsion,
- Coalescence and flocculation of water droplets in the middle,
- Sedimentation: settling of cells at the bottom.

However, the biodemulsification mechanism is still a topic of interest as biodemulsifiers are yet to be fully comprehended and it should be further studied.

2.5.1.1. Sedimentation/Creaming

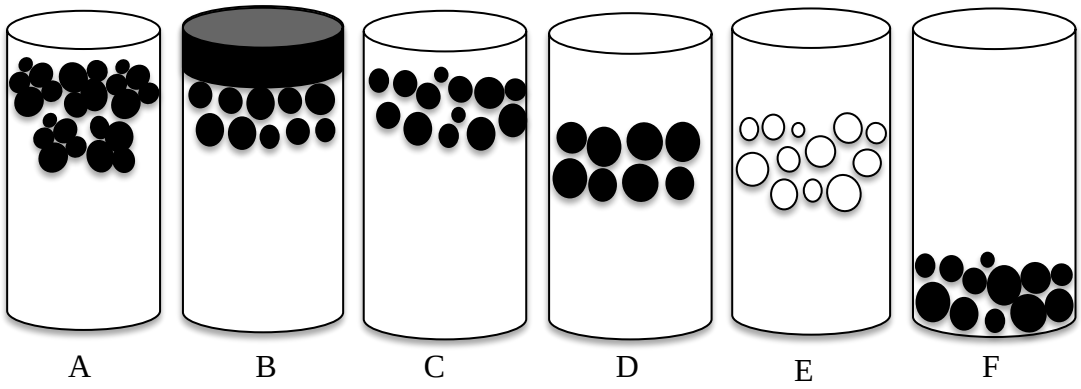

Figure 2.7: A: Flocculation, B: Coalescence, C: Creaming, D: Ostwald ripening, E: Phase inversion and F: Sedimentation (Tadros, 2004, Schuster, 1996)

Sedimentation is the settling of droplets (see Figure 2.7) due to the force of gravity and this is likely to occur when the density of the dispersed liquid is higher than that of the continuous

phase. Creaming is the opposite, namely the migration of dispersed liquid due to buoyancy. Hence with O/W emulsions, creaming is more likely to occur due to lower oil density (Tadros, 2004; Schuster, 1996).

Evaluation of the rate of sedimentation/creaming is done according to the following (Tadros, 2004; Schuster, 1996).

- Observation of the emulsion separation with the aid of graduated cylinders within a temperature-controlled environment.
- Measurement of the emulsion turbidity and height.
- Measurement of ultrasonic velocity, absorption and height.
- Observation of emulsion changes due to application of centrifugation within a controlled gravitational force environment, where the gravitational force does not exceed the critical value (see Equation 2.4).

$$g = x(2\pi v)^2$$

Equation 2.4: Centrifugal gravitational force

Where: g is the gravitational force, v is the number of revolutions per second and x is the mean distance of the centrifuge tube from the axis of rotation.

The data obtained can then be used to calculate the degree of emulsion stability (see Equation 2.5). Degree of emulsion stability is the measure of the opaque phase remaining after a period of time (Tadros, 2004).

$$\frac{t}{V} = \frac{1}{bV_\infty} + \frac{t}{V_\infty}$$

Equation 2.5: Degree of emulsion stability

Where: V is the volume of oil separated, t is the time, V_∞ is the extrapolated volume at infinite time, and b is a constant.

If the particle radius of a produced emulsion is greater than 100 nm and the change in density is greater than 0,1 then the creaming/sedimentation effect will exceed the Brownian diffusion (see Equation 2.6). If, however the particle radius of the produced emulsion is greater than 1 μm, the change in density is greater than 0,2 and there is no thickener; sedimentation/creaming occurs within hours or less and at a rate that is greater than 0,44 μm/s (Tadros, 2004).

$$\frac{4}{3}\pi r^3 \Delta\rho g L \gg kT_{abs}, \quad (if\ R > 100\ nm; \Delta\rho > 0{,}1)$$

Equation 2.6: Brownian diffusion

Where r is the particle radius, $\Delta\rho$ is the change in emulsion density, g is the gravitational force, L is the height of the container, k is the Boltzmann constant and T_{abs} is the absolute temperature.

Emulsions can be dilute, concentrated or complex and they are categorised based on the volume fraction (ϕ) of the dispersed phase in the emulsion. Dilute emulsions have a volume fraction of less than 0,01 and adhere to Stoke's law (See Equation 2.7) (Tadros, 2004). Thus, the sedimentation/creaming rate is calculated as follows:

$$v = \frac{2\Delta\rho g r^2}{9\eta_0}$$

Equation 2.7: Stoke's rate of sedimentation/creaming for dilute emulsions

Where: v is the rate of sedimentation/creaming, r is the particle radius and ρ is the density of the dispersed liquid, g is the gravitational force, and η_0 is the viscosity of the continuous liquid.

Concentrated emulsions have a volume fraction (ϕ) between 0,1 and 0,2. However, the sedimentation/creaming rate of concentrated emulsions is lower than that of dilute emulsions. Thus, if the emulsion has a volume fraction of 1, the rate will be only 35% of the Stoke's rate (see Equation 2.8) (Tadros, 2004).

$$v_c = v(1 - 6.55\phi)$$

Equation 2.8: Rate of sedimentation/creaming for concentrated emulsions

Where: v_c is the rate of sedimentation/creaming for concentrated emulsions, v is the rate of sedimentation/creaming and ϕ is the volume fraction.

Lastly, complex emulsions have a volume fraction that is greater than 0,2. The rate as a function of volume fraction, decreases exponentially to a point where it approaches zero (Tadros, 2004).

2.5.1.2. Flocculation

Flocculation is the system whereby the Van der Waals forces are greater than the repulsive energy between droplets thus resulting in the grouping of droplets (see Figure 2.7). The rate of flocculation is affected by the droplet sizes, and thus can be investigated by measurement of turbidity over a period of time and/or droplet counting with the aid of optical microscopy. The rate of flocculation for diluted emulsions is given in Equation 2.9 following, which is obtained by plotting the turbidity as a function of time. The rate constant (k) is then calculated as the slope of the turbidity vs time plot (Tadros, 2004).

$$\tau = n_0 A V_1^2 (1 + 2 n_0 k t)$$

Equation 2.9: Rate of flocculation for dilute emulsions

Where: τ is the rate of flocculation, n_0 is the number of droplets at $t = 0$, A is the optical constant, V_1 is the volume of the droplets, k is the rate constant and t is the time.

2.5.1.3. Ostwald ripening

Ostwald ripening is the result of solubility difference between large and small droplets within an emulsion (Schuster, 1996). The smaller droplets unite with the larger droplets (see Figure

2.7). The volume fraction of the dispersed phase of the emulsion affects the rate of Ostwald ripening (ϑ) because of the interaction of the diffusing droplets.

The rate of Ostwald ripening, also referred to as the LSW theory, is given by Equation 2.10 (Tadros, 2004; Taylor, 1992; Taylor, 1995), and evaluated by plotting the cubed critical radius (r_c) as a function of time (t). Droplets that are at a critical radius do not experience any change in size (Taylor, 1992). The LSW theory assumes that there is no interaction between the particles and is thus limited to emulsions with low volume fractions (Taylor, 1992).

$$\vartheta = \frac{dr_c^3}{dt} = \frac{8DS(\infty)V_m}{9RT_{abs}}$$

Equation 2.10: Rate of Ostwald ripening

Where: ϑ is the rate of Ostwald ripening, r_c is the critical radius of a droplet (no radius changes), D is the diffusion coefficient of the dispersed phase in the continuous phase, $S(\infty)$ is the infinite radius, V_m is the molar volume of the dispersed phase, R is the gas constant, T_{abs} is the absolute temperature and t is the time.

2.5.1.4. Coalescence

Coalescence is the outcome of the reduction and interference of the film between the droplets, thus promoting the joining of droplets of the same medium (see Figure 2.7) (Schuster, 1996). Before coalescence, approaching droplets of the same medium cause fluctuation forming waves in the surrounding medium. The peak of these fluctuation forms wave-generating points where the distance between the separated droplets is shorter, resulting in points of substantial Van der Waals forces. As the distance decreases, the film eventually ruptures, and coalescence occurs (Tadros, 2004). The rate of coalescence, a first order function based on the Cockbain's theory, is as follows (Fang et al., 2016; Tadros, 2004; Schuster, 1996):

$$n = \frac{n_0}{Kt}(1 - e^{-Kt})$$

Equation 2.11: Rate of coalescence

Where: K is the rate constant of coalescence, n is the number of droplets at time t, t is the time and n_0 is the number of droplets at time $t = 0$.

The half-life of the emulsion droplets is regarded as the coalescence speed and is evaluated with the given equation. The coalescence speed decreased when exposed to an increase in temperature whilst the rupture rate constant increased (Fang et al., 2016).

$$t_{1/2} = \frac{ln2 + C}{k}$$

Equation 2.12: Droplet coalescence speed

Where: $t_{1/2}$ is the stabilization half time, k is the coalescence constant and C is the regression factor.

2.5.1.5. Phase inversion

Phase inversion is an activity where the dispersed phase interchanges with the continuous phase (see Figure 2.7). It occurs either catastrophically or transitionally due to changing conditions that significantly affect the HLB of the emulsion system (Tadros, 2004; Tadros et al., 2004). Catastrophic phase inversion occurs when there is an increase of volume fraction of the dispersed phase. Whereas transitional phase inversion occurs due to the change in temperature and/or introduction of electrolytes to the emulsion system. Rate of phase inversion is evaluated by measuring either the emulsion conductivity or viscosity as a function of the emulsion volume fraction (Tadros, 2004).

2.6. Biodemulsifier production and biodemulsification kinetics

2.6.1. Biodemulsifier production kinetics

The preparation kinetics of a lipopeptide type of biodemulsifier produced by *Candida tropicalis* strain JM-1 for the destabilisation of cold rolling plant emulsion wastewater were investigated. The results obtained showed that the growth characteristic of the strain was constant with both the non-optimized and optimized culture (Li, 2011).

The strain achieved maximum cell growth under certain fermentation conditions which was explained well by the Logistic equation and not the classic Monod equation. The growth kinetic model, the dry cell weight (C_x) as a function of time (t), was established as follows (Li, 2011):

$$C_x = \frac{2.15}{1 + 20.47e^{-0.174t}}$$

Equation 2.13: Candida tropicalis strain JM-1 growth kinetic model

The kinetic model was found to be a precise description of the growth and stationary phases, but minor errors were visible between actual and calculated values during the lag and death phases. The researchers went even further and established the lipopeptide production model (see Equation 2.14), which is the polynomial model shown below. The actual values fitted the lipopeptide production (C_P) model values well during both the growth and stationary phases (Li, 2011).

$$C_P = \frac{-t^7}{7237948787} + \frac{t^6}{36485123} - \frac{t^5}{480910} + \frac{t^4}{13520} - \frac{62t^3}{50931} + \frac{43t^2}{4870} - \frac{69t}{3205} + \frac{44}{4171}$$

Equation 2.14: Lipopeptide production model

There seems to be much work reported on biodemulsification/biodemulsifiers but hardly any studies on biodemulsification kinetics as shown in Table 2.6. The microbial growth kinetics is important for the production of biodemulsifiers but it also important to know the rate of biodemulsification. There is therefore a need for studies on biodemulsification kinetics; knowledge concerning the rate of biodemulsification will definitely be a great contribution to the oil industry.

2.6.2. Biodemulsification kinetics

Biodemulsification has been reported to occur either due to microbial extracellular products or intracellular products. This implies that depending on the microorganism used and the conditions that it is exposed to, biodemulsification can occur with or without microorganisms. In that case, it cannot be simply concluded that biodemulsification kinetics will follow microbial growth trends; however, if microorganisms are present it would have to be considered as a possibility. Therefore, various published microbial growth profiles and common kinetic models will be investigated (Mitchell et al., 2004).

2.6.2.1. Empirical reaction models

Investigation of kinetic parameters is a complex task due factors such as difficulty with analysis of indirect measurements of system parameters. Though simulated systems are generated to enable direct measurements of parameters, the results are not always representative of the actual system. However, they do somehow shed light on the kinetics of the system. Empirical reaction models include exponential, linear (see Equation 2.15), logistic and two-phase models which do not describe the outcome of substrate concentrations on the microbial growth (Mitchell et al., 2004).

$$X = Kt + X_0$$

Equation 2.15: Linear model

Where: X is the microbial biomass, K is the linear growth rate, t is the time and X_0 is the initial biomass.

These models however through non-linear regression describe the experimental microbial growth profiles on the assumption that the microbial growth depends only on the system parameters. The Logistic model (see Equation 2.16) describes the entirety of a microbial growth curve with a single function, an advantage that some of the well-known models do not have since they tend to describe a single phase or exclude other phases of the growth profile (Mitchell et al., 2004).

$$X = \frac{X_m}{1 + \left(\left({}^{X_m}/_{X_0}\right) - 1\right) e^{-\mu t}}$$

Equation 2.16: Logistic model

Where: X is the microbial biomass, μ is the specific growth rate constant, t is the time, X_m is the maximum possible biomass and X_0 is the initial biomass.

The Two-phase model was designed to describe reactions with two major phases, namely exponential and deceleration. The initial phase of the Two-phase model is described by the exponential model (Equation 2.17) for as long as the reaction has not yet reached the deceleration period $(where\ t < t_a)$ (Mitchell et al., 2004).

$$X = X_0 e^{\mu t}$$

Equation 2.17: Exponential model

Where: X is the microbial biomass, μ is the specific growth rate constant, t is the time, and X_0 is the initial biomass.

The deceleration phase occurs at time (t_a) and the specific growth rate of this phase is represented by the Equation 2.18 below. The deceleration model accounts for the exponential phase growth rate. However, the application of this model is limited by the hasty exponential phase that does not produce substantial biomass for experimental analysis (Mitchell et al., 2004).

$$X = X_A e^{\left[\frac{\mu L}{k}\left(1 - e^{-k(t - t_a)}\right)\right]}, t \geq t_a$$

Equation 2.18: Two-phase model deceleration phase specific growth rate

Where: X is the microbial biomass, μ is the specific growth rate constant, t is the time, X_A is the microbial biomass at $t = a$, t_a is the time when growth profile changes from exponential to decelerating, L is the specific growth rate ratio and X_0 is the initial biomass.

2.6.2.2. Microbial growth models

Microbial growth kinetics refers to the relationship between the specific microbial growth rate of microorganism(s) and the substrate concentration(s). Microbial growth can be significantly described with four parameters, namely; maximum specific growth rate (μ_{max}), substrate saturation constant (K_s), growth yield $Y_{X/s}$ and predicted substrate concentration at 0 dilution rate $(s_{\min})$ (Kovárová-Kovar et al., 1998).

In some instances, microbial growth may not be controllable with a single nutrient and this is due to the fact that microorganisms within a natural environment are generally exposed to a mixture of substrates. A change of environment for microorganisms does not guarantee the same kinetic properties (Kovárová-Kovar et al., 1998) because the microorganisms tend to make relevant adjustments for their survival. For example, when gram negative or positive microorganisms are exposed to various substrate concentration, they significantly adjust

their kinetic parameters by applying some of the strategies mentioned below (Kovárová-Kovar et al., 1998):

- Alternation between different transport systems with varying affinity,
- A particular uptake system resulting with varying kinetic parameters as substrate concentration is varied and
- Catabolic and anabolic capacity variations.

It is common knowledge in the microbiology field, that the growth rate is subject to the growth medium composition. Studies have shown that simultaneous utilization of more than one carbon source is found when bacteria and yeast are cultivated under batch conditions with high substrate concentrations. Thus, the growth rate will be dependent on several substrates instead of just one. However, there are studies that have shown the growth rate of microbial cells being solely dependent on one specific substrate (e.g. glucose, ammonia, etc.) (Kovárová-Kovar et al., 1998).

Kinetic parameters are not necessarily constant, due to the adaptive nature of microorganisms and how their background can affect their performance. Hence in some instances, microorganisms are conditioned by exposure to certain environments over a period such that they apply the abovementioned strategies and produce different results. Due to the complex nature of microbial growth, Monod warned that microbial growth be studied with the consideration of prescribed laws (Kovárová-Kovar et al., 1998).

There are various microbial growth models used, and those that paved way for many other growth models such as the Monod model (see Equation 2.19). The Monod model, generally criticised due to its lack of accounting for hindered microbial growth at excessive substrate concentrations, is known for the introduction of the limiting substrate concept, which later paved way for microbial growth kinetics. However, it is predominantly used to describe microbial growth by relevant industries. The Monod model describes the relationship between the limiting substrate (S) concentration and the growth rate (μ). It however disregards the substrates required for cell maintenance especially during cell degradation, although it is capable of describing systems that are complex in nature, such as multiple enzyme systems (Kovárová-Kovar et al., 1998; Saravanan et al., 2008; Button, 1993).

Therefore, when describing microbial growth in conditions with higher substrate concentration, the Haldane model (see Equation 2.20) is applied, which is based on the Monod model but considers the dissociation of the substrate (K_i). Another model that is also considered for altered substrate concentration is the Han-Levenspiel which considers the critical inhibitor concentration (S_m) (Saravanan et al., 2008).

MVKH2 is a novel model (see Equation 2.22) that considers both the growth and death of the microorganisms. The death or deterioration of microbial cells during the stationary phase is represented by the integral term. Combined with the Aiba specific growth rate model (see

Equation 2.23), the MVKH2 model was found to perform best in the prediction of the specific growth rate constant of the microorganisms. However, the application was on aromatic hydrocarbon degradation, which is not far off the application of hydrocarbon-consuming microorganisms used for the production of biodemulsifiers (Khoshdel et al., 2016).

