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MICROBIAL ENHANCED OIL RECOVERY FROM OILY SLUDGE USING A NOVEL PLUG FLOW REACTOR SYSTEM

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ABSTRACT

Title: Microbial Enhanced Oil Recovery from Oily Sludge Using a Novel Plug Flow Reactor System

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The refining and transportation of crude oil often results in an inevitable accumulation of recalcitrant waste products such as oily sludge. Remediation processes such as landfarming and anaerobic landfilling have been recently ruled as inappropriate disposal methods, due to the length of time it takes for remediation to reach completion, as well as the uncontrollable release of harmful organics into the soil, atmosphere and potentially groundwater. This study focused on a biological treatment method for oily sludge using a plug flow reactor system.

Biosurfactant-producing bacteria, *Bacillus cereus, Klebsiella oxytoca and Cronobacter dublinensis* were isolated from petroleum-contaminated and uncontaminated soil samples. Optimal growth of the bacteria was observed when incubated at ±30°C and biosurfactant activity was confirmed using the drop-collapse test. Oily sludge was simulated in the laboratory using fumed silica as intermediate compound to form Pickering emulsions. The recovery of oil involved the comparison of a plug flow system and a batch system. The plug flow system achieved up to 85% oil recovery from oily sludge while the batch system only achieved a maximum of 30% elution of the aqueous phase. The effect of waterflooding, in conjunction with biosurfactant-producing bacteria on the batch system had negligible effect while on the plug flow system, an increase of ±10% in percentage recovery was observed.

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GC-MS analysis of oil before and after the recovery process showed a reduction of concentration of certain compounds, suggesting possible degradation by bacteria or loss due to volatilization. TOC analysis showed a reduction in TOC of residual sludge, after completion of recovery process, suggesting degradation activity by bacteria trapped in the sludge matrix after the completion of the recovery process. Fifteen days after completion of recovery process, residual sludge from the plug flow system had a low TOC value of 1.03mg/L while sludge from the batch system had a much higher value of 7.119mg/L. The high percentage recovery of oil, coupled with the reduction of sludge TOC to negligible values suggest that MEOR is a feasible method for treatment of oily sludge and can be further investigated as a waste treatment procedure in the petroleum industry.

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DECLARATION

I Oluwademilade Martha Fayemiwo, declare that the thesis which I hereby submit for a Master of Science (Applied Science) in Environmental Technology degree at the University of Pretoria is my own work and has not been previously submitted by me for any degree at this or other institutions.

Oluwademilade Martha Fayemiwo Date

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Dedicated to my awesome family

Alexander Fayemiwo

My awesome father, my life coach

Elizabeth Fayemiwo

My amazing mother, my super-woman

Olufunke Ogunleye and Oluwatosin Fayemiwo

The best siblings anyone could ask for.

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CHAPTER 1

INTRODUCTION

1.1 Background

Petroleum has been the driving force behind recent developments in human civilization for the past three centuries. This natural resource is the main source of revenue for many regions such as Africa, the Middle East, some parts of North America and Asia, and is limited in its availability. Several research efforts have focused on the extraction of heavy crude oil fractions from underground geological reservoirs (Perfumo *et al.* 2010). Like every large scale industrial process, the refining of crude oil results in an inevitable accumulation of waste products which include slop oil and oily sludge.

Slop oil is crude oil that is not pure enough to meet with the set standards for the final product specifications. According to Dave *et al.* (1994), slop oil is waste oil that exists as petrochemical complexes, containing not less than 240 hydrocarbons with more than half of those compounds being alkanes, with carbon chains ranging from C_5 to C_{11} compounds. The remaining components consist of C_{12} to C_{23} compounds and trace aromatic impurities. It is plausible that slop oil can be re-refined but this depends on the amount of bottom sediment and water found in it. The higher the amount of bottom sediment and water found in slop oil, the less likely it is to be re-refined. Instead, it is disposed using different processes that include shipment off the refinery site for possible hydrocarbon recovery. Other alternatives of disposal include deep well injection (also known as anaerobic landfilling) or landfarming at approved sites (US EPA report,

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2010). Oily sludge on the other hand is a pasty, highly viscous compound that is found mostly in refinery and storage tanks and is sometimes also found in oil transportation pipelines, causing a clog in refinery systems (Da Silva 2011). It is known to cause volume displacement and subsequent reduction in the efficiency of the refinery system due to its recalcitrant clay-like structure (US EPA report 1995). Oily sludge consists of a complex mixture of hydrocarbons, water, metals and suspended fine solid particles. Due to the complex nature of its composition, disposal of oily sludge is not straightforward since the oily component is not easily separated from the solid matrix (Elecktorowicz and Habibi 2005; Chirwa *et al.* 2013).

Unlike slop oil, oily sludge is not considered for re-refining due to the presence of heavy contaminants such as sand particles and metals (Abouelnasr and Zubaidy 2010). It is however often used as a starting material in delayed cokers that crack heavy long chain hydrocarbon molecules into smaller molecular gas and oil products as well as petroleum coke (Fanxiu 1998). Oily sludge can also be used as starting material for the production of bitumen or as incineration material in a rotary kiln. In cases where it is not considered as a raw material for the above-listed processes, oily sludge is shipped off the refinery site for landfarming at an approved disposal site (US EPA report, 2010).

The process of anaerobic landfilling involves the disposal of petroleum sludge in specially constructed pits, designed specifically for the purpose of sludge disposal. Until the early 90s, this process was considered the best possible disposal method but the fact that no treatment of the waste occurs led to outcries regarding the possibilities of groundwater contamination as well as extensive soil pollution (Read *et al.* 2001; Joseph and Joseph 2009; US EPA report 2010).

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Landfarming on the other hand involves the spreading of oily sludge across an approved land site where the degradation of hazardous compounds, in the form of PAHs, by indigenous soil bacteria is encouraged. Landfarming was considered a safe and cost-effective mode of petroleum waste disposal until studies revealed that this waste treatment method usually takes years to complete, and some heavy oil fractions still remain in high concentrations in the soil even after years of bacteria activity, leaving considerable amount of time for possible infiltration of these hazardous wastes into groundwater (Hejazi *et al.* 2003; Marin *et al.* 2005).

In order to safely dispose of oily sludge in particular, it is imperative that the total organic carbon content of the waste be reduced to a negligible amount, hence the proposal for oil recovery as a waste treatment method. Oil recovery is not an entirely new concept; generally, its definition in the petroleum industry spans across the extraction step of crude oil refining to the treatment of waste and inevitable by-products accumulated during refining, storage and transportation of crude oil and its derivatives. It is estimated that currently, oil recovery methods utilized in extraction of crude oil can only recover about 30% to 40% of the oil available in reservoirs (Lazar *et al.* 2007; Sen 2008; Brown 2010). This has led to various studies that aim at encouraging the use of different downstream processing techniques in a process known as Enhanced Oil Recovery (EOR).

With regards to waste treatment, the recovery of oil from oily sludge not only reduces the overall organic carbon content but it also helps to recover crude oil fractions that can either be re-refined or used in other industries that utilize petroleum products (US EPA report 2010; Brown 2010). The recovery of oil from petroleum sludge using a variety of physical and chemical methods has been reported, with many of such studies reporting little or no success. Furthermore, the recovery of oil from petroleum sludge using a biological system such as Microbial Enhanced Oil

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Recovery (MEOR) has been sparsely reported in literature. MEOR involves the use of biosurfactant-producing bacteria as emulsifying and demulsifying agents. Biosurfactants are metabolites produced by some bacteria, yeasts and fungi. Biosurfactants are amphipathic surface active molecules that are widely used across a range of industries-cosmetics, detergent production and have recently become one of the most studied classes of compounds in the petroleum industry. Biosurfactants are characterized by their ability to reduce surface and/or interfacial tension between immiscible liquid emulsions (e.g. oil-water emulsions), and oil-solid mixtures. Although the feasibility of MEOR has been theoretically reported in few studies, there has not been much success in this area of study (Banat 1995; Joseph and Joseph 2009; Brown 2010).

The use of MEOR has been explored in the petroleum industry, mainly as a method to aid the extraction of crude oil from oil reservoirs. Its use as a waste treatment method for the recovery of oil from oily sludge is only beginning to gain momentum of recent.

1.2 Problem statement: Treatment of Petroleum Sludge

The disposal of petroleum sludge is carried out using either anaerobic landfilling, or landfarming. Both processes were previously approved as appropriate disposal methods until recent rulings by the US Environmental Protection Agency (US EPA report, 2010). According to the report by the US EPA, petroleum sludge is a form of hazardous waste due to its high content of PAHs, therefore its disposal into the environment without prior treatment is unadvisable. In addition, the cost of these disposal methods, in terms of construction and maintenance have been reported to be too high (Bhattacharyya and Shekdar 2003; Joseph and Joseph 2009).

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The treatment of petroleum sludge is a challenging process that has been the focus of research studies recently. Combinations of physical, chemical and more recently, biological methods have been explored as possible appropriate methods for the treatment of petroleum sludge. Physical and Chemical methods have however started to lose popularity owing to the high cost of construction as well as possible contamination due to chemical residue. Biological treatment methods are fast gaining approval as they are known to leave no harmful residues and are often cheaper than physical or chemical methods.

1.3 Project Objectives

The aim of this study was to explore the possibility of using a biological system to treat petroleum sludge. The use of bacteria as remediation organisms has gained a lot of momentum in other pollution areas which include bioremediation of acid mine drainage areas by sulfur-eating bacteria (Garcia *et al.* 2001; Hard *et al.* 1997; Utgikar *et al.* 2002). The use of biosurfactant-producing bacteria as agents of bioremediation in the petroleum industry is fast becoming an area of research that is leading to the development of new processes and technologies. Although the success rate regarding the use of microorganisms in the petroleum industry is still debatable, this area of research cannot be ignored (Lazar 1997; Brown 2010).

1.3.1 Primary objective

The main objective of this study was to design a system that could enhance the recovery of oil from petroleum sludge using biosurfactant-producing bacteria. The system is aimed at achieving recovery at a low energy and financial cost while reducing the concentration of harmful PAHs in

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the waste. A hydrodynamic approach was used in a continuous flow plug flow system to achieve continuous recovery of oil from sludge. The design of such a system had to take into consideration the dynamics of refineries and it was imperative that the design would fit into the refinery system, be cost-effective, time-efficient, easy to operate and to use with no harmful chemical residue left behind. The aim of designing such a system was to investigate the possibility of reducing the concentration of harmful PAHs to almost negligible amounts, and if possible, to zero.

1.3.2 Secondary objectives

The spin-off objectives of this study included the characterization of isolated bacteria species and the determination of their suitability in the petroleum industry. The reduction of the total organic carbon (TOC) content of petroleum sludge was also an important objective as it was considered imperative to reduce the TOC of petroleum sludge to conspicuously negligible amounts that would be safe for disposal into the environment. The isolation of the biosurfactants produced by the bacteria isolated for the course of this study was also an objective which was expected to lead to the characterization of the isolated compounds in order to possibly understand the physiology of the production of biosurfactants by these bacteria.

1.4 Thesis outline

This thesis is presented starting with a background literature review that explains the various processes that have been employed as treatment methods for oily sludge including landfarming and its ill effects on different orders of life, oil recovery (physical and chemical methods) as well

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as review of studies that have reported success with MEOR. The research methodology employed in this study include bacteria isolation, gas chromatographic characterization of oil samples, analysis of experimental findings as well as the use of existing kinetic models to describe the data obtained during the oil recovery process.

1.5 Research Findings

This study uncovered two rarely-reported biosurfactant producers, *Cronobacter dublinensis* and *Klebsiella oxytoca* from the *Enterobacteriaceae* family. One member of the Bacillus species, *Bacillus cereus* was also found to be a producer of biosurfactant. A continuously fed plug flow reactor was able to recover 85% of oil trapped in petroleum sludge within a period of five days (120 hours) while a full batch system investigated for the sake of comparison was only able to recover approximately 22% of oil within the same time period. The analysis of the oil samples before and after the recovery process showed that there was more recovery than degradation; it was feared during the course of the study that degradation might occur during the incubation of bacteria with petroleum sludge as the bacteria could choose to use the hydrocarbons present in the petroleum sludge as their carbon and energy source. While this could still achieve the aim of biotreatment, it is important to note that the process of degradation can take years to complete. The TOC analysis of residual sludge after completion of recovery showed high bacteria activity and reduction of organic carbon in residual sludge to a negligible amount.

1.6 Significance of Research

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The ability to recover oil from petroleum sludge could avert the strain involved in the disposal of this waste product. It is estimated that refineries produce thousands of tons of sludge with no suitable avenue for disposal. The recovery of oil from petroleum sludge is an area of research that has faced a lot of difficulty given that the plausible use of biosurfactants in the petroleum industry is still debatable, considering the problems faced such as low biosurfactant yields and inability of biosurfactant-producing microbes to endure the environmental conditions available in refineries. The successful recovery of oil from petroleum sludge which was achieved during the course of this study is not a replacement for landfarming but rather an effective biotreatment procedure that speeds up the process of landfarming. Though it may not re-refined, the oil recovered can be used in other industrial processes such as paint production, feed stream for industries involved in the production of road tar and cosmetic industries .