The Michaelis-Menten model (See Equation 2.24), which assumes equilibrium, was designed to evaluate a process that is catalysed by a single enzyme. It is applicable when the rate of active substrate transportation controls microbial growth and in this instance K_s and K_M are considered to be similar. Using the linearised Michaelis-Menten model (see Equation 2.25), a plot of $(C_{A_0} - C_A)/\ln(C_{A_0}/C_A)$ vs $t/\ln(C_{A_0}/C_A)$ results in the Michaelis constant, rate of reaction and rate constant values. The slope of the plot equates to kC_{E0}, the x-intercept is $K_M/kC_{E0}$and the y-intercept is $-K_M$ (Button, 1993; Doran, 2013; Levenspiel, 1999).

Table 2.7: Microbial growth models

Model name	Model equation	Description
Monod	$\mu = \frac{\mu_{max} S}{S + K_s}$ **Equation 2.19: Monod model**	μ is the specific growth rate $(1/hr)$ S is the limiting substrate concentration (mg/L) μ_{max} is the maximum specific growth rate $(1/hr)$ K_s is the half saturation coefficient (mg/L)
Haldane	$\mu = \frac{\mu_{max} S}{S + K_s + \left({}^{S^2}/_{K_i}\right)}$ **Equation 2.20: Haldane model**	μ is the specific growth rate $(1/hr)$ μ_{max} is the maximum specific growth rate $(1/hr)$ K_s is the biomass affiliation to the substrate (mg/L) K_i is the substrate dissociation constant (mg/L) S is the substrate concentration (mg/L)
Han-Levenspiel	$\mu = \frac{\mu_{max}\left(1 - {}^{S}/_{S_m}\right)^n}{S + K_s - \left(1 - {}^{S}/_{S_m}\right)^m}$ **Equation 2.21: Han-Levenspiel model**	μ is the specific growth rate $(1/hr)$ μ_{max} is the maximum specific growth rate $(1/hr)$ K_s is the biomass affiliation to the substrate (mg/L) S is the substrate concentration (mg/L) S_m is the critical inhibitor concentration above which reaction stops (mg/L) n is an empirical constant m is an empirical constant
MVKH2	$\frac{dX}{dt} = \mu - \gamma + k_d \left\lvert \int_0^t X(t)dt \right\rvert$ **Equation 2.22: MVKH2 model**	X is the biomass concentration $\left({}^{cells}/_{g}\right)$ μ is the microbial specific growth rate constant γ is the stationary constant k_d is the death constant $\left({}^{1}/_{day^2}\right)$ t is the time (day)
Aiba	$\mu = \frac{\mu_{max} S\left(e^{-S/K_i}\right)}{S + K_s + {}^{S^2}/_{K_i}}$ **Equation 2.23: Aiba model**	μ is the specific growth rate $\left({}^{1}/_{day}\right)$ μ_{max} is the maximum specific growth rate $\left({}^{1}/_{day}\right)$ K_s is the biomass affiliation to the substrate (μM) K_i is the substrate dissociation constant (μM) S is the substrate concentration (g)

Michaelis-Menten	$-r_A = \frac{kC_{E0}C_{\mathrm{A}}}{K_M + C_{\mathrm{A}}}$ **Equation 2.24: Michaelis-Menten model**	C_A is the reactant A concentration C_{E0} is the total enzyme K_M is the Michaelis constant $-r_A$ is the rate of reaction k is the rate constant
Linearised Michaelis-Menten	$\frac{C_{A_0} - C_A}{\ln\left({C_{A_0}}/{C_A}\right)} = -K_M + kC_{E0}\frac{\mathrm{t}}{\ln\left({C_{A_0}}/{C_A}\right)}$ **Equation 2.25: Linearised Michaelis-Menten model**	C_{A_0} is the reactant A concentration at $\mathrm{t} = 0$ C_A is the reactant A concentration K_M is the Michaelis-Menten constant k is the rate constant C_{E0} is the total enzyme t is the time.

Sources: (Amin, 2012; Doran, 2013; Khoshdel et al., 2016; Kovárová-Kovar et al., 1998; Levenspiel, 1999; Mitchell et al., 2004; Saravanan et al., 2008)

3. Materials and method

3.1. Microorganism

B.licheniformis STK 01 strain, obtained from decaying wood (Amodu et al., 2014) was maintained on a nutrient agar plate at 4 °C and sub-cultured every week.

3.2. Materials

Table 3.1: List of chemicals used in the study

Uses	Chemical name	Supplier
Preparation and maintenance of microorganism	Ammonium nitrate (NH_4NO_3)	Merck
	Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	Merck
	EDTA	Sigma-Aldrich
	Ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$)	Merck
	Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	Merck
	Dipotassium phosphate (K_2HPO_4)	Merck
	Nutrient agar	Sigma-Aldrich
	Potassium dihydrogen orthophosphate (KH_2PO_4)	Merck
	Sodium hydroxide (NaOH)	Merck
	Yeast extract	Merck
Carbon sources	Diesel (50ppm)	Local garage
	Fructose	Sigma-Aldrich
	Glucose	Merck
	Motor oil	Local garage
	Paraffin	Merck
	Sucrose	Sigma-Aldrich
Simulated emulsion	Paraffin	Merck
	Span 60	Sigma-Aldrich
	Tween 60	Sigma-Aldrich

3.3. Method

3.3.1. Preparation of microorganism

Nutrient agar (16 g) was dissolved into 500 mL of distilled water using a heated magnetic stirrer. Once dissolved, the nutrient agar was autoclaved at 121 ºC for 15 min. After cooling to a warm temperature (it solidifies at room temperature), the nutrient agar was poured into

petri dishes. Once it was cool and solid, *B.licheniformis STK 01* was streaked (from storage) onto a nutrient agar plate using an inoculating loop. The inoculated nutrient agar plate was incubated at 37 ºC for 24 hrs. Aseptic techniques were used in the handling of the microorganisms.

3.3.2. Preparation of glassware

Erlenmeyer flasks were closed with cotton wool covered with foil, and glass cylinders were closed with foil. The glassware was autoclaved (see Figure 3.1) at 121 ºC for 15 min.

Figure 3.1: Autoclave used in the study for glassware and nutrient media sterilisation

3.3.3. Preparation nutrient media

Nutrient media were prepared by adding 4 g of NH_4NO_3, 1 g of yeast extract, 4 g of K_2HPO_4, 6 g of KH_2PO_4, and 0,2 g of $MgSO_4 \cdot 7H_2O$ (Hou et al., 2014b) to 1L of distilled water and stirred till dissolved using a magnetic stirrer. The nutrient was autoclaved at 121 °C for 15 min. Trace mineral solution was prepared by dissolving 1 g of $CaCl_2 \cdot 2H_2O$, 1 g of $FeSO_4 \cdot 7H_2O$ and 1,4 g of EDTA in 1L of distilled water. The solution was then filter sterilised with a 0,2 µm syringe filter. After cooling, the pH was measured (see Figure 3.2) and buffered to pH 7 using NaOH. Trace mineral solution (1 mL) was then added to the nutrient media.

3.3.4. Preparation of carbon sources

The paraffin, motor oil and diesel were slightly warmed on a heating plate and individually filter sterilised with a 0,2 µm syringe filter into a sterilised Scott bottle for storage. 10%

solutions of glucose, fructose and sucrose were separately made using a magnetic stirrer and distilled water. The solutions were then autoclaved at 121 °C for 15 min.

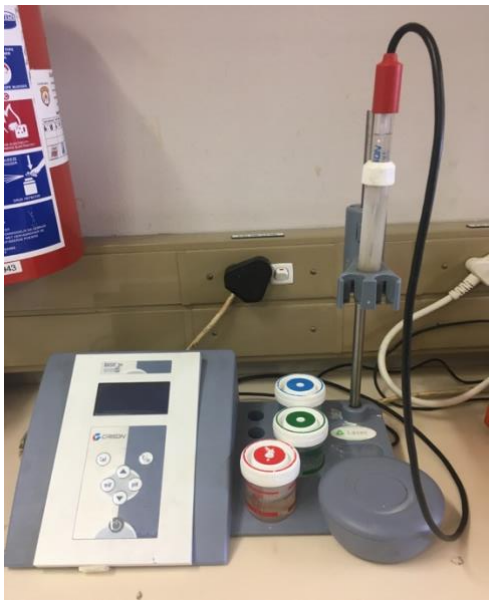

Figure 3.2: pH meter used in the study

3.3.5. Cultivation of *B.licheniformis* STK 01 biodemulsifier

48 mL of the prepared nutrient media was transferred into a sterilised 100 mL graduated conical flask. The nutrient media was then inoculated with *B.licheniformis* STK 01 using an inoculation loop. Lastly, 2 mL (4 v/v%) of carbon source was pipetted into the conical flask. The conical flask with inoculated growth media was placed in an incubator shaker at 37 °C and 160 rpm for 24 hrs.

43 mL of the freshly prepared nutrient media was transferred into a sterilised 100 mL graduated conical flask, followed by a 2 mL of carbon source and 5 mL of aged growth media. The conical flask with the prepared nutrient media and aged growth media was placed in an incubator shaker (see Figure 3.3) at 37 °C and 160 rpm for 24 hrs. Thereafter, the cultivated biodemulsifier was used for demulsification tests. The experiment was conducted in triplicate, in order to minimise result errors.

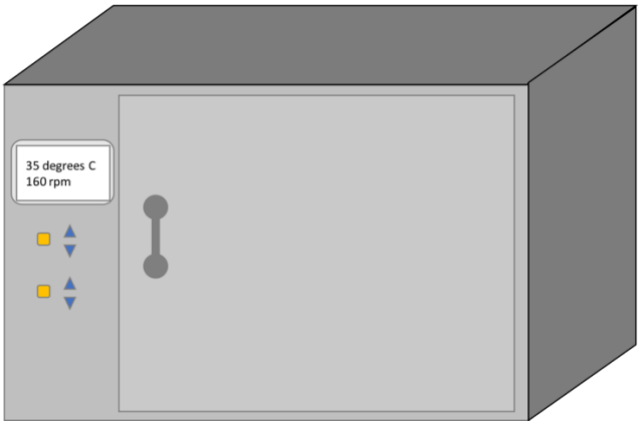

Figure 3.3: Incubator shaker used for both biodemulsifier production and emulsion biodemulsification process

3.3.6. Preparation of emulsion

The emulsion was prepared by dissolving 8,7 g of Tween 60 in 1 L of distilled water on a low heated magnetic stirrer to produce a Tween/water solution. 1,2 mL of Span 60 was also dissolved in 1 L laboratory grade paraffin on a low heated magnetic stirrer to produce a Span/oil mixture. 5 mL of the Span/oil was transferred into a 15 mL conical centrifuge tube using a 5 mL pipette, followed by 5mL of Tween/water solution and then mixed for 1min using a vortex mixer (see Figure 3.4). Volume readings of the produced emulsion were then noted. The experiment was conducted in triplicate, in order to minimise the result errors.

Figure 3.4: Vortex mixer used in the study

3.3.7. Demulsification and data analysis technique

10% (v/v) of the cultivated biodemulsifier was pipetted into the emulsion within a volumetric glass cylinder. The glass cylinders were then placed in an incubator at 37 °C for 24 hrs. Over time, the oil would separate from the emulsion and float to the top and water would gravitate to the bottom and accumulate with the excess water, whilst the emulsion volume

would slowly reduce and remain at the centre (see Figure 3.5). Readings of the changes (oil separated, remaining emulsion and accumulating water) were measured periodically.

$$\boldsymbol{Biodemulsification\ capability} = \left(\mathbf{1} - \frac{\boldsymbol{V_t^E}}{\boldsymbol{V_{t=0}^E + V_{t=0}^{CS}}}\right) \times \mathbf{100\%}$$

Equation 3.1: Equation used to calculate the biodemulsification capability

For the biodemulsification capability calculation, the remaining emulsion volume V_t^E at time t was divided by the sum of the initial emulsion volume $V_{t=0}^E$ and the biodemulsifier volume $V_{t=0}^{CS}$ added. The result was then subtracted from one and converted to a percentage.

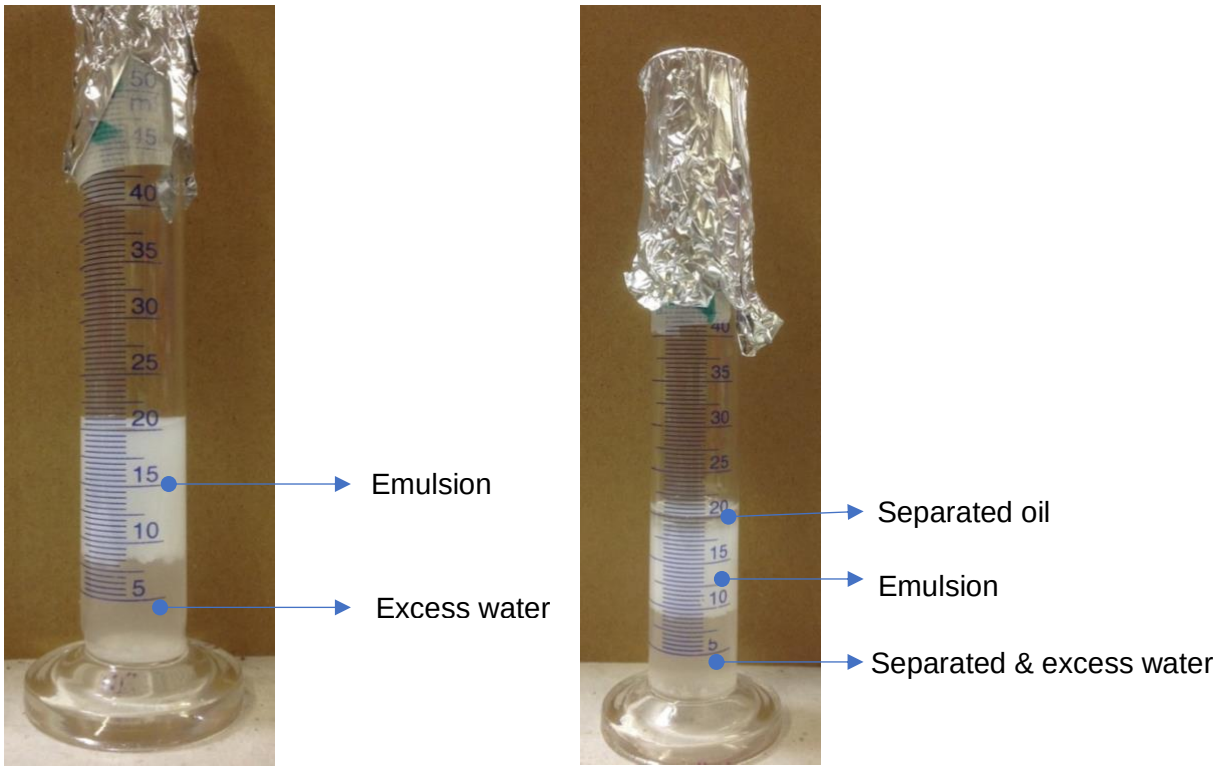

Figure 3.5: Simulated emulsion with *B.licheniformis* STK 01 biodemulsifier A-before treatment (t=0hr) and B-after treatment (t=1hr)

3.3.8. Cultivation of *B.licheniformis* STK 01 biodemulsifier for the temperature effect study

96 v/v% of the prepared nutrient medium (see Section 3.3.3) was transferred into a sterilised graduated conical flask. The nutrient medium was then inoculated with B.licheniformis STK 01 using an inoculation loop. Lastly, 4 v/v% of carbon source was pipetted into the conical flask. The conical flask with inoculated growth medium was placed in an incubator shaker at 37 °C and 160 rpm for 24 hrs.

86 v/v% of freshly prepared nutrient media was transferred into a sterilised graduated conical flask. Followed by 4 v/v% of motor oil and 10 v/v% of aged growth media pipetted into the conical flask. The conical flask with the nutrient media mix was placed in an

incubator shaker (see Figure 3.3) at 37 °C and 160 rpm for 24 hrs. Thereafter, the cultivated biodemulsifier was used for demulsification tests at various temperatures (37 °C, 47 °C, 57 °C and 67 °C), and 10 v/v% of the biodemulsifier was added to the simulated emulsion. The experiment was conducted in triplicate, in order to minimise the result errors.

4. Effect of carbon source on the produced biodemulsifier

4.1. Introduction

Biosurfactants have been a topic of interest in recent years, due to their application in various industries (Huang et al., 2014; Huang et al., 2016b). It has been shown that biosurfactant production is largely dependent on the available substrate in the microbial environment, as a variety of microorganisms possess the ability to adapt to different substrates found in their environment (Das, 2001; Haghighat et al., 2008; Liu et al., 2011a).

Even though a combination of substrates and conditions significantly affect the demulsifying capabilities of a strain, growth medium carbon sources are known to play an even more significant role, due to their ability to change the cell surface composition (Liu et al., 2011b; Huang et al., 2014; Li et al., 2012). An *Alcaligenes* sp. S-XJ-1 strain has been reported to grow on various carbon sources under the same conditions (Liu et al., 2010). Although the microbes were grown on the same concentration of carbon sources, it was shown that biodemulsifying cells produced from the hydrophobic carbon source had the highest demulsifiying ratio (Liu et al., 2010).

A different study with a similar strain (*Alcaligenes* sp. S-XJ-1) investigated the effects of waste frying oil (WFO) and/or paraffin as carbon sources for the production of a biodemulsifier (Liu et al., 2009). The study found that the WFO was not a suitable carbon source for the production of a biodemulsifier; furthermore WFO did not make any significant contributions to the biodemulsifier capability when combined with paraffin, whilst paraffin as a carbon source produced a biodemulsifier with a high demulsification ratio. It was concluded that the alkanes of the paraffin were difficult for the microorganism to utilize, thus promoting the cell surface changes of the cells in order that they survive in the hydrophobic conditions. This resulted with a biodemulsifier that possessed properties which enabled it to separate the emulsion (Liu et al., 2011b).

However, it was found that some carbon sources, despite poor demulsifying capabilities, supported cell biomass compared to hydrophobic carbon sources. This higher demulsifying ratio by hydrophobic carbon source is attributed to the fact that its presence affects the microbial cell surface composition (Liu et al., 2011b; Huang et al., 2014; Nadarajah et al., 2002).

It has been shown that cells grown on hydrophilic carbon sources (e.g. glucose) were found to contain a higher oxygen concentration, while the cells grown on the hydrophobic carbon source (e.g. paraffin) contained elemental nitrogen and functional groups (See Table 4.1) (Liu et al., 2011b; Huang et al., 2014). Additionally, fewer carbonyl groups and increased elemental nitrogen was proposed to contribute positively to the biodemulsifying capability of

a biodemulsifier. Furthermore, the hydrophilic carbon source cell surfaces contained polysaccharides whereas the hydrophobic carbon source cell surfaces had proteins or peptides. It can thus be concluded that the biodemulsifying capability of cultivated cells is significantly affected by the cell surface composition (Liu et al., 2010).

Table 4.1: Effect of carbon source on the cell surface composition

Carbon source	Outcome	%DE ratio
Alkane	• The produced cells contained abundant elemental nitrogen and basic functional group. • The cell surface was rich in proteins or peptides	91%
Fatty acid ester	• The produced cells had relatively abundant surface lipid • The cell surface contained more acidic functional group	53%
Carbohydrate	• The produced cells had a high oxygen concentration ($^{CO}/_{C} \sim 0{,}28$) • The cell surface contained more polysaccharides	31%

Sources: (Huang et al., 2014; Liu et al., 2011b)

The majority of the strains found to have demulsifying capabilities thrived when cultivated with hydrophobic carbon source (e.g. cetane, tetradecane, crude oil, kerosene, etc.). However, there seems to be a carbon source preference for different strains. Subsequently, an effective carbon source ought to have the ability to contribute to the modification of the cell surface towards the promotion of biodemulsification (Liu et al., 2011b; Huang et al., 2014).

Biodemulsifiers are advantageous over chemical demulsifiers due to their amphiphilic nature, environmental friendliness, ability to adapt under harsh conditions and biodegradability. Although the fact that hydrophobic carbon sources tend to be more expensive than soluble carbon sources might be a disadvantage (Liu et al., 2011b), the environmental benefit may outweigh this especially if a greener process were to be designed. Therefore, for industrial application of biodemulsifiers to be attractive, it is important to encourage research and developmental studies that will identify the significant parameters that would facilitate the feasibility and sustainability of biodemulsifier production (Liu et al., 2011b; Huang et al., 2014; Huang et al., 2016b).

4.2. Experimental procedure

Six different carbon sources were used to cultivate *B.licheniformis* STK 01 biodemulsifiers that were then used to demulsify simulated oil-water emulsions. The simulated oil-water

emulsion was produced by mixing surfactants Tween 60 and Span 60 with paraffin and water (refer to Section 3.3.6). The mixture was then centrifuged which resulted in a simulated emulsion (see Figure 3.5 A). The biodemulsification ratio of the different biodemulsifiers was measured using Equation 2.3. The materials and methods of this procedure are outlined in Chapter 3.3.

4.3. Results and discussion

4.3.1. Effect of insoluble carbon sources

The effect of insoluble carbon sources (also known as hydrophobic carbon sources) on the biodemulsification capability of *B.licheniformis* STK 01 was investigated. The insoluble carbon sources investigated were motor oil, diesel and paraffin. The results show that motor oil was the most suitable insoluble carbon source compared to diesel and paraffin. Diesel and paraffin cultivated cells did however possess biodemulsification capabilities.

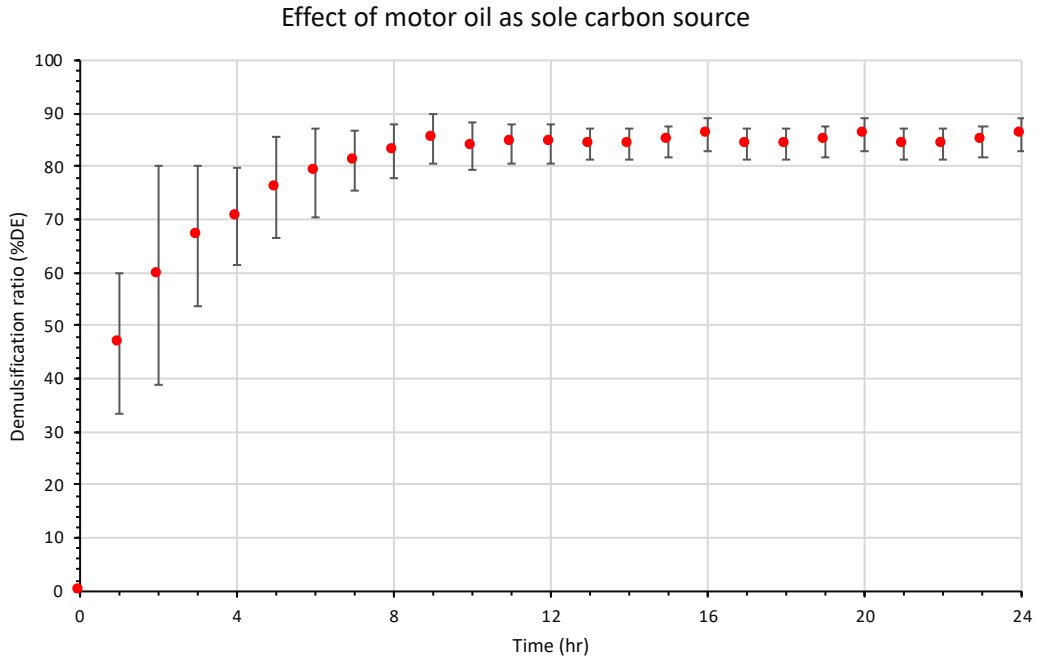

Figure 4.1: Demulsification ratio of emulsion by *B.licheniformis* STK 01 biodemulsifier produced using motor oil carbon source

Figure 4.1 shows the trend of biodemulsification simulated emulsion (paraffin and water, see Chapter 3.3.6) using motor oil cultivated biodemulsifer. The demulsification ratio increased exponentially, approaching a maximum value of 85.2% at the 9th hour. That is, the biodemulsifier exponentially demulsified the emulsion during the initial 9 hrs. The rate at which the biodemulsification occurred from there onwards was quite slow as the maximum value occurred between the 9th and 24th hrs, a period in which the biodemulsifier only

achieved an additional 0,9% of demulsification. Therefore, a 12 hour biodemulsification period can be assummed for maximum biodemulsification.

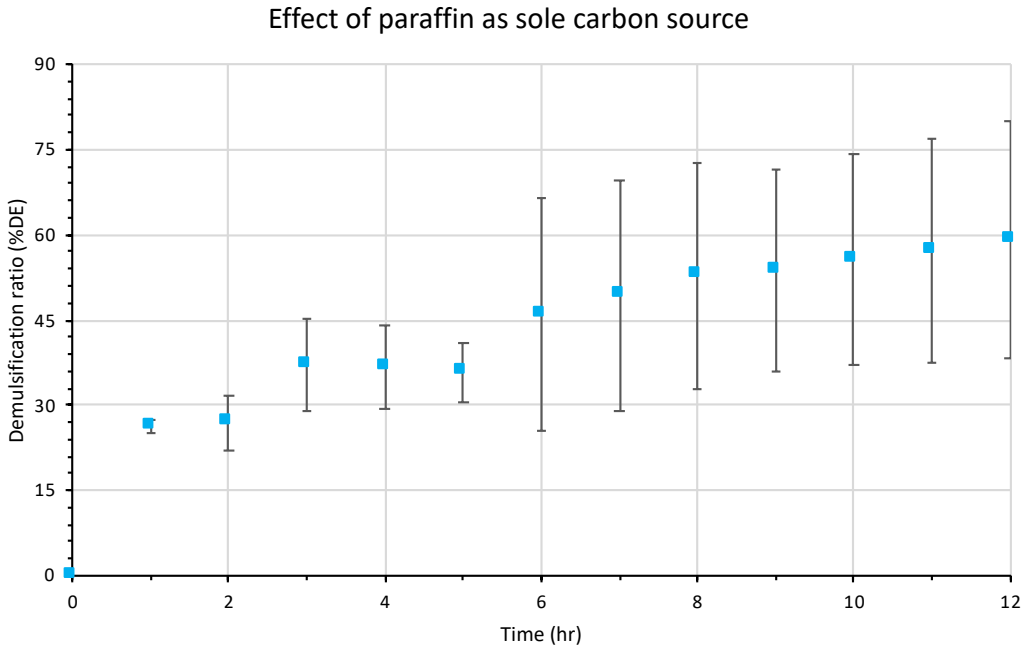

Figure 4.2: Demulsification ratio of emulsion by *B.licheniformis* STK 01 biodemulsifier produced using paraffin carbon source

Figure 4.2 and Figure 4.3 show similar trend as Figure 4.1 for biodemulsification simulated emulsion using parafin and diesel respectively, It was found that paraffin, diesel and motor oil cultivated cells were able to demulsify 61,9%, 73,7% and 86,1% respectively of the simulated emulsion within a 24 hr period.

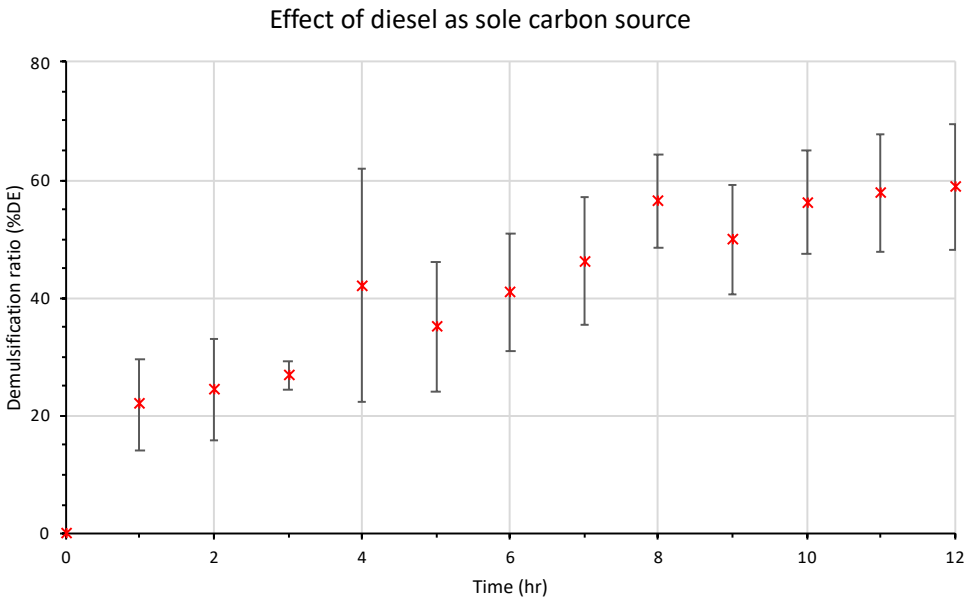

Figure 4.3: Demulsification ratio of emulsion by *B.licheniformis* STK 01 biodemulsifier produced using diesel carbon source

It can also be observed that beyond the 12th hour, the demulsification ratio increases marginally by 2,8 and 14,8% for paraffin and diesel as carbon source respectively. The effect of these insoluble carbon sources is quite clear in Figure 4.4. It can be seen that the demulsification ratio increases with the insoluble carbon source in the following order:

Motor oil > Diesel > Paraffin

The results obtained in the case of paraffin, being the lowest performing hydrocarbon, is in contrast with the literature, as it has been reported to be the most suitable carbon source for a biodemulsifier (Hou et al., 2014a; Li et al., 2012; Liu et al., 2010). However, that conclusion was not far-fetched since paraffin cultivated cells were able to demulsify 59,1% of the emulsion within 12 hrs, without any optimisation studies implemented. This implies that there was still a possibility of obtaining a higher biodemulsification performance from paraffin through biodemulsifier production studies.

Interestingly, the performance sequence of the insoluble carbon sources is in line with their molecular weight, meaning that motor oil as the heaviest, produced the biodemulsifier that demulsified the emulsion the most within the specified period; whereas, paraffin is the lightest in terms of molecular weight, and produced the lowest performing biodemulsifier. Therefore, these results suggest that the more complex an insoluble carbon source, the higher the performance capability of the resultant biodemulsifier, which somewhat correlates with a study that concluded that crude oil was the most suitable carbon source for biosurfactant production (Haghighat et al., 2008), crude oil being a complex combination of hydrocarbons.

Motor oil cultivated biodemulsifier was found to give the highest demulsification value over the experimental period. The control experiment (see Figure 4.4) gradually destabilised over the 12 hr period to a %DE value of 6,3%, eventually reaching a %DE value of 12,7% over a 24 hr period, which may be assumed to be insignificant in this context, since the treated samples all achieved a biodemulsification ratio of more than 60% within 24 hrs.

Figure 4.4 shows a closer look at the biodemulsification trend of the various insoluble carbon sources over a 12 hr period and that of the control sample. It depicts the biodemulsification achieved by the paraffin cultivated biodemulsifier which seems to initially follow an exponential trend during the initial 8hr period, during which 52,8% demulsification was achieved. This was subsequently followed by a more linear demulsification period till the 12 hr mark.

The diesel trend in Figure 4.4 shows the biodemulsification by the diesel cultivated biodemulsifier which also exponentially demulsified the emulsion during the initial 8 hr period to 56,4% demulsification. This then linearly demulsified an additional 2,5% of the emulsion during the remaining 4 hr period, a trend that seems to also be followed by the motor oil cultivated biodemulsifier shown in Figure 4.4. A demulsification value of 82,9% was

achieved during the initial 8 hr period which depicts an exponential period, followed by restitution period where only 1,4% of demulsification was achieved over a 4 hr period.

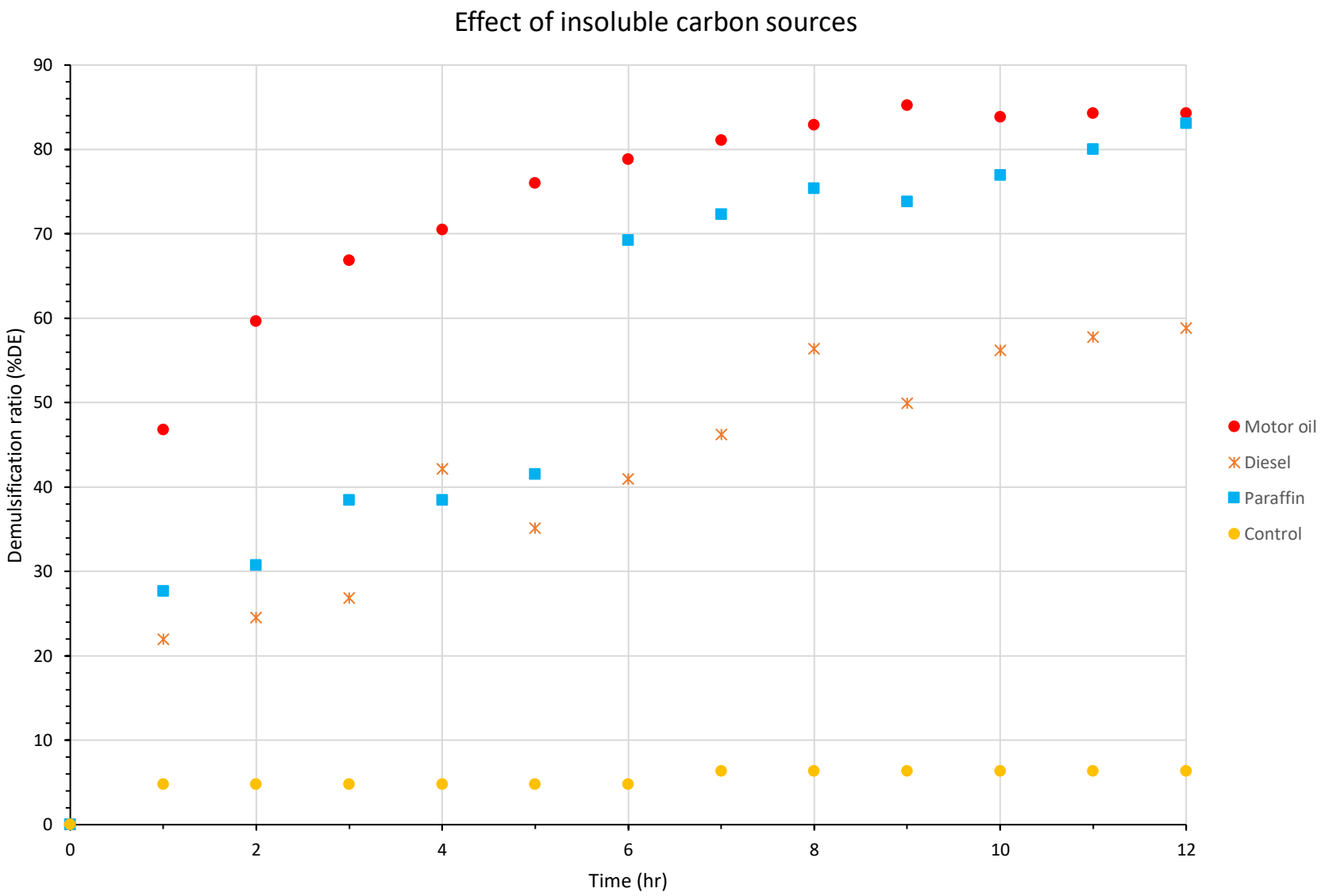

Figure 4.4: Effect of insoluble carbon source on demulsification ratio of emulsion by *B.licheniformis* STK 01 biodemulsifier

4.3.2. Effect of soluble carbon sources

The effect of soluble carbon sources on the biodemulsification capability of *B.licheniformis* STK 01 was investigated and the results showed that glucose was the most suitable soluble carbon source compared to fructose and sucrose. However, glucose only achieved a %DE of 46,4 within 12 hrs (see Figure 4.5), which is quite low compared to a study that found that glucose grown *Bacillus Mojavensis* biodemulsifier achieved a %DE of 60% (Li et al., 2012).