This study has addressed a significant area of research regarding one of the most important industries in the world and the success rate achieved shows that MEOR is plausible for sludge treatment and can be further investigated for use in oil recovery from reservoirs and wells, another area of research that has recently gained a lot of momentum.

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CHAPTER 2

LITERATURE REVIEW

2.1: Bioremediation of Oily Sludge: General Overview

The accumulation of waste products in the petroleum industry has instigated a lot of studies focused on the remediation of these wastes. Various methods have been proposed, ranging from landfarming to the recovery of oil from petroleum wastes which are often in the form of slop oil or oily sludge. Slop oil is easily treated, considering it can easily be re-refined or shipped off the site for hydrocarbon recovery. Oily sludge on the other hand, is more difficult to handle and is currently the focus of many remediation studies. It is well known that oily sludge consists of a complex mixture of poly-aromatic hydrocarbon (PAH) compounds. These compounds often exist in viscous forms that are not easily accessible for degradation by microorganisms. PAHs consist of two or more fused Benzene rings in linear, angular or cluster arrangements. Examples of such compounds include compounds include Naphthalene, Benzopyrene, and Anthracene etc. PAHs have been discovered to be toxic compounds that have carcinogenic and/or mutagenic properties; hence they pose a real danger to the environment (Wilson and Jones, 1993).

The typical concentrations in oily sludge of some harmful PAHs have been reported by the US EPA in a report published in 2010. Pyrene was reported to be present at a concentration of 9.43mg/L, C3-pyrene/fluoranthene was present at 16.86mg/L while confirmed carcinogenic PAHs like chrysene and benzopyrene were found to be present at 14.90mg/L and 22.71mg/L respectively. The high concentrations of these PAHs highlight the irrefutable dangers associated

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with the disposal of oily sludge into the environment and also reinforce the disapproval of landfarming as a disposal method for oily sludge.

2.2 Ecological Impacts of Landfarming

Landfarming was once considered a cost-effective and safe disposal method for petroleum wastes. Early studies in the 70s and 80s reported that landfarming was an acceptable and safe mode of disposal (Bossert *et al.* 1984); however, recent studies have shown that landfarming is potentially harmful to the environment and to organisms living in ecosystems associated with landfarming sites. Landfarming is based on the ability of indigenous microbes to biologically degrade harmful PAHs contained in oily sludge; The inefficiency of this process as a petroleum waste treatment method has been demonstrated by studies that show that landfarming requires a considerable amount of time (ranging from 5 to 15 years) for complete degradation of hydrocarbons to occur (Hejazi *et al.* 2003; Bartha 1986).

Table 2-1 shows the estimated half-time required for the complete biodegradation of aliphatic and aromatic compounds found in petroleum wastes. In comparison with photo-oxidation, biodegradation takes a considerably longer amount of time to reach completion. As can be seen from table 2-1, the aliphatic compounds are easily degraded within a short period of time, relative to the aromatic hydrocarbons which are recalcitrant and take much longer, with some of them taking more than 600 days- enough time for these compounds to potentially seep through the soil and contaminate groundwater.

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Table 2-1: Estimated Half-time for degradation of hydrocarbons in oily sludge

Hydrocarbon	**Photo-oxidation($t_{1/2}$ days)**	**Biodegradation ($t_{1/2}$ days)**
	Aliphatic	
Hexane	2.0	4.7
Octane	1.3	6.4
Nonane	1.1	7.4
Dodecane	0.8	11.8
Hexadecane	0.5	21.7
Eicosane	0.4	39.9
	Aromatic	
Benzene	5.5	4.5
Ethylbenzene	1.8	5.0
Naphthalene	0.5	5.6
Methylnaphthalene	0.2	8.9
Anthracene	0.3	123
Chrysene	0.2	343
Coronene	0.2	643

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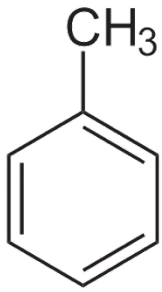
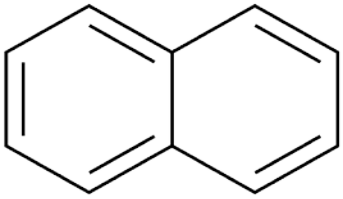
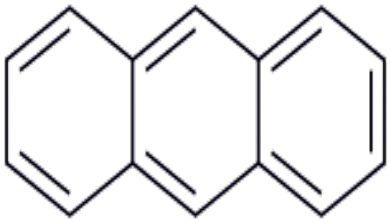


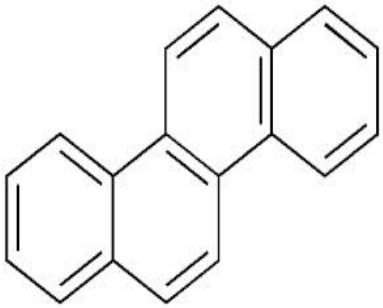
a.) Toluene (5.78 mg/L)



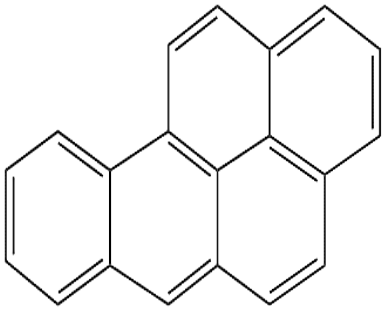
b.) Naphthalene (9.83 mg/L)



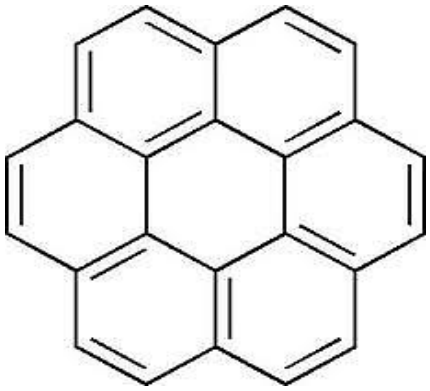
c.) Anthracene (13.97 mg/L)



d.) Chrysene (16.85 mg/L)



e.) Benzopyrene (22.71 mg/L)



f.) Coronene (22.89 mg/L)

Figure 2-1: Chemical structures and typical concentrations of some PAHs found in oily sludge (US EPA report, 2010).

The lack of efficiency of landfarming in the removal of PAHs in oily sludge was demonstrated during a study carried out by Dréau *et al.* (1997). Dréau et al. (1997) investigated the hydrocarbon balance of a site in France that had been contaminated with petroleum wastes for 36

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years (1956-1992). The results from the study showed that over a period of two years during which the site was not used as a disposal site anymore, the short chain aliphatic compounds with a maximum carbon chain of 25 were no longer present. These chains were either broken down by bacteria in the petroleum wastes or they were more volatile than the other compounds in the waste and evaporated into the air. The use of GC analysis however revealed that many aromatic compounds were still present in the soil in high concentrations highlighting the dangers of landfarming. A similar study by Al-Awadhi *et al.* (1996) showed that landfarming takes a considerable amount of time to achieve acceptable levels of hydrocarbon degradation in the soil. Liu *et al.* (2008) reported only 58.2% sludge degradation after 360 days (approximately one year).