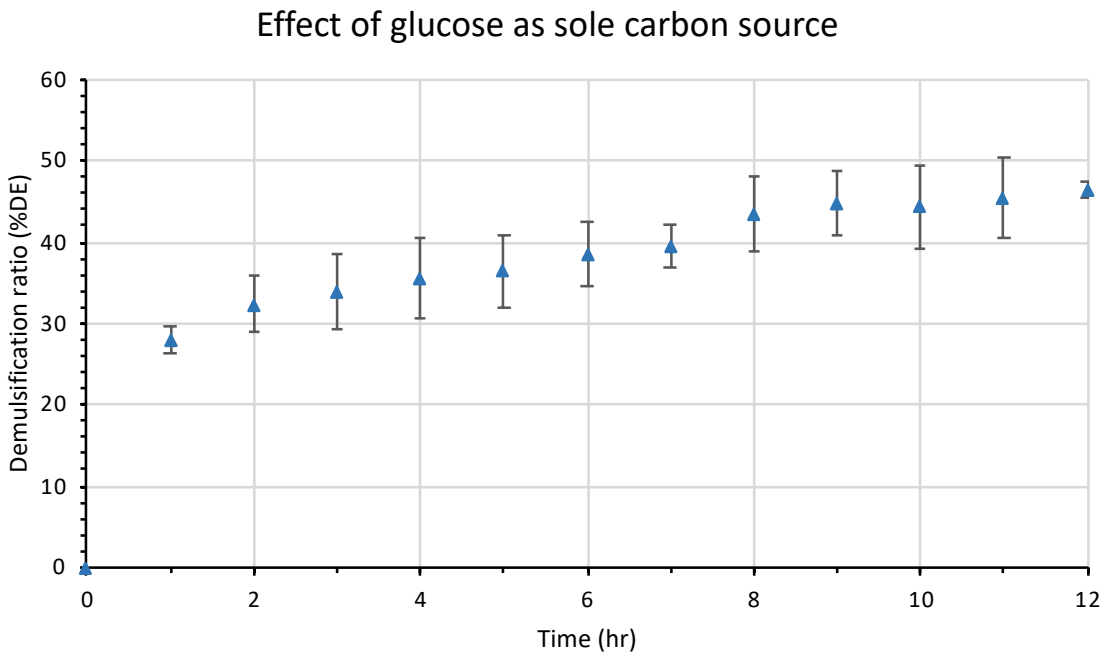

Figure 4.5: Demulsification ratio of emulsion by *B.licheniformis* STK 01 biodemulsifier produced using glucose carbon source

Fructose and sucrose achieved %DE values that were quite close, 45,1% and 44,7% respectively (see Figure 4.6 and Figure 4.7). Interestingly, glucose and fructose are both monosaccharides and both have a molecular mass of 180,16 g/mol, whereas sucrose is a disaccharide and has a molecular mass of 342,3 g/mol. However, there is a 7,8% difference between the achieved %DE of glucose and fructose, and a difference of only 0,4% between fructose and sucrose. Since glucose and fructose have the same amount of carbon, hydrogen and oxygen molecules, it can be assumed that the molecular structure affected the bioavailability of the carbon source to the cells.

The results show that soluble carbon sources could potentially be the preferred carbon sources if optimisation studies managed to give a higher %DE. Furthermore, they concur with literature that showed that cells grown on a soluble carbon source did possess some biodemulsification capabilities (Huang et al., 2014; Liu et al., 2011b), though not necessarily the best.

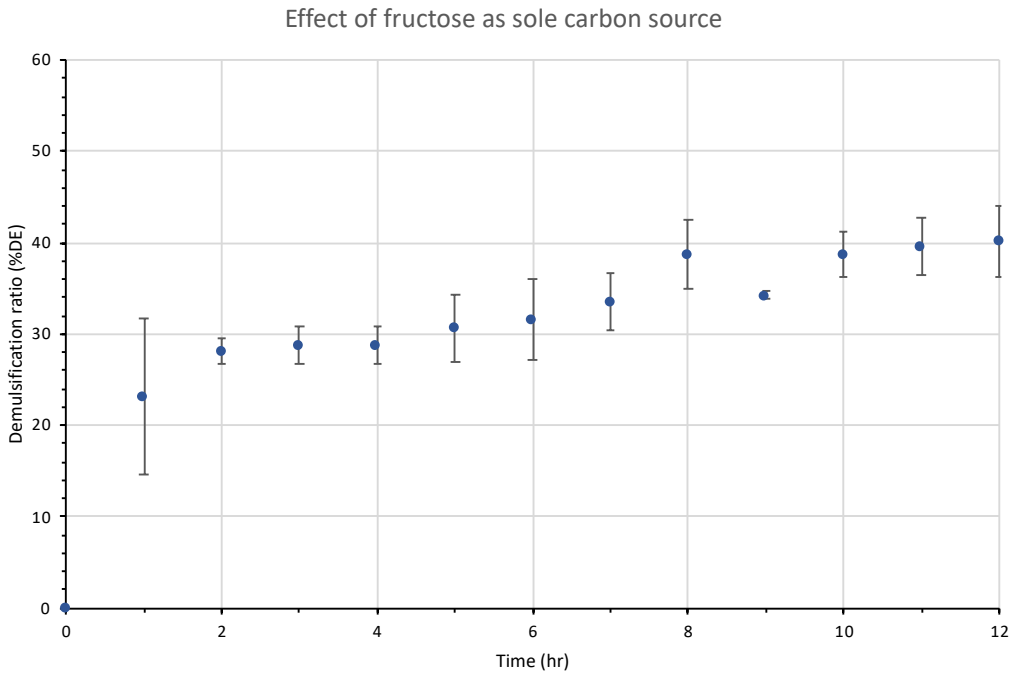

Figure 4.6: Demulsification ratio of emulsion by *B.licheniformis* STK 01 biodemulsifier produced using fructose carbon source

It also shows that there is probably a substance that is produced by the cells that does not fully promote biodemulsification capabilities such as polysaccharides (Huang et al., 2014; Liu et al., 2011b). Thus, a hydrophilic (soluble) carbon source in this case, essentially prohibited biodemulsification.

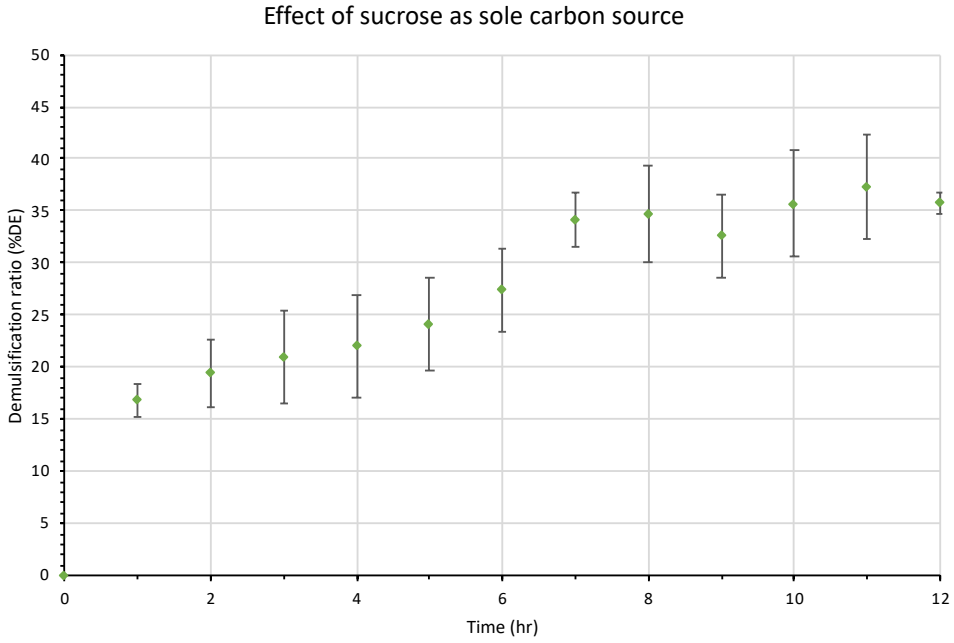

Figure 4.7: Demulsification ratio of emulsion by *B.licheniformis* STK 01 biodemulsifier produced using sucrose carbon source

Figure 4.8 shows the effect of the produced biodemulsifiers on the simulated emulsion samples and the simulated emulsion stability (control). It shows that once the simulated emulsions were exposed to the produced biodemulsifiers, there was exponential biodemulsification of the samples. The exponential biodemulsification occurred during the initial 8 hrs for both fructose and glucose and 7 hrs for sucrose, meaning that majority of the achieved biodemulsification was achieved within the mentioned periods.

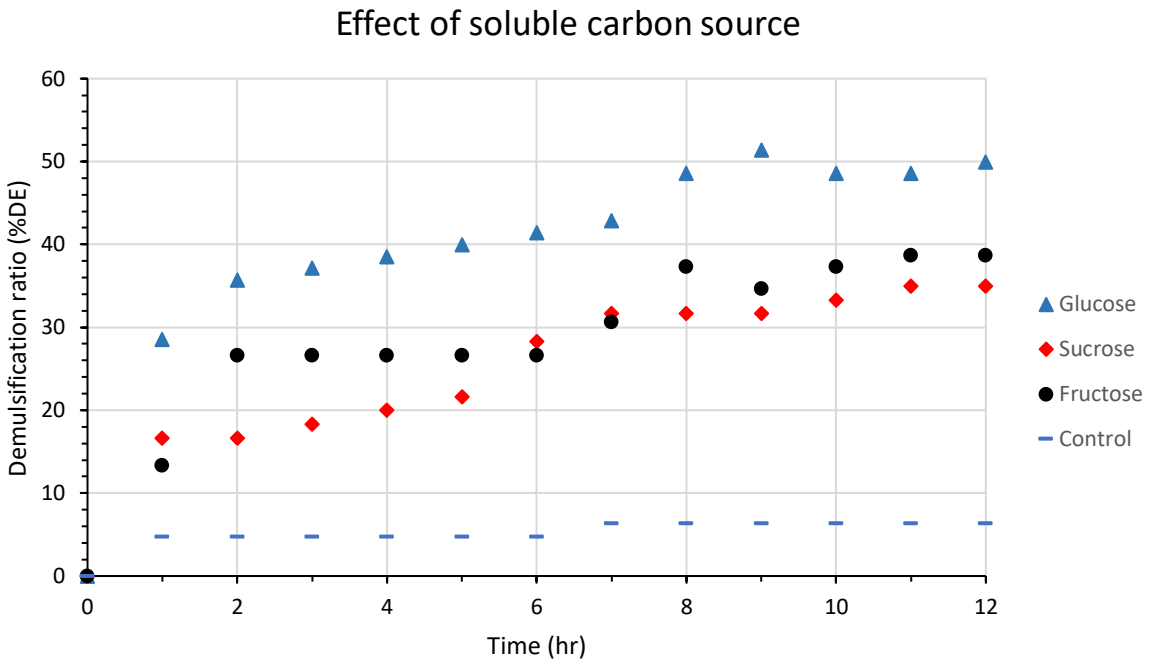

Figure 4.8: Effect of soluble carbon source on demulsification ratio of emulsion by *B.licheniformis* STK 01 biodemulsifier

Fructose-produced biodemulsifier exponentially demulsified the emulsion during the initial 8 hrs (38,7% demulsification ratio), accomplishing only 6,43% further demulsification within the remaining 16hrs, therefore, a restitution phase over the 8-12 hr period. Glucose-produced biodemulsifier followed a similar trend, achieving 43,4% demulsification within the initial 8hrs and a further 9,5% demulsification within the subsequent 16 hrs. This shows exponential demulsification of the simulated emulsion within the 8th hour.

Additionally, sucrose-produced biodemulsifier exponentially demulsified the simulated emulsion within 7 hrs, accomplishing only 34,2% demulsification. It further accomplished an additional 10,5% demulsification within the remaining 17hrs.

4.3.3. The effect of carbon sources

An investigation of the effect of carbon sources, namely diesel, motor oil, paraffin, glucose, fructose and sucrose, on the production and efficiency of a *B.licheniformis* STK 01 biodemulsifier was conducted. It was found that all cultivated cells possessed biodemulsification capabilities, though at varying efficiencies. The most efficient in this case

was the motor oil cultivated cells followed by diesel, paraffin, glucose, fructose then sucrose respectively (see Table 4.2). It is important to note that the same simulated emulsion was used in all the experiment so that a common basis can be established.

The results show a clear distinction between the achieved %DE of soluble and insoluble carbon sources. All soluble carbon sources achieved a %DE value of less than 53% over a 24 hr period whereas the insoluble carbon sources achieved a %DE of more than 61%. This showing a difference of about 9% between the highest soluble carbon source %DE achievement and the lowest insoluble carbon source %DE achieved value.

Table 4.2: %DE of *B.licheniformis* STK 01 grown on various carbon sources

Carbon source	**%DE – 24hrs**
Sucrose	44,7 ± 5,5
Fructose	45,1 ± 8,9
Glucose	52,9 ± 4,8
Paraffin	61,9 ± 15,7
Diesel	73,7 ± 3,1
Motor oil	86,1 ± 3,1

Results shown in Table 4.2 show the similar biodemulsification trend between the various cultivated biodemulsifiers. It further shows the extensive difference between the motor oil-cultivated biodemulsifier and all other cultivated biodemulsifiers. The other biodemulsifiers were within the 44%-74% %DE range while motor oil biodemulsifier was well above 80%. However, they all seem to have exponentially demulsified the simulated emulsion during the ±8 hr period, thereafter followed by a slower demulsification period, referred to as the restitution period. The graph further shows that sucrose-cultivated cells had a slower start compared to those cultivated with fructose, however showing a steeper demulsification at the 5_{th} hr, then closely mimicking fructose biodemulsifier thereafter.

A comparison of the soluble and insoluble carbon source results shows that *B.licheniformis* STK 01 prefers insoluble carbon sources in order to attain biodemulsification capabilities, hence confirming the conclusions of various other studies that found that microorganisms with biodemulsification capabilities had a tendency to require hydrocarbon substrates. Furthermore, the results concurred with studies that found that hydrophilic carbon sources inhibited the production of biodemulsifiers (Liu et al., 2010; Mohebali et al., 2012), since the glucose, sucrose, and fructose grown cells did have some extent of biodemulsifier capabilities which were quite low compared to that of hydrophobic carbon sources. It can be concluded that hydrophilic carbon sources do not fully inhibit the production of

biodemulsfiers but do however contribute negatively to the production of an efficient biodemulsifier.

The results clearly show that microorganism biodemulsification production is affected by the growth medium nutrients that it is exposed to. Additionally, that the performance of a biosurfactant, whether intra- or extracellular, is significantly affected by the nutrients, more especially the carbon source that it is exposed to during the growth/incubation period. This correlates with the stance taken in literature that the biodemulsification capability is significantly affected by the composition of the growth media (Coutinho et al., 2013).

It is deduced from literature that since hydrophobic carbon sources have higher biodemulsification values, the cells probably produce proteins or peptides such as lichenysin, a biosurfactant known to be produced by *B.licheniformis* strains. The presence of such proteins and peptides has been known to enhance the biodemulsifying capability of a strain by increasing the CSH which promotes the adsorption of the cell to the oil-water interface (Doran, 2013; Haghighat et al., 2008; Huang et al., 2009; Huang et al., 2010b). However, this is to be further investigated.

Although all cells cultivated on the various carbon sources did possess biodemulsification capability, it was obvious that the insoluble carbon sources were the most suitable. The hydrophobic carbon sources contributed more positively to the production of a *B.licheniformis* STK 01 biodemulsifier. This correlates with literature which reported the hydrophobic carbon source preference for biodemulsifier production (Liu et al., 2010; Liu et al., 2011b) and contradicts literature which states that glucose is the best suitable carbon source (Li et al., 2012).

Lastly, despite the low %DE values of the produced *B.licheniformis* STK 01 biodemulsifiers for some of the carbon sources, it shows that the *B.licheniformis* cells are capable of consuming different types of carbon sources (Haghighat et al., 2008). Therefore, the strain can be concluded as a biodemulsifying strain.

4.4. Summary

In this study, the effect of various carbon sources for the production of a *B.licheniformis* STK 01 biodemulsifier was investigated. The objective was to determine the most suitable carbon source that would achieve efficient biodemulsification capability of the produced biodemulsifiers. The results show that carbon sources do play a significant role in the production of biodemulsifier, and it was observed that carbon sources did produce biodemulsifiers that were able to demulsify an emulsion at varying efficacies. The insoluble carbon source produced better performing biodemulsifiers compared to those cultivated with soluble carbon sources. Motor oil was found to be the most suitable carbon source for the production of a biodemulsifier followed by diesel, paraffin, glucose, fructose and sucrose

respectively. Glucose was most suitable among the soluble carbon sources since it is known to be the most commonly used industrial carbon source.

Furthermore, the results showed that the heaviest (by molecular weight) insoluble carbon source produced the highest performing biodemulsifier whilst the lightest insoluble carbon source produced the lowest performing biodemulsifier. Thus, it was concluded that the carbon source complexity affected the bioavailability of the substrate, in turn affecting the performance of the biodemulsifier produced.

Lastly it was found that there was a clear distinction between the biodemulsifiers produced by soluble carbon sources compared to those produced by insoluble carbon sources, showing that the growth media composition, more especially carbon sources, significantly affected the ability of the produced biodemulsifier. Thus, hydrocarbons were concluded to be the most suitable carbon sources for the production of biodemulsifiers, with motor oil being the most efficient.

5. Investigation of *B.licheniformis* STK 01 biodemulsifier kinetics

5.1. Introduction

Biodemulsification is the use of microorganism and/or biological products for the separation of oil from oil/water emulsions. The concept is gaining recognition because of the appealing properties of biosurfactants such as environmental friendliness. However, it is yet to be fully implemented due to the limited existing studies. Although various studies on biodemulsification have been done, only certain topics have been covered so far. These topics include the characterization of demulsifying microorganisms, optimisation of biodemulsifier performance and/or production, interfacial properties, biodemulsifier production kinetics, etc.

However, there are some publications that have investigated the half-life (which they also referred to as the demulsification speed) of the demulsification of emulsion by strains such as *Micrococcus* sp. cultivated with tetradecane. The half-life of the biodemulsification process was determined and it was concluded that all treated (washed with n-pentane, n-hexane, kerosene or chloroform-methanol-water) cells followed first order kinetics. However, that is as far as they went regarding the kinetics, as further elaboration was not divulged (Das, 2001).

Another study investigated the biodemulsification of both W-O and O-W emulsions by *Pseudomonas Aeruginosa* MSJ cultivated by various carbon sources and the physiological characteristics. Including the effect of the physiological characteristics on the biodemulsification ability. It determined the half-lives of the various biodemulsifications and it was also concluded that both the cultivated cells and the supernatants (biosurfactants) followed first order kinetics (Coutinho et al., 2013).

A different study investigated the optimised production medium for the *Bacillus Mojavensis* XH1 biodemulsifier and further characterised the produced biodemulsifier. The study managed to determine and improve the half-life of the biodemulsification process by cultivated *Bacillus Mojavensis* XH1 biodemulsifier (Li et al., 2012). A similar study was also undertaken but it pursued the optimisation studies of the cultivation conditions of *Alcaligenes* sp. S-XJ-1 biodemulsifier. Their aim was to optimise the biodemulsifier production yield. The study also determined the half-life of the biodemulsification process (Liu et al., 2010).

The core fundamentals of the biodemulsification process are yet to be fully comprehended. Therefore, the question still remains, what is the rate of biodemulsification. It is not clear whether hydrophobicity or hydrophilicity of various carbon substrates will affect their biodemulsification rates. Kinetics of biodemulsification of these category of substrates would require further investigation as there is limited information regarding this subject. Results of

such studies would not only provide understanding of the kinetics but may also provide a basis for development of design of such process.

5.2. Experimental procedure

Motor oil was used to cultivate *B.licheniformis* STK 01 biodemulsifier that was then used to demulsify simulated oil-water emulsions. The simulated oil-water emulsion was produced as described in Section 3.3.6. The biodemulsification test was conducted at four different temperatures, in order to investigate the temperature effect, namely 37 °C, 47 °C, 57 °C and 67 °C. The biodemulsification ratio of the biodemulsifier was measured as described in Section 3.3.7 and the materials and methods of this procedure are outlined in Chapter 3.3.

Stoichiometric calculations were done on the experimental results obtained. With the aid of Solver, the numbers of moles of the emulsion(n_a), oil (n_b) and water (n_c) were calculated (refer to Chapter 7.1). These were then used to calculate the various concentrations and find the rate of reaction.

Table 5.1: Reaction orders and equations used for integral method data analysis

Name	Reaction	Equation
Zero order		$C_{A0} - C_A = kt$
1st order	$A \Rightarrow products$	$-ln(C_A/C_{A0}) = kt$
2nd order	$A + B \Rightarrow products$ $2A \Rightarrow products$	$ln\left(\frac{C_B C_{A0}}{C_{B0} C_A}\right) = (C_{B0} - C_{A0})kt$
		$ln\left(\frac{C_B}{C_A}\right) = (C_{B0} - C_{A0})kt + \left(\frac{C_{B0}}{C_{A0}}\right)$
		$\frac{1}{C_A} = kt + \frac{1}{C_{A0}}$
3rd order	$A + B + D \Rightarrow products$	$\frac{(2C_{A0} - C_{B0})(C_{B0} - C_B)}{C_{B0} C_B} + ln\left(\frac{C_{A0} C_B}{C_A C_{B0}}\right) = (2C_{A0} - C_{B0})^2 kt$
		$\frac{1}{{C_A}^2} = 8kt + \frac{1}{{C_{A0}}^2}$
		$\frac{1}{{C_A}^2} = 2kt + \frac{1}{{C_{A0}}^2}$

Source: (Levenspiel, 1999)

The trial-and-error method was then applied to fit the concentration/time values to known reaction order plots (see Table 5.1), which is generally referred to as the integral method of analysis. A method applicable where the rate of reaction is a function of the concentration of a reactant and quite useful in obtaining rate constants. The resulting reaction order was then

verified using the polynomial fit method (differential method of analysis). The rate parameters were then determined.

5.3. Results and discussion

5.3.1. Kinetics of *B.licheniformis* STK 01 biodemulsifier produced with soluble carbon sources

Sucrose-cultivated *B.licheniformis* STK 01 biodemulsifier were found to be the least performing biodemulsifier. The biodemulsifier achieved a biodemulsification ratio of 44,7% within a 24 hr period, which is nearly half of the 86,1% achieved by the motor oil-cultivated biodemulsifier. The kinetics of the sucrose-cultivated biodemulsifier was investigated and it was found that the integral method endorsed the first order kinetics (see Figure 7.1 in Appendix).

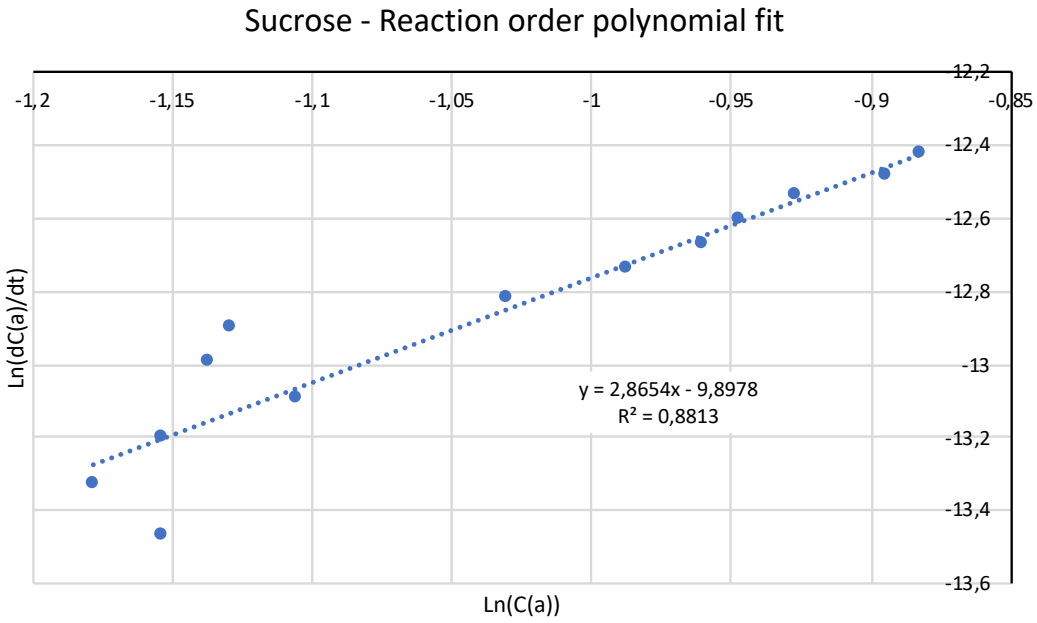

Figure 5.1: Sucrose-cultivated *B.licheniformis* STK 01 biodemulsifier kinetics

It was however concluded that based on the polynomial fit, the sucrose-cultivated biodemulsifier adhered to third order kinetics as shown in Figure 5.1. Therefore, the specific biodemulsification rate constant k_s is $5{,}029 \times 10^{-5}\ dm^6/mol^2 s$. The rate of biodemulsification ($-r_s$) by the sucrose-cultivated *B.licheniformis* STK 01 biodemulsifier is represented by Equation 5.1.

$$-r_s = -\frac{dC_a}{dt} = 5{,}029 \times 10^{-5}\ dm^6/mol^2 s \times {C_a}^3$$

Equation 5.1: Rate of biodemulsification of sucrose-cultivated *B.licheniformis* STK 01 biodemulsifier

Fructose-cultivated *B.licheniformis* STK 01 biodemulsifier kinetics were investigated. It was found that according to the polynomial method, the data seemed to follow a fifth order trend,

however with a lower R^2 value of 76,8% (see Figure 7.2 in Appendix A). It was therefore concluded according to the integral method alone that the biodemulsifier adhered to third order kinetics, where the R^2 was 91,8% (see Figure 5.2).

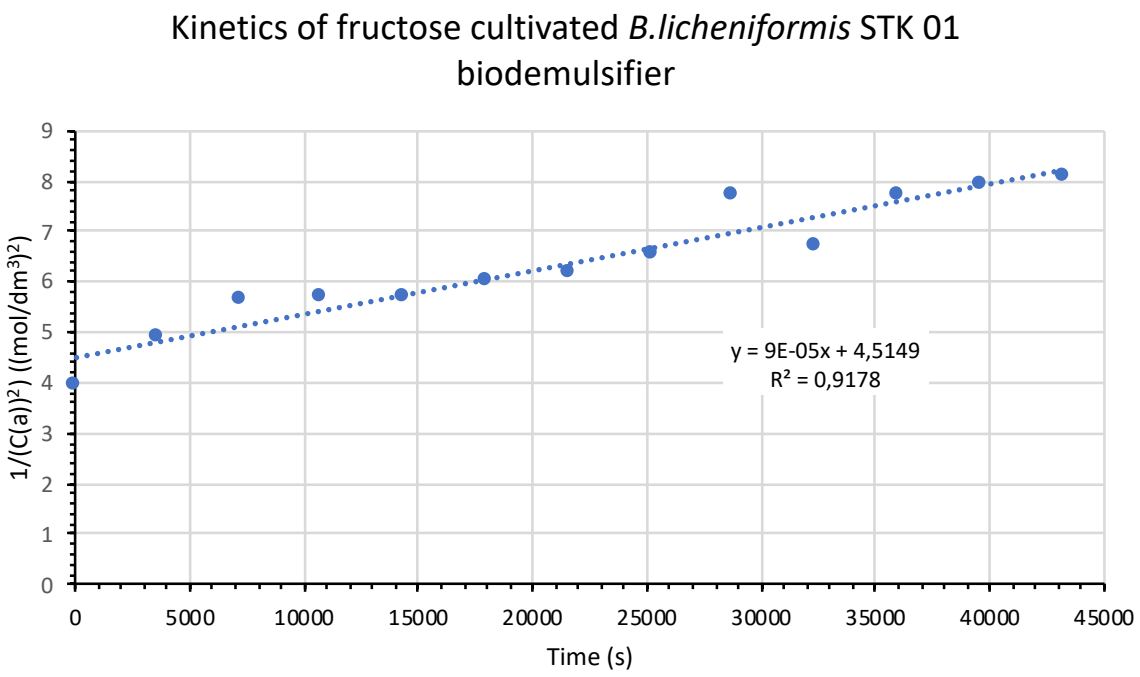

Figure 5.2: Kinetics of fructose-cultivated *B.licheniformis* STK 01 biodemulsifier

Thus, like sucrose-cultivated biodemulsifier, the rate of biodemulsification $(-r_f)$ by fructose-cultivated biodemulsifier is according to the following equation. Where the specific rate of biodemulsification constant (k_f) is $9 \times 10^{-5}\ dm^6/mol^2 s$.

$$-r_f = 9 \times 10^{-5}\ dm^6/mol^2 s \times {C_a}^3$$

Equation 5.2: Rate of biodemulsification of fructose-cultivated *B.licheniformis* STK 01 biodemulsifier

Furthermore, the kinetics of the glucose-cultivated *B.licheniformis* STK 01 biodemulsifier was investigated and it was not possible to correlate the integral method with polynomial fit. According to the polynomial fit, the data were of seventh order kinetics, although the linear trend line had an even lower R^2 of 53,4%, thus an error of +40% (see Figure 7.3 in Appendix A).

It was then concluded based on only the integral method that the biodemulsifier obeyed third order kinetics, with an R^2 value of 95% (see Figure 5.3). Therefore, the specific rate of biodemulsification constant (k_g) is $10 \times 10^{-5}\ dm^6/mol^2 s$ and the rate of biodemulsification is as described by Equation 5.3.

$$-r_g = 10 \times 10^{-5}\ dm^6/mol^2 s \times {C_a}^3$$

Equation 5.3: Rate of biodemulsification of glucose-cultivated *B.licheniformis* STK 01 biodemulsifier

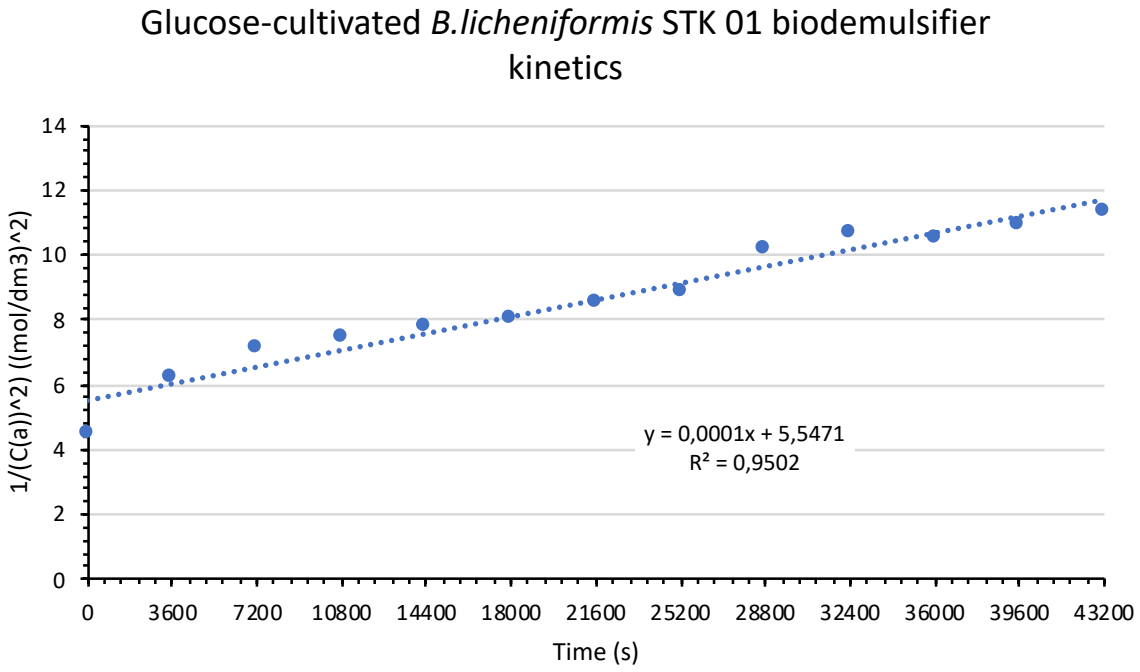

Figure 5.3: Glucose-cultivated *B.licheniformis* STK 01 biodemulsifier kinetics

It is therefore concluded that all studied soluble carbon sources in this case adhered to third order kinetics, which does not correlate with existing literature that has found that biodemulsification follows first order kinetics. Furthermore, this is not aligned with the results of the insoluble carbon sources (see Section 5.3.2) that were studied in this case, which were found to comply with first order kinetics (Coutinho et al., 2013; Das, 2001).

5.3.2. Kinetics of *B.licheniformis* STK 01 biodemulsifier produced with insoluble carbon sources

The diesel-cultivated biodemulsifier showed good biodemulsification capability, achieving 73,7% of biodemulsification within 24 hrs. With the trial and error method, a plot of $\ln\frac{C_a}{C_{a0}}$ vs t gave a linear trend line with an R^2 of 94%, thus endorsing first order kinetics (see Figure 5.4 below). However, a plot of $\frac{1}{C_a}$ vs t also gave a linear trend line with an R^2 of 93,3% (see Figure 7.4 in Appendix A) commending second order kinetics, which was quite close to the latter. The polynomial method was then applied, and it was found that the first order polynomial plot had a higher R^2 of 90,5% (see Figure 7.5 in Appendix A).