2.3 Effects of Landfarming on Microorganisms

The effects of landfarming on the environment have been widely studied; however, not a lot of research has been done regarding the impact of landfarming on native microbial communities at the sites. A case study by Genouw *et al.* (1994) reported that there was no considerable side effect of landfarming on the microbial life present in soil subjected to landfarming conditions. However, in a separate study, it was reported that the continuous tilling of soil in order to stimulate biodegradation by indigenous bacteria affected soil biota and led to lesser efficiency of removal of petrochemical pollutants (Taranaki Council report 2012).

The loss of efficiency during landfarming can be attributed to a number of factors such as insufficient supply of nutrients in landfarming sites, static conditions that do not allow for proper distribution of nutrients as well as uncontrollable environmental factors such as pH, temperature

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and soil salinity. In the above studies, the physiology of the soil microorganisms was not evaluated. Some investigations however revealed that the physiology of microorganisms in landfarming sites might play a role in the efficiency or lack thereof of the process. A study highlighted anoxic stress on the microorganism as a factor that affected the physiology of microorganisms during landfarming. The feasibility of degradation requires an acclimatisation period by the bacteria, which may vary from weeks, to months and in some cases even years (Wilson and Jones 1993).

The metabolism of recalcitrant PAH compounds depends on the presence of certain metabolic pathways, enzymes and genes in microorganisms (Guerin and Jones 1988a; 1988b; Guerin and Boyd 1992; Grifoll *et al.* 1992, 1994, 1995). Many studies that have investigated the physiology of hydrocarbon-degrading bacteria show that bacteria initially oxidise aromatic hydrocarbons to *cis*-dihydrodiols in order to allow for easier access for degradation (Aitken *et al.* 1998, Juhasz &Naidu 2000). A report by Samanta *et al.* (2002) also supported these studies, stating that certain regulations (enzymatic and genetic) as well as physiological systems need to be present in bacteria for successful degradation to occur. The first step according to this report is the production and action of an enzyme called di-oxygenase. This enzyme incorporates oxygen atoms into Benzene rings, making them more accessible as di-hydroxylated compounds. These di-hydroxylated compounds can be further cleaved to form intermediates of the TCA cycle, making it easier for microorganisms to use these compounds as a source of carbon and energy. Many naphthalene-degrading microorganisms for example possess the necessary enzymes to degrade PAHs; however, there are a number of microorganisms in soil that do not possess these enzymes or pathways for complete degradation of PAHs (Kanaly and Harayama 2000; Sabaté *et al.* 1999).

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Table 2-2: Some common PAH degrading genera

Bacterial genus	Genes	Compounds degraded
	Gram Positive	
Rhodococcus sp.	*nar, iso*	HMW, BTEX
Norcadiaides	*phd*	HMW
Mycobacterium spp.	*nid, pdo*	HMW, LMW
Terrabacter spp.	*dbf, fad*	HMW, HMW
Arthrobacter sp.	*ISP*	HMW
	Gram Negative	
Pseudomonas spp.	*nah, bmo, tou*	LMW, BTEX, BTEX
Commonamonas spp.	*phd*	LMW
Ralstonia sp.	*nah, nag, tbu, phl*	LMW, LMW, BTEX, BTEX
Burkholderia sp.	*phn, tmo*	LMW, HMW, LMW
Alcaligenes sp.	*phn, gst*	LMW, HMW, LMW
Novosphingobium spp.		
Sphingomnonas spp.	*phn, arc, adh, nah*	LMW, HMW, BTEX
Porphyrobacter sp.		
Rhizobium sp.		

LMW: naphthalene, phenanthrene, anthracene. HMW: fluorene, fluoranthene, pyrene, benzo[a]pyrene, chrysene. BTEX: Benzene, toluene, Ethylbenzene, xylene (Ewers 2009).

2.4 Impact of Landfarming on Higher Order of Life

The release of petroleum hydrocarbons into the atmosphere has generally been considered to be negligible; in other words, it was believed that the release of volatile and semi-volatile compounds into the air had no negative impact on the health of humans in such locations. A

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study by Hejazi *et al.* (2003) proved otherwise; the study involved the investigation of the risks posed by landfarming to the health of humans residing in arid regions. The study was carried out in Saudi Arabia and it revealed that most hydrocarbons contained in petroleum wastes tend to volatilize easily, facilitating an increase in the concentration of volatile organic compounds in the air. It was speculated that the presence of these compounds in the atmosphere could affect the health of on-site workers. Benzene, a well-known carcinogen was determined to be one of the predominant pollutants emitted from petroleum and petrochemical wastes (McMichael 1988; Farris *et al.* 1993).

A report by Chiarenzelli *et al.* (1998), suggested that remediation methods could lead to the release of semi-volatile compounds into the atmosphere, causing an increased risk of affecting human health. Herskowitz *et al.* (1971) reported a neuropathic condition associated with the continuous inhalation of n-hexane by three petroleum site workers. In a study carried out in 1997, Greenberg reported the health risk associated with increased exposure to toluene, a very common hydrocarbon typically found in petroleum wastes. The effects of toluene on humans, reported in this study included a complete dysfunction of the central nervous system. Owing to this, an inhalation reference concentration of 0.4mg toluene/m^3 was developed by the U.S. EPA. Solomon and Janssen (2010) investigated the health effects of the Gulf of Mexico oil spill and reported potential direct threats posed by petroleum compounds to the health of humans who could inhale them. These reports highlight the negative effects landfarming could have on human life; the inevitable release of volatile and semi-volatile petroleum hydrocarbons into the air could result in severe neurological disorders and generally affect the quality of life of such individuals.

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2.5 Environmental Factors that affect Landfarming

There have been suggestions that landfarming may be affected by environmental factors that affect bacteria activity adversely. These suggestions have been reinforced by studies that focused on evaluating the effects of environmental factors on bacteria activity and subsequently on the effectiveness of landfarming. In 1979, Dibble and Bartha investigated the effects of soil moisture content, soil pH, availability of micronutrients, organic supplements, mineral nutrients, and the incubation temperature on the ability of indigenous soil bacteria to degrade PAHs. It was reported that the biodegradation of oily sludge was optimal at temperatures ranging from 20°C to 28°C. At temperatures above 30°C, increase in degradation was negligible. Optimal degradation rates were observed at soil moisture contents that ranged from 30% to 90%, pH values between 7.5-7.8 and at higher carbon ratios when carbon quantity was compared to nitrogen and Phosphorus. This study supported the notion that indigenous microbes might be affected by environmental parameters in landfarming but unfortunately, the waste disposal method does not in any way give control over these factors.