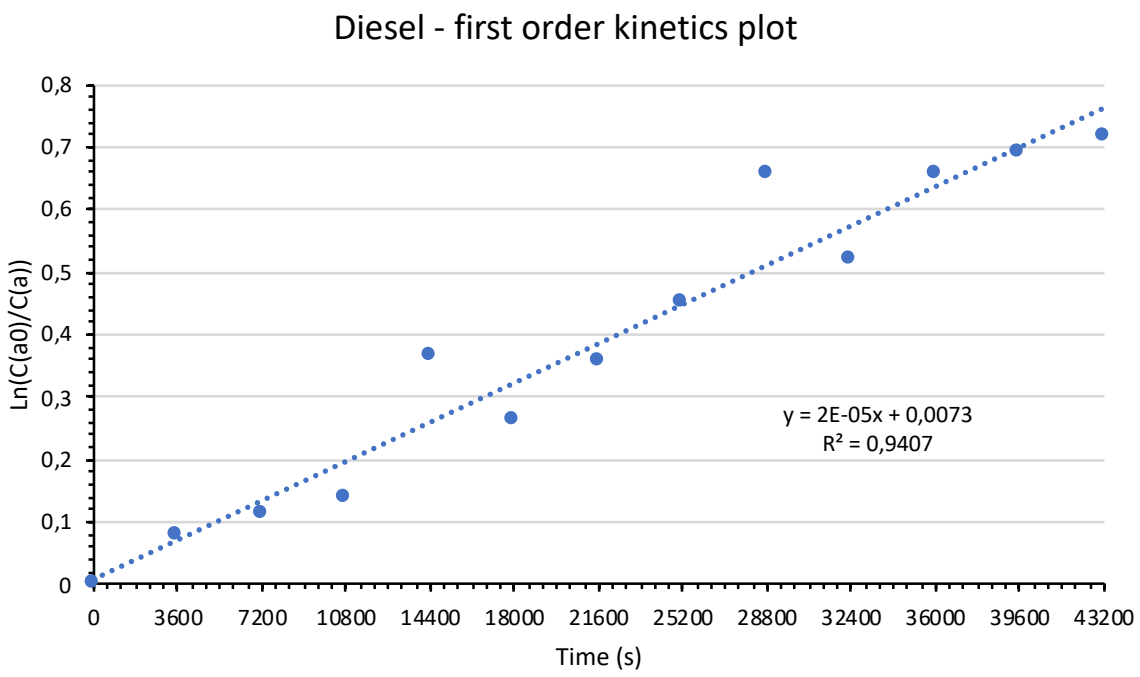

Figure 5.4: Kinetics of diesel-cultivated *B.licheniformis* STK 01 biodemulsifier

It was therefore concluded that the diesel-cultivated *B.licheniformis* STK 01 biodemulsifier followed first order kinetics. Thus the rate constant (k_d) for the rate of biodemulsification by *B.licheniformis* STK 01 biodemulsifier is $2{,}447 \times 10^{-5}\ s^{-1}$ (see Table 5.2). And the rate of biodemulsification $(-r_d)$ by diesel-cultivated biodemulsifier is according to Equation 5.4.

$$-r_d = 2{,}447 \times 10^{-5}\ s^{-1} \times C_a$$

Equation 5.4: Rate of biodemulsification of diesel-cultivated *B.licheniformis* STK 01 biodemulsifier

Paraffin-cultivated *B.licheniformis* STK 01 biodemulsifier was found to be the lowest performing of the studied insoluble carbon sources. It only achieved a biodemulsification ratio of 61,9% within a 24 hr period. Paraffin-cultivated *B.licheniformis* STK 01 biodemulsifier kinetics were investigated. The integral method was applied, and a first order kinetics fit was obtained with an R^2 of 96,4%, second order fit with an R^2 of 97,7% and a third order fit with an R^2 of 97,4% (see Figure 7.6 and Figure 7.7 and Figure 7.8 in Appendix A) thus endorsing that the biodemulsifier followed second order kinetics.

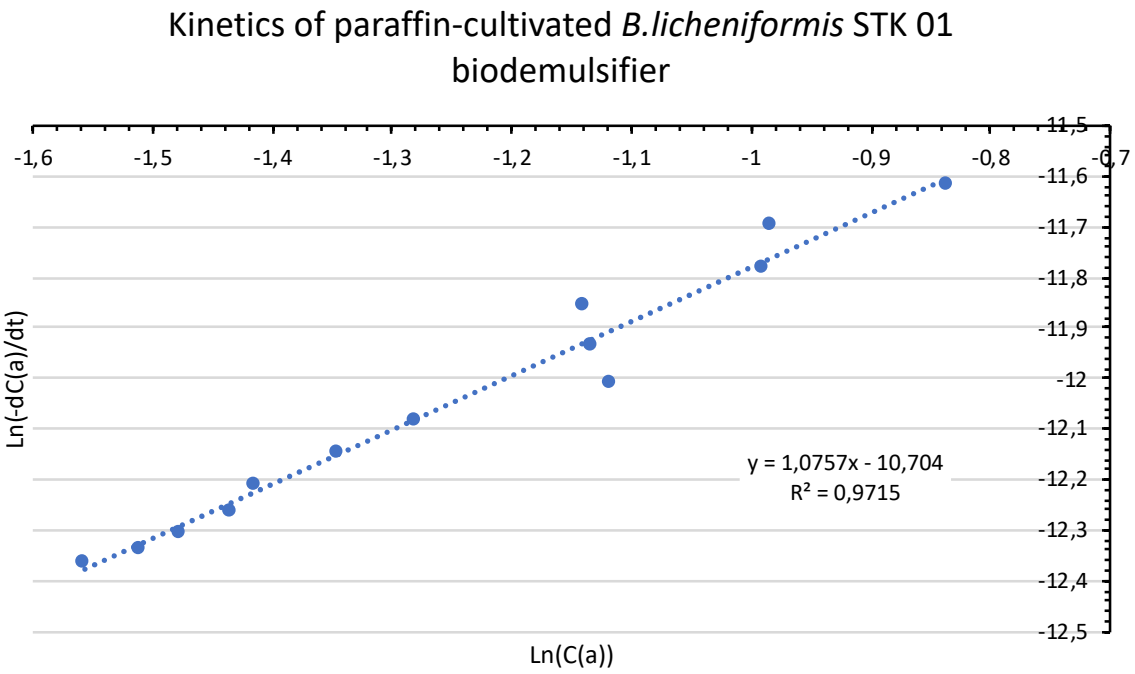

Figure 5.5: Kinetics of paraffin-cultivated *B.licheniformis* STK 01 biodemulsifier

However, a polynomial fit was done, and it confirmed first order kinetics with an R^2 of 97,2% (see Figure 5.5). It was therefore confirmed that the biodemulsifier follows first order kinetics and it is represented by the rate of biodemulsification below Equation 5.5, with a specific biodemulsification of rate constant (k_p) of $2{,}245 \times 10^{-5}\ s^{-1}$.

$$-r_p = 2{,}245 \times 10^{-5}\ s^{-1} \times C_a$$

Equation 5.5: Rate of biodemulsification of paraffin-cultivated *B.Licheniformis* STK 01 biodemulsifier

Motor oil-cultivated *B.licheniformis* STK 01 biodemulsifier was found to be the most suitable biodemulsifier due to its 86,1% demulsification ratio within a 24 hr period. The integral method (see Figure 7.9) and polynomial fit (R^2 of 94,2%) was applied to the experimental data and it was found that the *B.licheniformis* STK 01 biodemulsifier adheres to first order kinetics (see Figure 5.6). The specific biodemulsification rate constant (k_m) is $11{,}561 \times 10^{-5}\ s^{-1}$.

Since motor oil was found to be the most suitable carbon source and the produced biodemulsifier to be the most effective and best performing, it is therefore concluded that the rate of biodemulsification by *B.licheniformis* STK 01 biodemulsifier can be determined with the aid of Equation 5.6.

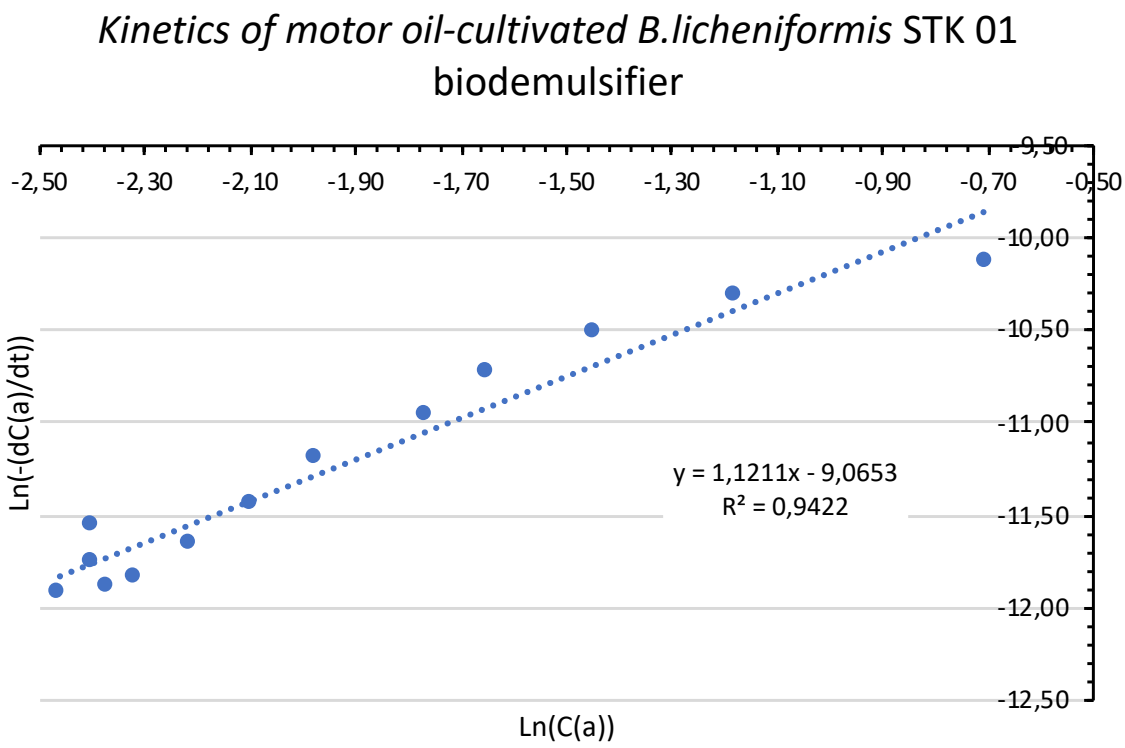

Figure 5.6: Kinetics of motor oil-cultivate *B.licheniformis STK 01* biodemulsifier

The finding that all *B.licheniformis* STK 01 biodemulsifiers cultivated on insoluble carbon sources followed first order kinetics correlates with the existing literature (Coutinho et al., 2013; Das, 2001, Li et al., 2012; Liu et al., 2010). Studies in existing literature have found that *Bacillus mojavensis* XH1 biodemulsifier, which belongs to the same genera as *B.licheniformis* STK 01, followed first order kinetics.

$$-r_m = 11{,}561 \times 10^{-5}\ s^{-1} \times C_a$$

Equation 5.6: Rate of biodemulsification of motor oil-cultivated *B.licheniformis* STK 01 biodemulsifier

The *Bacillus mojavensis* XH1 biodemulsifier was cultivated on a combination of glucose and paraffin carbon sources, whereas *B.licheniformis* STK 01 biodemulsifier was cultivated on diesel. However, this study has gone further and found that the specific biodemulsification rate constant for diesel, paraffin, and motor oil are as stated in Table 5.2.

Table 5.2: Insoluble carbon sources specific biodemulsification rate constants

Carbon source	**Specific Biodemulsification rate constant** (s^{-1})	
Diesel	k_d	$2{,}447 \times 10^{-5}$
Paraffin	k_p	$2{,}245 \times 10^{-5}$
Motor oil	k_m	$11{,}561 \times 10^{-5}$

5.3.3. The effect of temperature on the biodemulsification of emulsion by *B.licheniformis* STK 01 biodemulsifier

The effect of temperature on the biodemulsification process by *B.licheniformis* STK 01 biodemulsifier was investigated on the assumption that this can be studied independently. A plot of $\ln(k_m)$ and $\frac{1}{T_{abs}}$ should give a linear plot in order that it be defined by the Arrhenius equation.

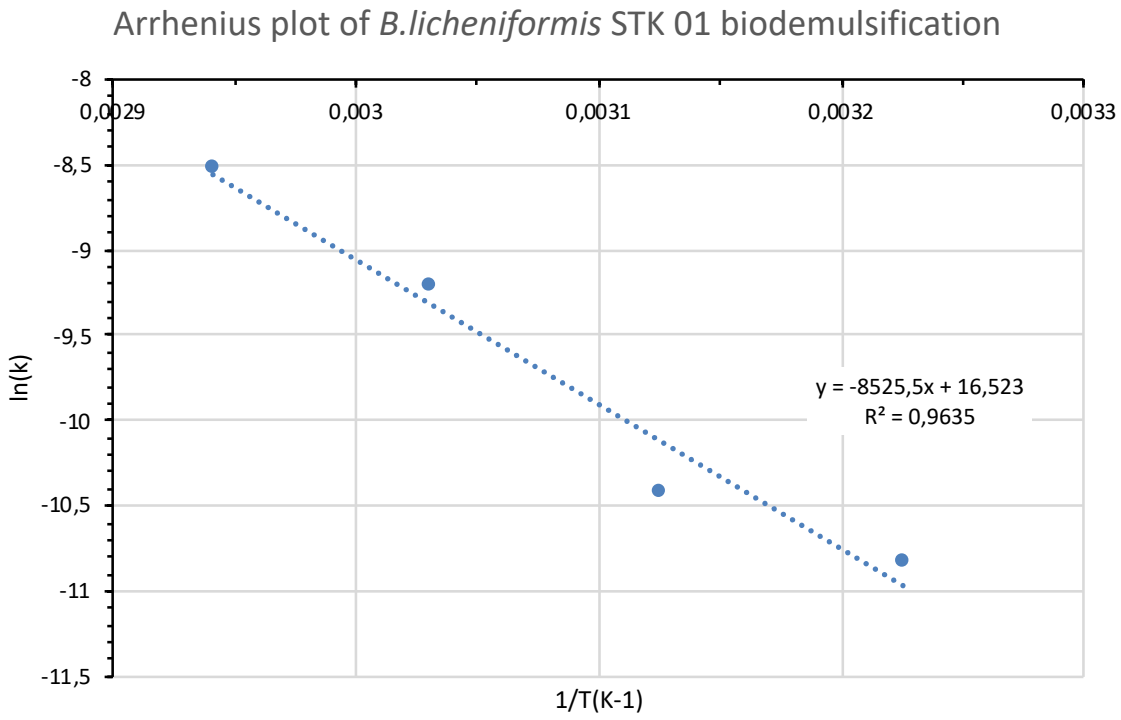

Figure 5.7: The effect of temperature on the rate of biodemulsification by *B.licheniformis STK 01* biodemulsifier

The relationship between the specific rate constant of biodemulsification (k_m) and temperature is defined by Equation 5.7 with an R^2 value of 96,3% as shown in Figure 5.7. It was then found that the activation energy (E) and frequency factor (A) of the biodemulsification reaction was 70,88 KJ/mol and 14× 10^6 s^{-1} respectively.

$$\ln(k_m) = 16{,}523 - 8525{,}5\frac{1}{T}$$

Equation 5.7: Relationship between the rate of biodemulsification and temperature

5.4. Summary

The kinetics of various *B.licheniformis* STK 01 biodemulsifiers cultivated with different carbon sources were investigated. It was found that all biodemulsifiers cultivated with soluble carbon sources, namely glucose, sucrose and fructose, adhered to third order kinetics. This contradicts literature that found that the biodemulsification reaction obeyed first

order kinetics. The specific biodemulsification rate constants of glucose (k_g), fructose (k_f) and sucrose (k_s) are $10\times 10^{-5}\ dm^6/mol^2s$, $9\times 10^{-5}\ dm^6/mol^2s$ and $5{,}029\times 10^{-5}\ dm^6/mol^2s$ respectively.

The results show that the studied insoluble carbon sources, namely motor oil, paraffin and diesel adhered to first order kinetics. This finding endorses existing literature which found that biodemulsification followed first order kinetics. Furthermore, the kinetic parameters were investigated, and it was established that the specific biodemulsification rate constants of motor oil (k_m), diesel (k_d) and paraffin (k_p) are $11{,}561\times 10^{-5}\ s^{-1}$, $2{,}447\times 10^{-5}\ s^{-1}$ and $2{,}245\times 10^{-5}\ s^{-1}$ respectively.

Lastly, the relationship between temperature and the rate of biodemulsification by *B.licheniformis* STK 01 biodemulsifier was established using the Arrhenius equation. It was determined that the value of the frequency factor (A) to be $14\times 10^{6}\ s^{-1}$ and that of the activation energy (E) to be 70,88 KJ/mol.

6. Conclusion and recommendations

6.1. General conclusions

The objective of the study was to investigate the effect that the carbon source has on the biodemulsification capability of the produced biodemulsifier of *B. Licheniformis* with a view to determine most suitable carbon source for the production of a *B.licheniformis* STK 01 biodemulsifier. Lastly, to investigate the kinetics of the biodemulsification reaction by *B.licheniformis* STK 01 biodemulsifier, which may be necessary in design of biodemulsification process.

This is not the first study on the topic of biodemulsification, however existing literature has mostly focused on biodemulsification capability, characterization of demulsifying microorganisms, biodemulsifier production kinetics, optimization, and physical and chemical properties. This leaves a gap for literature on the fundamental principles of the biodemulsification reaction, hence, the objective to investigate biodemulsification kinetics. Even though a few studies (Coutinho et al., 2013; Das, 2001) have stipulated the kinetics of the biodemulsification process, the studies were not detailed and failed to provide the kinetic parameters of the reaction. Hence literature still speculates on the mechanism that is followed by the biodemulsification process which is generally dictated by the kinetics and is yet to be concluded.

The results obtained showed that *B.licheniformis* STK 01 was able to consume various carbon sources and produce a biodemulsifier. Also, that the carbon source played a significant role in the biodemulsification capability of the produced biodemulsifier. The insoluble carbon sources were found to produce biodemulsifiers with a higher biodemulsification capability compared to those cultivated with soluble carbon sources. The results were quite distinct in that all biodemulsifiers cultivated with soluble carbon source achieved less than 53% of demulsification over a 24 hr period, while biodemulsifiers cultivated with insoluble carbon sources achieved demulsification ratios above 60% within the 24 hr period.

Motor oil-cultivated *B.licheniformis* STK 01 biodemulsifier was found to the most efficient biodemulsifier, achieving a biodemulsification ratio of 82,9% within 8hrs and 86,1% within a 24 hr period. Like all produced biodemulsifiers in this study, the biodemulsifier exponentially demulsified the emulsion within the initial 8hr period, then reached a period of restitution where low demulsification changes were observed. Furthermore, results showed that the more complex molecular structure positively contributed to the biodemulsification capability.

The biodemulsifiers produced with soluble carbon sources were found to adhere to third order kinetics, which contradicted existing literature, whilst biodemulsifiers cultivated with

insoluble carbon sources were confirmed to obey first order kinetics, correlating with existing literature. Furthermore, equations describing the rate of biodemulsification, the effect of the temperature on the biodemulsification rate and the specific rate of biodemulsification constants were determined. For motor oil-cultivated biodemulsifier, the specific rate of biodemulsification constant (k_m) is $11{,}561 \times 10^{-5}\ s^{-1}$ and the activation energy (E) is 70,88 KJ/mol.

6.2. Recommendations and future work

The study was aimed at investigating the effect of carbon source on the produced biodemulsifier and the biodemulsification kinetics. The scope of this study did not include all parameters that form part of the production of a biodemulsifier and the actual biodemulsification process. For this reason, the following are recommendations drawn from this study for future investigation.

- It is evident that *B.licheniformis* STK 01 is capable of producing an efficient biodemulsifier, and the assumption is that it produces lichenysin like other *B.licheniformis* strains about which it is not known if they are intra- or extracellular. Hence, a study on the type of biodemulsifier produced is recommended, in order to fully establish the kinetics study and eventually assist with the upscaling and production feasibility studies.
- A cultivated mixture of *B.licheniformis* STK 01 cells and biological products (biosurfactant, etc.) were used for the demulsification of the simulated emulsions. Thus, this study is unable to confirm whether the biodemulsifier was the actual cells or the biological products. A study on the actual biodemulsifying components is therefore recommended, to shed light on the mechanism of the biodemulsification process.
- The produced *B.licheniformis* STK 01 was able to achieve a relatively high demulsification ratio of 82,9% within 8hrs, albeit considering the disadvantage of high production cost. A high efficiency rate would be necessary in order to offset some of the disadvantages. It is then recommended that optimisation studies on the production of *B.licheniformis* STK 01 biodemulsifier be conducted.

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7. Appendices

7.1. Appendix A: Calculations

7.1.1. Hydrophilic-Lipophilic balance of emulsion

$$\%(A) = \frac{(HLB_{Oil} - HLB_B) \times 100}{HLB_A - HLB_B}$$

$$\%(Tween60) = \frac{(6 - 4{,}7) \times 100}{14{,}9 - 4{,}7}$$

$$\%(Tween60) = 12{,}74$$

Thus 0,1274 mL per 100 mL of water

$$\%(Span60) = 100 - 12{,}74 = 87{,}26$$

Thus 0,8726 g per 100 mL of oil

7.1.2. Biodemulsification capability

$$Biodemulsification\ ratio = \left(1 - \frac{V_t^E}{V_{t=0}^E + V_{t=0}^{CS}}\right) \times 100\%$$

$$\%DE = \left(1 - \frac{0{,}666\ \text{mL}}{5{,}5\ \text{mL} + 1\ \text{mL}}\right) \times 100\%$$

$$\%DE = 87{,}9\%$$

7.1.3. Solver calculations for motor oil samples

$$Emulsion \rightarrow Oil\ + Water$$

$$aA \rightarrow bB\ + cC$$

7.1.3.1. Mass balance

$$\boldsymbol{In = Out}$$

Therefore: $m_{A_i} = m_A + m_B + m_C$

Table 5.3: Stoichiometry equations applied

	aA (Emulsion) ⟶	bB (Oil)	cC (Water)
Initial	$m_{a0} = \rho_a V_{a0}$ $n_{a0} = \frac{m_{a0}}{M_a}$	$m_{\mathrm{b0}} = \rho_b V_{\mathrm{b0}}$ $n_{\mathrm{b0}} = \frac{m_{\mathrm{b0}}}{M_b}$	$m_{\mathrm{c0}} = \rho_c V_{\mathrm{c0}}$ $n_{\mathrm{c0}} = \frac{m_{\mathrm{c0}}}{M_c}$
Change	$-n_{a0}\mathrm{X}$	$+\frac{a}{b}n_{a0}\mathrm{X}$	$+\frac{a}{c}n_{a0}\mathrm{X}$
Final	$n_a = n_{a0}(1 - \mathrm{X})$ $C_a = \frac{n_a}{V_T}$	$n_b = \frac{a}{b}n_{a0}\mathrm{X}$ $C_b = \frac{n_b}{V_T}$	$n_c = \frac{a}{c}n_{a0}\mathrm{X}$ $C_c = \frac{n_c}{V_T}$

Oil (B):

Mass

$$m_b = \rho_b V_b$$

$$m_b = 0{,}845\ g/mL \times 3{,}73\ mL$$

$$m_b = 3{,}155\ g$$

No of Moles

$$n_b = \frac{m_b}{M_b}$$

$$n_b = \frac{3{,}155\ g}{338{,}4\ g/mol}$$

$$n_b = 9{,}32 \times 10^{-3}\ mol$$

Water (C):

Mass

$$m_c = \rho_c V_c$$

$$m_c = 0{,}998\ g/mL \times 1{,}3\ mL$$

$$m_c = 1{,}297\ g$$

Moles

$$n_c = \frac{m_c}{M_c}$$

$$n_c = \frac{1{,}297\ g}{18{,}02\ g/mol}$$

$$n_c = 7{,}20 \times 10^{-2}\ mol$$

Emulsion (A):

Conversion factor

$$X = 1 - \frac{V_{ai}}{V_a}$$

$$X = 1 - \frac{1{,}13\ mL}{6{,}17\ mL}$$

$$X = 0{,}816$$

7.1.3.2. Solver equations

Solve $a, b\ and\ c$, if n_{ai} is as follows:

$$n_{ai} = \frac{n_c a}{Xc}$$

$$n_{ai} = \frac{n_b a}{Xc}$$

$$a > 0$$

$$b > 0$$

$$c > 0$$

7.1.3.3. Molecular mass of emulsion

$$M_a = \frac{\mathrm{m}_a}{n_a}$$

$$M_a = \frac{5{,}455\ \mathrm{g}}{5{,}34 \times 10^{-3}\ mol}$$

$$M_a = 1020{,}95\ \mathrm{g/mol}$$

7.1.3.4. Concentration of emulsion

$$C_a = \frac{\mathrm{n}_a}{V_T}$$

$$C_a = \frac{9{,}82 \times 10^{-4}\ mol}{10{,}8\ ml}$$

$$C_a = 9{,}06 \times 10^{-4}\ mol/ml$$

7.1.4. Kinetics calculations

7.1.4.1. Arrhenius equation

$$\ln\,(k_m) = \ln\,(A) - \left(\frac{E}{R} \times \frac{1}{T}\right)$$

Linear equation obtained from the plot of $\ln\,(k_m)$ vs $\frac{1}{T}$

$$\ln\,(k_m) = 16{,}523 - 8525{,}5\frac{1}{T}$$

Thus:

7.1.4.2. Activation energy

$$\frac{E}{R} = 8525{,}5$$

$$E = 8525{,}5\ K \times 8{,}314\ J/molK = 70{,}88\ KJ/mol$$

7.1.4.3. Frequency factor

$$\ln(A) = 16{,}523$$

$$A = e^{16{,}523} = 14 \times 10^{6}\ s^{-1}$$

7.2. Appendix B: Supplementary data

7.2.1. Sucrose supplementary data

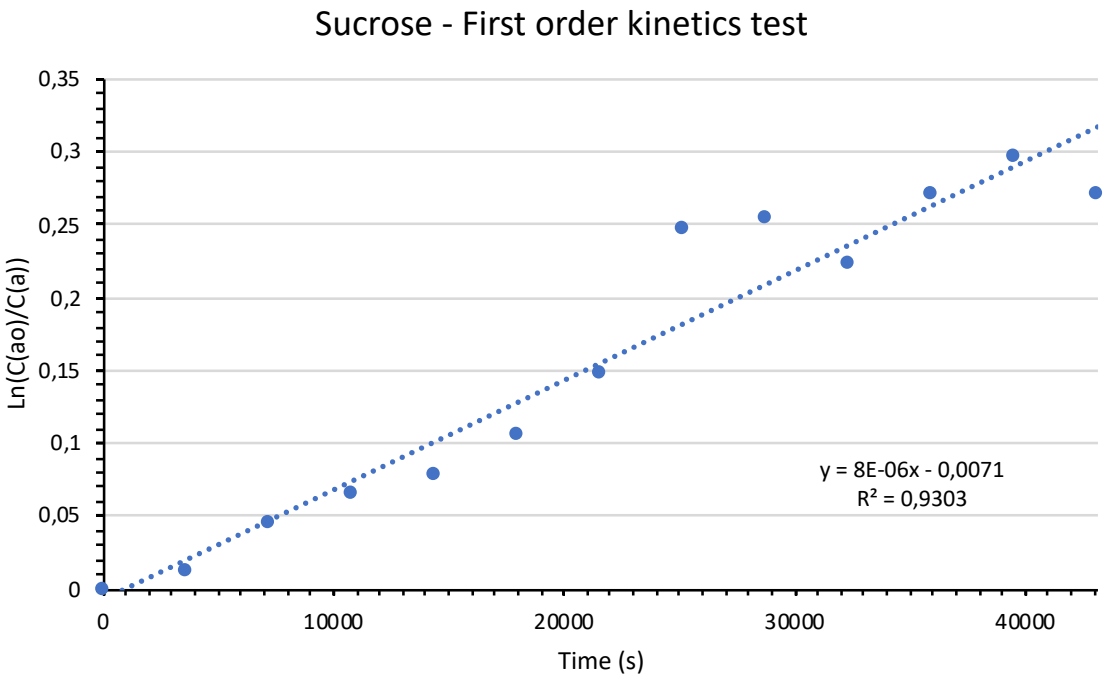

Figure 7.1: First order kinetics polynomial fit for sucrose-cultivated B.licheniformis STK 01 biodemulsifier

7.2.2. Fructose supplementary data

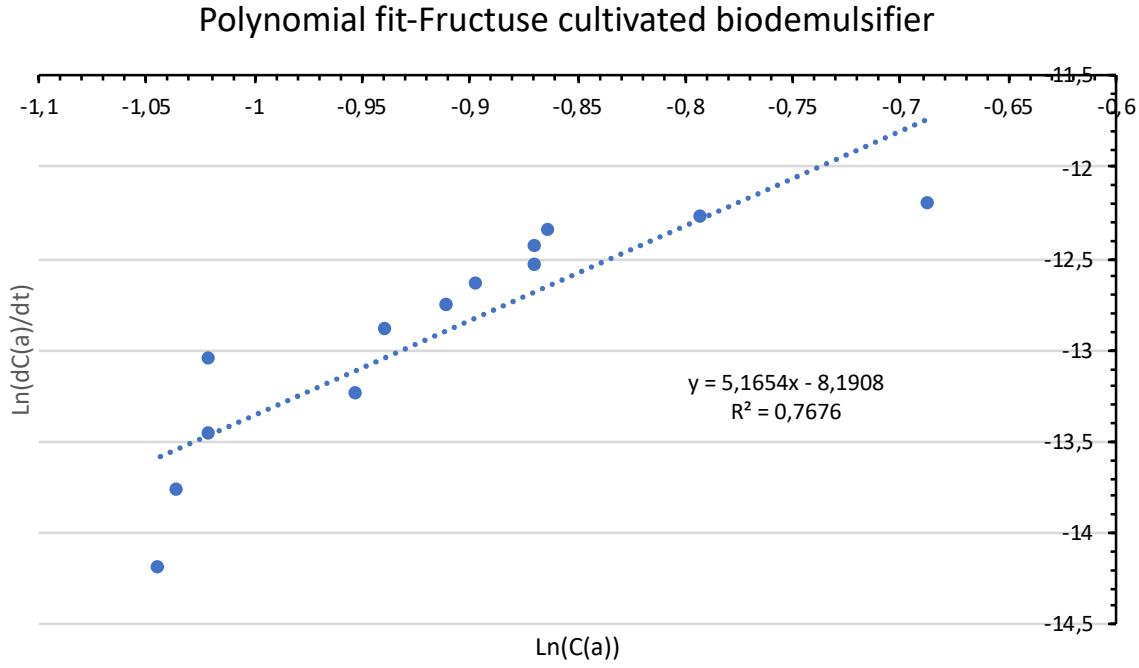

Figure 7.2: Fifth order kinetics polynomial fit for fructose-cultivated B.licheniformis STK 01 biodemulsifier

7.2.3. Glucose supplementary data

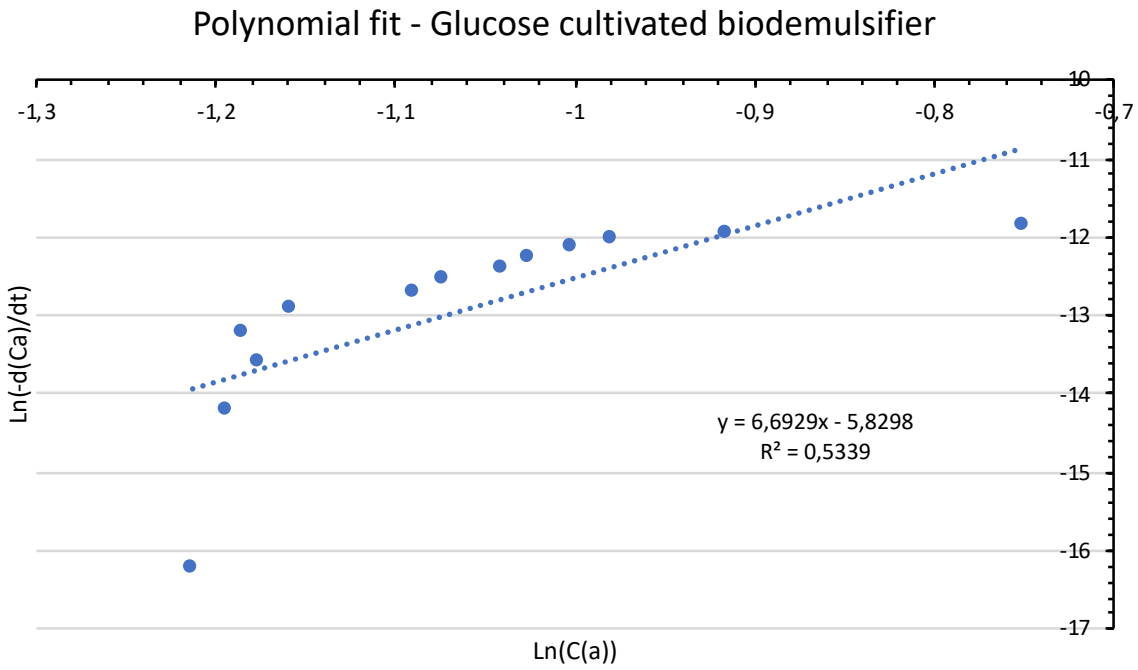

Figure 7.3: Seventh order kinetics polynomial fit for glucose-cultivated *B.licheniformis STK 01* biodemulsifier

7.2.4. Diesel supplementary data

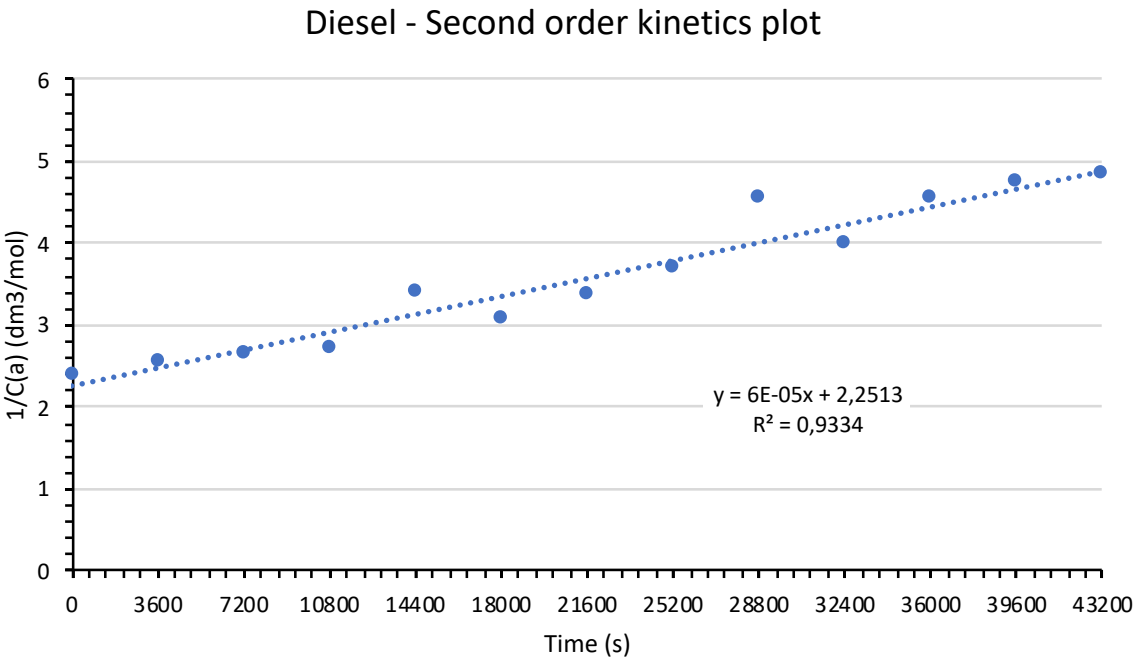

Figure 7.4: Second order kinetics plot for diesel-cultivated *B.licheniformis STK 01* biodemulsifier