In a study by Lazar *et al.* (1997), motion was considered as a crucial factor that possibly affected the degradation of compounds contained in oily sludge disposed via landfarming. The report showed that the efficiency of the process varied from 16.75% degradation to about 95% degradation, depending on the moving conditions (Erlenmeyer flasks placed on rotary shakers at 200rpm). The study however reported that the degradation of hydrocarbons varied from 16.85% to only 51% under static conditions- the condition experienced in landfarming sites.

In 2001, Vasudevan and Rajaram demonstrated the inefficiency of landfarming as well as the lack of control over environmental parameters when they carried out bioremediation of sludge

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which had been disposed on to landfarming sites, in glass troughs. The pair used a bacteria consortium, inorganic nutrients, compost and a bulking agent (wheat bran) to support the growth of the bacteria consortium. Though the results showed 76% hydrocarbon removal from the contaminated soil, it also showed that landfarming on its own is not effective enough to reduce hydrocarbon content in contaminated soil; there is a need for additional factors and nutrients to increase biodegradation.

The above-mentioned studies have revealed extenuating factors where landfarming is concerned; besides being unable to strictly control environmental and other physical factors, the metabolic pathways of the bacteria present in the soil cannot always be manipulated. Owing to this discovery, the reliability of landfarming has reduced drastically as it is now clear that the process has varying degrees of efficiency depending on factors that are beyond human control. Besides the fact that landfarming is not as effective as it was thought to be when it was initially proposed as a method of petroleum waste treatment, it can also be regarded as a waste of useful oil fractions that can possibly be utilized in other industries besides the petroleum industry. The irregularity of landfarming efficiency, coupled with the ill effects it has on the environment, has led to the investigation of alternative methods that will aid in the treatment of oily sludge.

2.6 Oil Recovery

The recovery of oil from petroleum sludge is not an entirely new concept. Oil recovery is one of the major steps during the extraction of crude oil from oil reservoirs (Sen 2008). This concept is now gaining attention as a possible remediation method where oily sludge is concerned. `The

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feasibility of oil recovery as well as its long-term favourable effects on the environment has gained a lot of attention, making it the cynosure for many remediation studies (Joseph and Joseph 2009; Brown 2010; Lazar *et al.* 2007; Donaldson *et al.* 1989; Finnerty and singer 1983; Mehta and Jain 2009). Several investigations have been carried out from as far back as the 80s, with many of them focusing on physical and chemical methods through which oil can be separated from oily sludge.

One such study was conducted by Mishra, (1989). The method was based on the possibility of separating oil from water and other solids in sludge based on the physical properties of the components; physical properties that were taken into consideration included the weight and density of the components as well as their hydrophobicity. Other researchers attempted to separate components of the sludge matrix based on their size differences (Lathe and Ruthven 1956). The removal of coarse particles from sludge was achieved using sieves (sieve bends or vibro-sieves). In cases where the sludge components were too fine to be separated based on particle size, the densities of the different components were taken into consideration. The density separation approach can be controlled, allowing one to decide what will be separated as light fractions which are mainly hydrophobic substances and what will emerge as the heavier fractions which are mainly the hydrophilic substances in the mixture (Mishra 1989).

In May 1999, Jean *et al.* described the freeze and thaw method they used in separating oil from sludge. After freezing and thawing, the sludge resulted in three distinct layers. The top layer was an oil layer followed by a middle water layer and at the bottom, a semi-solid layer characterised by sediments. GCMS analyses of the different layers exposed a considerable amount of reusable alkanes in the oil layer. A similar study by Chen and He (2003) also investigated the freeze and thaw method as a plausible oil recovery technique. Demulsification in this study was however

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not achieved by simply freezing and thawing. Instead, the use of surfactant molecules to destabilize the ice lattices was employed. In 2012, Zhang *et al.* investigated the freeze and thaw technique further by employing it alongside the use of ultrasound treatment to break the recalcitrant structure of oily sludge. It was concluded that a combination of ultrasound treatment with freezing and thawing achieved an oil recovery rate of 80% but each treatment carried out on its own achieved less than half of that percentage.

The use of chemicals such as solvents as oil recovery agents has also been extensively studied. Abouelnasr and Zubaidy (2010) investigated the use of two solvents- methyl ketone (99% purity) and LPG condensate as oil recovery agents. The results showed that the system, which involved the mixing of sludge with the solvents, followed by filtration to collect the solid residue and subsequent distillation to recover the separate oil and solvent fractions only recovered between 24% and 38.8% of the oil contained in sludge. Several other studies have been carried out using solvents, with each reporting the same range of percentage recovery (Trowbridge and Holcombe 1995; Kam 2001, Ávila-Chávez *et al.* 2007; Abouelnasr and Zubaidy 2008; Taiwo and Otolorin 2009). El Naggar *et al.* (2010) investigated the use of petroleum cuts as solvents for oil extraction from oily sludge. The petroleum cuts used included n-heptane, ethylene dichloride, toluene, methylene dichloride and diethyl ether, naphtha cut and kerosene cut. An impressive recovery of 97% oil from dry and semi-dry petroleum sludge was reported and was explained by the ability of petroleum cuts to adhere to hydrocarbons, thereby causing easy elution.

Some studies have also focused on the recovery of oil from sludge and reservoirs using gas flooding as a technique. A report by Ostapovich & Pebdani (1993) described the recovery of oil by injecting oxygen-enriched gas down an injector well. The gas is ignited in the upper portion of the reservoir in order to create a combustion zone that will aid the reduction of oil viscosity

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and cause the oil to flow better. A similar method that involved the injection of steam into reservoirs to reduce the viscosity of heavy oil fractions was earlier described by Hartman & Shu (1986). Shen and Zhang (2003) investigated the feasibility of low temperature pyrolysis as a plausible method of oil recovery using sewage sludge and reported an oil recovery percentage of just 30%. In a separate study, Elektorowicz and Habibi (2005) proposed a separation phase technique that involved the combination of electrokinetic cells and electric potential. The study showed results that included 31.8% volatile hydrocarbon recovery and 42.2% non-volatile hydrocarbon recovery.

Although, many of these methods seemed plausible at the time they were proposed, the adverse implications were barely considered. Many of the methods described above are not cost effective on an industrial scale, and they tend to create a different problem- e.g. chemical residues, release of harmful substances etc. Some of the physical methods described (e.g. Mishra 1989) require the construction of specialized equipment with little or no assurance of successful oil recovery. Due to the incalculable danger these methods might have on the environment, research is now focused on inexpensive and relatively harmless methods of oil recovery from petroleum sludge in the form of biological waste treatment.