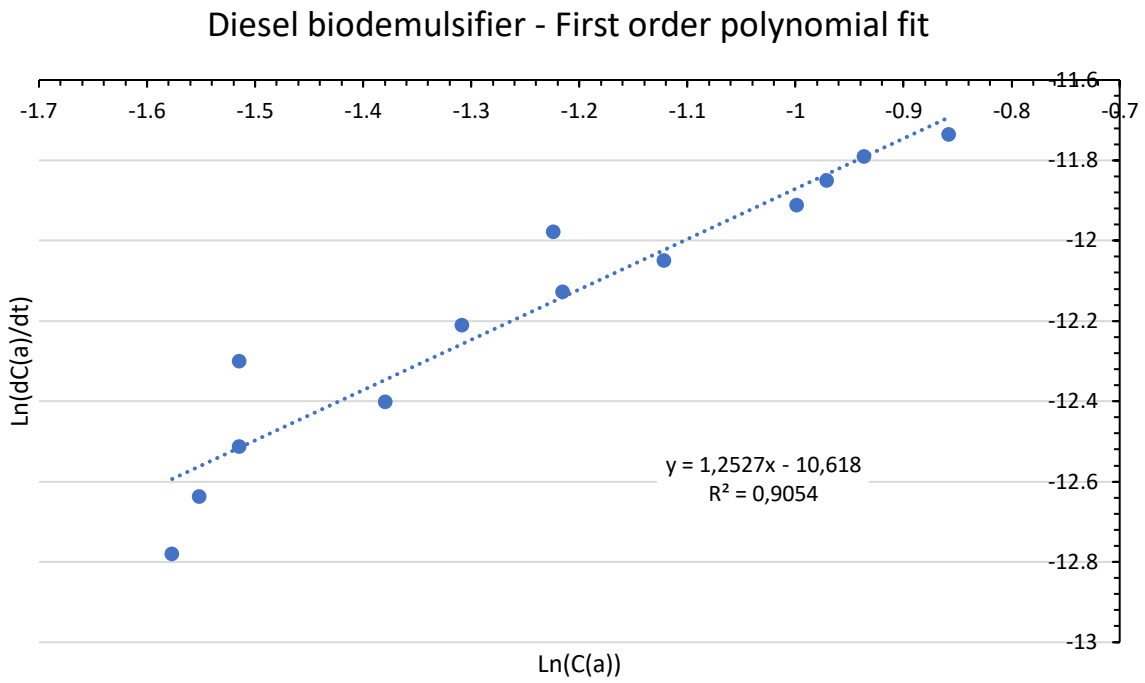

Figure 7.5: First order kinetics polynomial fit for diesel-cultivated B.licheniformis STK 01 biodemulsifier

7.2.5. Paraffin supplementary data

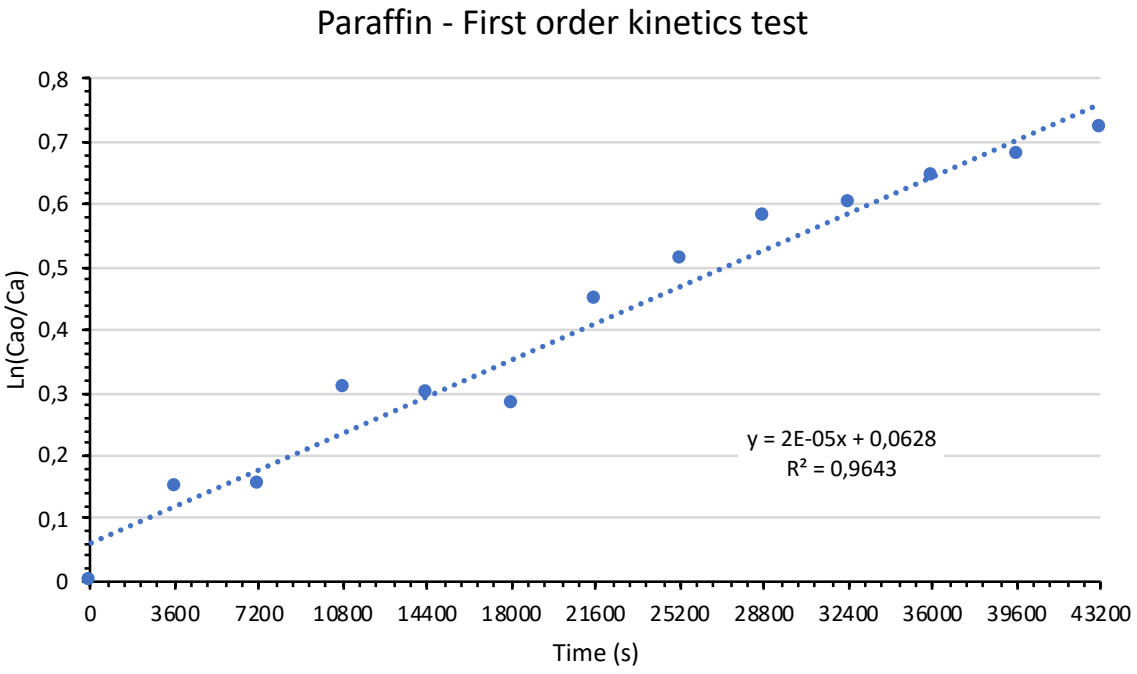

Figure 7.6: First order kinetics plot for paraffin-cultivated *B.licheniformis STK 01* biodemulsifier

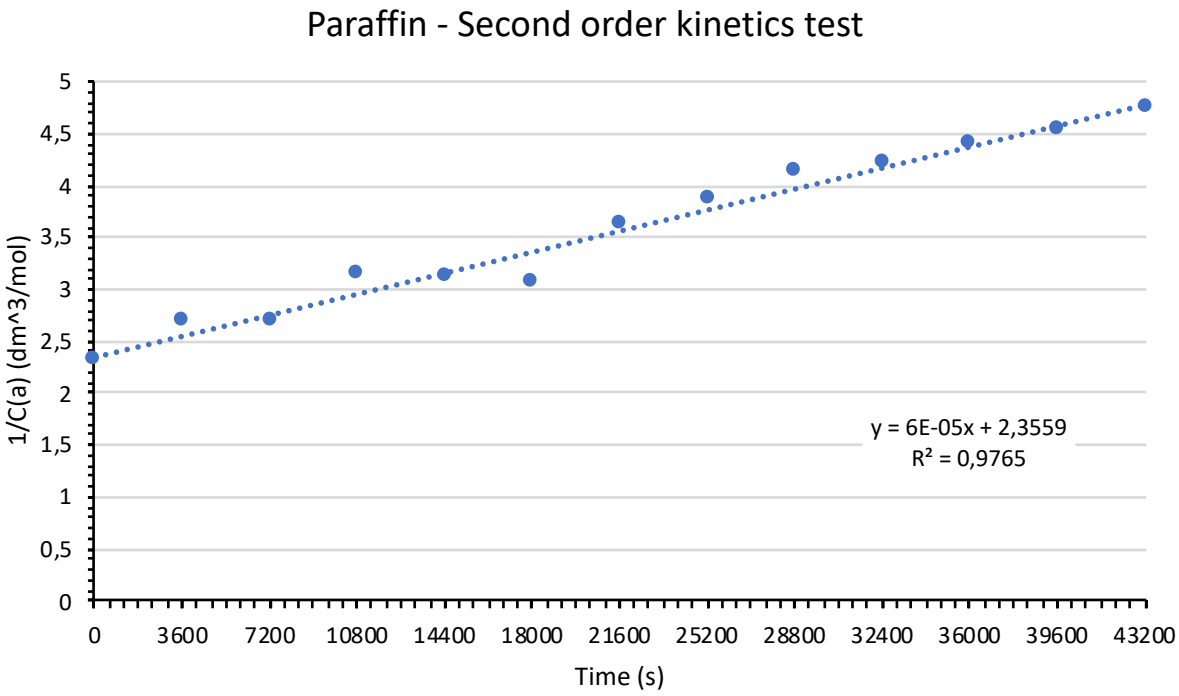

Figure 7.7: Second order kinetics plot for paraffin-cultivated *B.licheniformis STK 01* biodemulsifier

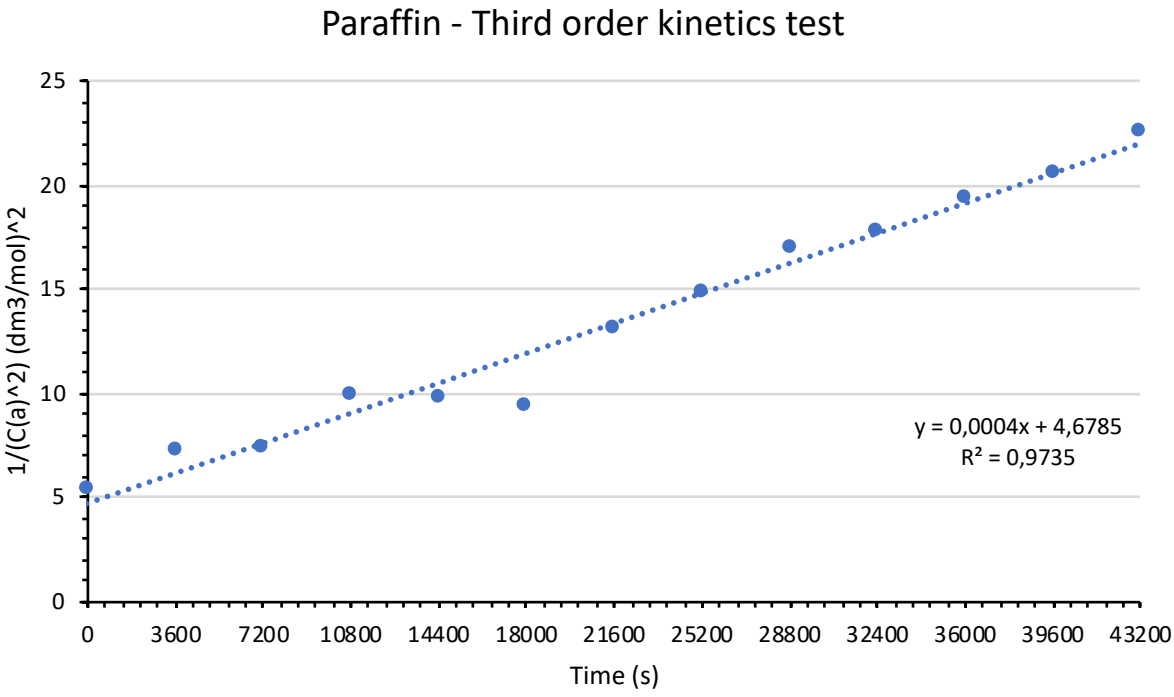

Figure 7.8: Third order kinetics plot for paraffin-cultivated *B.licheniformis STK 01* biodemulsifier

7.2.6. Motor oil supplementary data

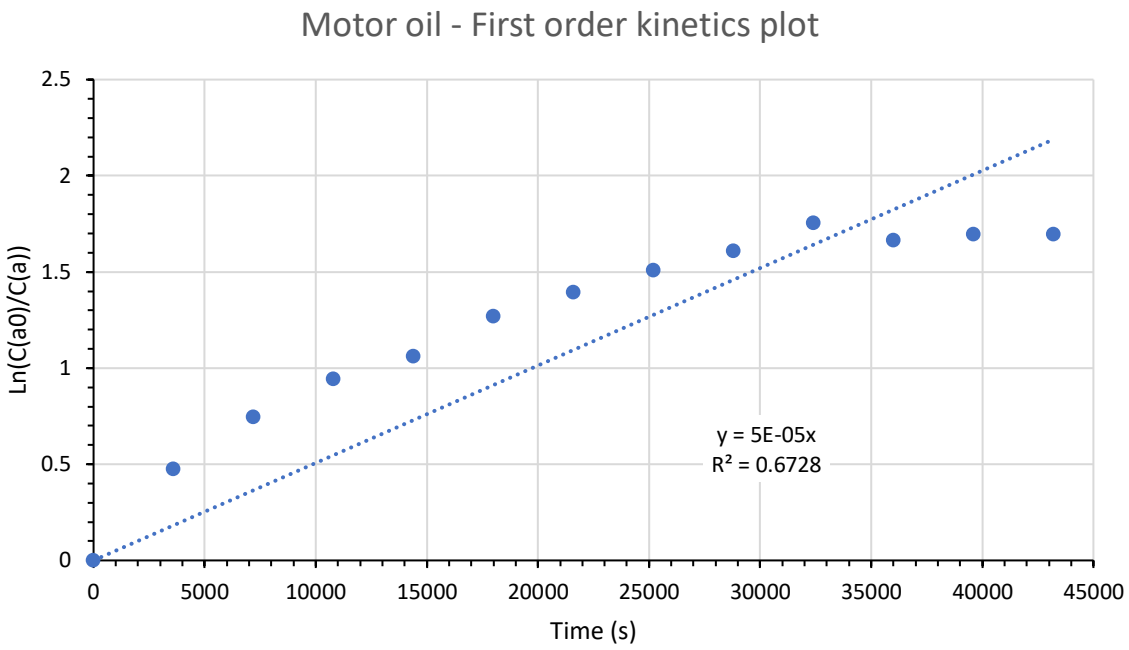

Figure 7.9: First order kinetics plot for motor oil-cultivated *B.licheniformis STK 01* biodemulsifier

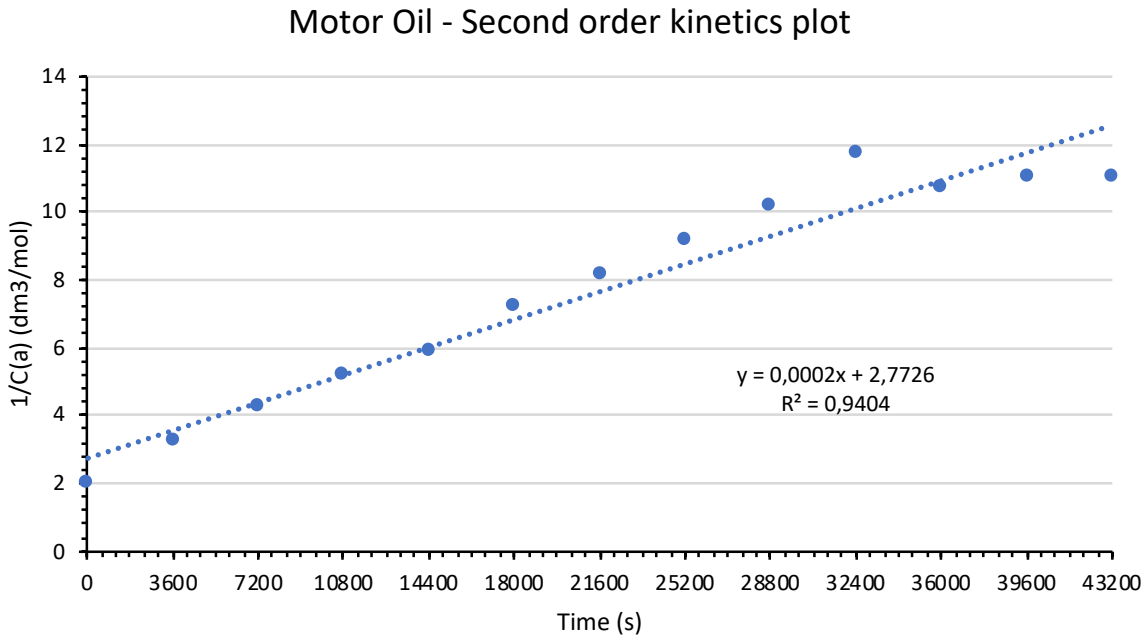

Figure 7.10: Second order kinetics plot for motor oil-cultivated *B.licheniformis STK 01* biodemulsifier

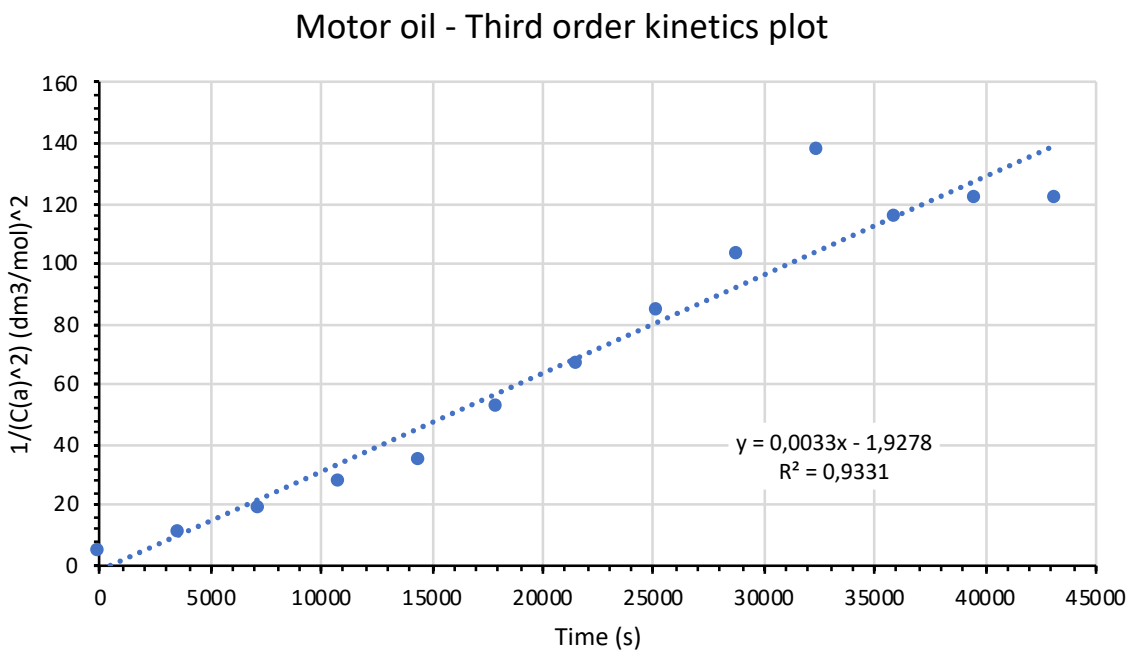

Figure 7.11: Third order kinetics plot for motor oil-cultivated *B.licheniformis STK 01* biodemulsifier