2.7 Surfactants and Biosurfactants

The use of biological oil recovery methods in oil recovery is considered more favorable than previously described physical and chemical methods. One biological method that is considered plausible is the use of biosurfactants. Surfactants are amphipathic compounds; in other words, they have both polar and non-polar sides, enabling them to interact with the two phases of immiscible emulsions as well as aid the possible degradation of recalcitrant compounds (Makkar

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and Rockne, 2003). The use of surfactants in oil recovery is not only used in the treatment of oily sludge waste. Surfactants are also applied on an industrial scale to oil reservoirs to aid the release of heavier oil fractions trapped in solid-oil emulsions (Perfumo *et al.* 2010). Currently, most of the oil recovered from reservoirs are light oil fractions and represent about one-third of the hydrocarbons contained in these reservoirs. The heavier oil fractions are trapped in viscous forms or in rocks and cannot be extracted using conventional oil recovery methods (Van Hamme *et al.* 2003; Lazar *et al.* 2007; Sen 2008). This has led to a need to enhance the recovery of oil from reservoirs (figure 2-2).

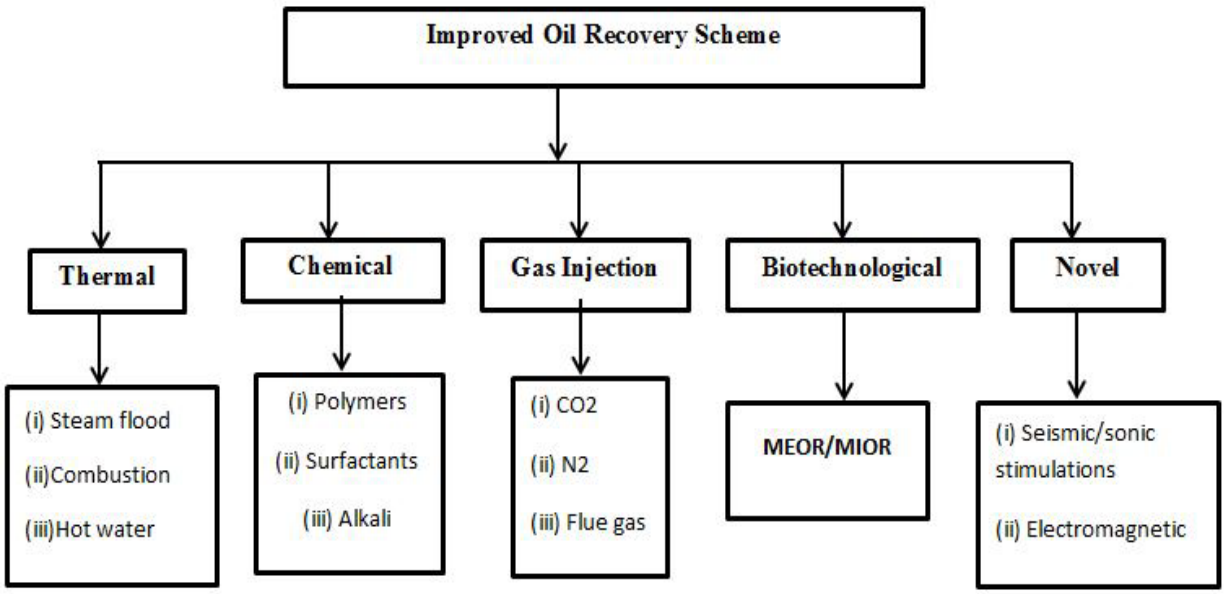

Figure 2-2: Flow sheet diagram showing techniques in enhanced oil recovery (Sen 2008).

Although the use of surfactants e.g. SDS seems plausible as viable agents of oil recovery from oily sludge, surfactants are synthetic compounds that pose a risk to the environment after oil recovery has been achieved. Surface active compounds are abundant in nature, ranging from the phospholipids that constitute the cell membranes of many organisms to the secondary

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metabolites (biosurfactants/bioemulsifiers) produced by a variety of bacteria, yeasts and fungi (Walter *et al.* 2010).

Biosurfactants, like surfactants are surface active compounds that are also able to reduce surface and interfacial tension, reduce viscosity and increase the feasibility of oil recovery processes such as rock wetting, micellar flooding, emulsification and de-emulsification (Guerra-Santos *et al.* 1984; Lazar *et al.* 2007; Brown 2010). Biosurfactants are preferred to their synthetic counterparts because they are non-toxic, non-hazardous, and biodegradable and generally tend to be environment friendly (Plociniczak *et al.* 2011).

The use of biosurfactants is considered to be one of the least expensive methods of oil recovery. Biosurfactants are popular in various industries for many processes such as emulsification, foaming, production of detergents and the production of cosmetic products amongst many others (Greek 1991).Owing to the 'bipolar' structure of surfactants, they are able to partition fluid mixtures that differ in polarity such as oil-water emulsions. Their structure also makes it possible for them to reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures, making them the perfect candidates for enhanced oil recovery (Singer, 1985; Shephord *et al.* 1995; Desai and Banat 1997).

Since the discovery of biosurfactants, a lot of work has been done to identify species that are able to produce these surface active compounds on cheap substrates.

2.7.1 Biosurfactant-producing species

The production of biosurfactants is generally an extracellular process in most bacteria; however in some yeasts, fungi and bacteria species, it is part of the cell membrane synthesis (Cerniglia 1997; Rahman and Gakpe 2008). There have been a number of studies regarding the production

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of biosurfactants and the microbial species that are able to produce this diverse group of compounds.

Biosurfactant production is a desirable characteristic of hydrocarbon-degrading bacteria (Bento *et al.* 2004). Most of these bacterial species are found in the soil and many studies have focused on determining if biosurfactants are produced by bacteria in soils that are uncontaminated with hydrocarbons or if they are only found in contaminated soils. A study carried out by Bento *et al.* (2004) focused on the characterisation of biosurfactant-producing bacteria species that were isolated from diesel-contaminated soil samples and characterized using 16S rRNA. The organisms were identified to be Bacillus species (*Bacillus cereus, Bacillus sphaericus, Bacillus pumilus, Bacillus fusiformis, Acinetobacter junii, pseudomonas sp.* and *Actinomyces sp*.). Most of these species are rod-shaped gram positive organisms.

Surfactin is a lipopeptide biosurfactant that is believed to be produced by *Bacillus* species. It is an eight member cyclic compound made up of seven amino acids and a β-hydroxydecanoic acid moiety. It was proposed that this particular biosurfactant is produced based on the presence a gene locus denoted as the *spf* locus (Nakano *et al.* 1992) and the presence of this gene, denoted as the *spf* gene is an indication of biosurfactant production in many Bacillus species. This formed the basis of a study carried out by Hsieh *et al.* (2004). The study involved the use of Polymerase Chain Reaction (PCR) as a method of identification of Bacillus species that contain the *spf* gene and hence produce surfactin. Polymerase Chain Reaction, popularly called PCR is an in vitro technique used to amplify DNA sequences with the use of oligonucleotide primers that are complementary to specific sequences in the target gene (Willey *et al.* 2008; Wilson and walker 2010). The study involved screening of 15 standard strains of *Bacillus subtilis* as well as 20 Bacillus sp. for the presence of the *spf* gene and subsequent production of surfactin. It was

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discovered that of the 15 standard strains that were screened, seven of them produced surfactin. Of the 20 Bacillus sp. collected and screened, 16 of them were discovered to surfactin producers; *B. amyloliquefaciens* and *B. circulans* were reported for the first time as surfactin producing organisms (Hsieh *et al.* 2004).

Table 2-3: Popular biosurfactant-producing species

16s rRNA gene sequence(Nearest relative in Genbank)	**Sequence Identity (%)**	**Genus**
Bacillus tequilensis	100	*Bacillus* sp.
Bacillus safensis	100	*Bacillus* sp.
Bacillus licheniformis	100	*Bacillus* sp.
Bacillus cereus	100	*Bacillus* sp.
Acinetobacter paryus	100	*Acinetobacter* sp.
Pseudomonas aeruginosa	100	*Pseudomonas* sp.
Pseudomonas oleovorans	100	*Pseudomonas* sp.
Pseudomonas protegens	100	*Pseudomonas* sp.
Serratia marcesens	100	*Serratia* sp.
Acintobacter junii	100	*Acinetobacter* sp.
Sphingobacterium spiritivorum	100	*Sphingobacterium* sp.
Comamonas sp.	100	*Comamonas* sp.
Buttiauxella sp.	100	*Buttiauxella* sp.
Marinobacter hydrocarbonoclasticus	100	*Marinobacter* sp.
Acinetobacter gyllenbergii	99	*Acinetobacter* sp.
Rhodococcus ruber	100	*Rhodococcus* sp.
Sinorhizobium meliloti	100	*Sinorhizobium* sp.
Marinobacter Pelagius	100	*Marinobacter* sp.
Corynebacterium falsenii	100	*Corynebacterium* sp.
Azorhizobium doebereinerae	100	*Azorhizobium* sp.
Haloplanus sp.	100	*Haloplanus* sp.

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Halopenitus persicus	100	*Halobacteriaceae* sp.
Acinetobacter calcoaceticus	100	*Acinetobacter* sp.

Besides *Bacillus* species, there are other groups of organisms that have been the focus of many studies. *Pseudomonas* species happen to be one of such groups; these organisms are straight or slightly curved rods that are gram negative. They are known for their ability to degrade a wide variety of organic molecules which is a possible explanation for why they are found in hydrocarbon-contaminated environments. They are very useful in the process of mineralization which is the breakdown of organic substances into inorganic molecules (Willey *et al.* 2008).

Pseudomonas species are very well known for the production of rhamnolipids and studies have shown that the presence of rhamnolipids is imperative for the growth of *Pseudomonas aeruginosa* on hydrocarbons (Diezel *et al.* 1996; Mulligan 2005; Olu-Arotiowa *et al.* 2008; Soberón-Chávez *et al.* 2005; Arino *et al.* 2005). It is further known that the production of these biosurfactants occurs when the organism is growing on hydrocarbons, glycerol, glucose or peptone. The highest production of biosurfactants occurred when hydrocarbons or glycerol (hydrophobic compounds) served as the carbon source (Guerra-Santos *et al.* 1984; Hisatsuka *et al.* 1971).

A study conducted by Guerra-Santos *et al.* (1984) revealed that the concentration of biosurfactants produced is dependent on the composition of the medium in which *P. aeruginosa* is cultivated. It was discovered that continuous culture cultivation led to production of higher concentrations of biosurfactant revealing that batch cultures are not advisable if one intends to obtain a sizeable amount of biosurfactants. The study went even further to determine the effect of the presence of Nitrogen sources and yeast extract on the production of biosurfactants. The

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nitrogen sources examined were ammonium and nitrate; it was discovered that biosurfactant production was poorer with ammonium. The effect of the presence of yeast in the medium was also reported. In the presence of yeast extract, the substrate which happened to be glucose in this study was completely used up and the biomass concentration was much higher. A higher production of biosurfactant was however favoured when the yeast extract was omitted from the medium. Another medium component that had a conspicuous effect on biosurfactant production was the concentration of Iron (Fe), a trace element added to the medium. At a high concentration of Fe (about 2mg of Fe per g of glucose), production of biosurfactants by *P. aeruginosa* was completely suppressed.

The interest in *Pseudomonas* species as suitable organisms for the production of biosurfactants have grown most probably due to their mineralization property. This property suggests that these organisms have the ability to grow on many non-metal contaminants, which can be generally grouped as PAHs. PAHs are rather complex and uneasy to degrade, however, certain groups of bacteria (Proteobacteria and high G+C gram positive bacteria have been studied for their ability to degrade these recalcitrant compounds. The Proteobacteria which are gram negative organisms are able to degrade low molecular weight PAHs such as Naphthalene and Phenanthrene while the high G+C gram positive organisms are able to degrade the high molecular weight PAHs (Zhang and Miller 1992; Rocha and Infante 1997).One study focused particularly on the ability of a soil *Pseudomonas* strain to produce biosurfactant while growing on PAH compounds. The bacterium was isolated from a sandpit that had received a lot of oil refinery waste products during the ‘60s. The organism was tested for its ability to grow on PAH compounds by cultivation with Naphthalene and observation of subsequent colour changes. In this study, it was reported that

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sufficient glycolipids were only secreted close to the end of the exponential phase (Deziel *et al.* 1996).

In 2001, Mishra *et al.* conducted a study on the contaminated soil at Mathura refinery situated about 150km south of Delhi, India. According to their report, the indigenous bacteria population in the soil was not adequate to initiate bioremediation. This was probably due to the presence of highly toxic compounds such as metals that were present in the crude oil wastes disposed, preventing the sufficient growth of hydrocarbon-degrading bacteria. The study revealed that enhancement of the bacterial consortium with nutrients resulted in better production of biosurfactants that in turn led to the bioremediation of the organic compounds in the soil. This study also revealed another organism that is capable of biosurfactant production and subsequent hydrocarbon degradation, *Acinetobacter baumannii.* This organism, like *Pseudomonas* belongs to the Proteobacteria group (Willey *et al.* 2008).

A study by Bodour *et al.* (2003) focused on the distribution of biosurfactant producers in contaminated and undisturbed arid soil samples. In this study, 20 soil samples were collected out of which 1305 colonies were obtained. After screening for biosurfactant production, 45 isolates were obtained. With the use of PCR and 16S rRNA sequencing, the isolates were identified revealing a new species of biosurfactant-producing bacteria- *Flavobacterium sp.* The results from this study went further to show that biosurfactant-producing bacteria can be found in most soils- contaminated or uncontaminated. With regards to the distribution of these organisms according to their gram characteristics, the gram positive bacteria were found mostly in soils that were either uncontaminated or contaminated with heavy metals while the gram negative organisms were found in soils that were contaminated with hydrocarbons or a combination of both hydrocarbons and metals.

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Das and Mukherjee (2007) focused on evaluating the biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa*. The results from this study reported that both strains proved effective in the bioremediation process but P. aeruginosa was found to be the more effective of the two strains in reducing the total hydrocarbon content of petroleum-contaminated soil from North-east India.

So far, *Pseudomonas* and *Bacillus* species are the most popular species for bioremediation of oily sludge. However, research has shown the production of biosurfactants in species such as Klebsiella, Lactobacillus, Serratia marcesens, Rhodococcus and Acinetobacter (Matsuyama *et al.*1985; Jenny *et al.* 1991; Batista *et al.* 2006; Soberón-Chávez *et al.* 2005; Velraeds *et al.* 1998; Zhi-feng *et al.* 2010; Jaysree *et al.* 2011; Anyanwu *et al.*2011; Bicca *et al.* 1999). There have also been rare discoveries of biosurfactant production in fungi such as *Aspergillus ustus* and *Ustilago maydis* (Kiran *et al.* 2009; Hewald *et al.* 2005). Biosurfactant-producing bacteria are believed to be found mainly in petroleum-contaminated soil samples (Willumsen and Karlson 1996; Rahman *et al.* 2002; Bodour *et al.* 2003; Barathi and Vasudevan 2001; Yateem *et al.* 2002) however, there have been studies that revealed the presence of biosurfactant-producing bacteria in uncontaminated soils and other environments including marine habitats (Jennings and Tanner 2000; Maneerat and Phetrong 2007; Das *et al.* 2008; Satpute *et al.* 2008; Das *et al.* 2009).

2.7.2 Identification of Biosurfactant-producing species

In order to successfully identify biosurfactant producing colonies, various tests can be carried out. Many of these tests are based on the physical characteristics and physiology of the microorganisms being tested. Studies that involved the isolation of biosurfactant-producing

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bacteria have relied mostly on tests such as hemolysis, emulsification assays and surface tension reduction (Banat 1995; Tabatabaee *et al.* 2005; Lin 1996).

The hemolysis test is based on the idea that biosurfactants can cause lysis of red blood cells. This principle is based on a test that was developed by Mulligan *et al.* (1984). It involves the cultivation of potential bacteria species on sheep blood agar plates. The test however is not absolutely accurate; the lysis of red blood cells cannot be attributed 100% to the presence of biosurfactants and may possibly be a result of enzymes produced by the bacteria during growth, enabling it to utilize blood cells (Schulz *et al.* 1990; Youssef *et al.* 2004; Plaza *et al.* 2006).

The emulsification test is widely used to identify biosurfactant producers and was developed by Cooper and Goldenberg (1987). This test measures the ability of the bacteria culture to form an emulsion with a hydrophobic compound or oil such as kerosene or selected hydrocarbons. The emulsion is calculated after 24 hours using the formula

$$E_{24} = \frac{h_{emulsion}}{h_{total}} \times 100\% \qquad (2\text{-}1)$$

Where $h_{emulsion}$ is the emulsion height that bacteria culture has formed with the selected hydrocarbon and h_{total} is the total height of the liquid in the vessel, which is usually a test tube. This test is considered reliable and has been applied in a number of screening studies.

The drop-collapse technique was first described by Jain *et al.* (1991) and was later modified by Bodour and Miller-Maier (1998). The principle of this method is based on the ability of biosurfactants to associate with both hydrophobic and hydrophilic compounds. The method involves adding a drop of bacteria culture to a surface covered with hydrocarbons or oil. If the

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drop collapses, there is likelihood that the culture contains biosurfactants. A beaded drop however suggests repulsion and the absence of biosurfactants.

The most popular identification of a biosurfactant producing bacterium is the direct measurement of surface tension of the bacteria culture with time. The popularity of this technique is based on the fact that it shows first-hand, the ability of the bacteria to reduce surface and interfacial tension or lack thereof (Batista *et al.* 2006; Willumsen and Karlson 1997). There are a variety of other methods that have been proposed by various authors which include the Du-Nouy-Ring method, the stalagmometric method, the micro plate assay, penetration assay, Hydrophobic interaction chromatography test, amongst many others (Dilmohamud *et al.* 2005; Smyth *et al.* 1978; plaza 2005; Maczek *et al.* 2007). A summary of the different possible screening methods is presented in Table 2-4 (Sen 2010; Walter *et al.* 2010).

Table 2-4: Comparison of screening methods for biosurfactant activity

Analytical Technique	**Qualitative Analysis**	**Quantitative Analysis**	**Analysis speed**	**Application in HTS**
Direct surface/interfacial tension measurement	++	+	min	-
Drop collapse assay	++	-	min	+
Micro plate assay	++	-	min	+
Penetration assay	++	-	min	+
Oil spreading assay	++	-	min	-
Emulsification capacity assay	+	-	d	+/-
Solubilization of crystalline Anthracene	+	-	d	+/-
Bacterial adhesion to hydrocarbons assay	+	-	min	-
Hydrophobic Interaction chromatography	+	-	h	-
Replica plate assay	+	-	d	-

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Salt aggregation assay	+	-	min	+/-
CTAB agar assay	+	-	d	-
Hemolysis assay	+	-	d	-

Qualitative analysis: ++= very efficient, += efficient; Quantitative analysis (of surface activity): += Yes, -+ No; Analysis speed (required time per sample) min= analysis within minutes, h=within hours, d=within days; Application in HTS: += Yes, - = No, +/- = not reported but principally applicable. HTS: High Throughput Sequencing. *Table taken from Sen (2010)

2.8 Microbial Enhanced Oil Recovery (MEOR)

MEOR is a tertiary oil recovery process that involves the use of microbial secondary metabolites in the form of biosurfactants to increase the efficiency of current oil recovery techniques. Currently, the primary and secondary oil recovery methods can only recover about one-third of the oil available in reservoirs, carving a niche for MEOR, not just as a plausible oil extraction method but also as a waste management process (Brown 2010; Donaldson *et al.* 1989). Microorganisms that can be exploited for the purpose of this process are abundant in various environments- oil reservoirs, storage tanks, oil-water separators, soil, marine environments etc. (Sen 2008). These organisms are able to produce biosurfactants while growing on cheap substrates, making them the perfect candidates for MEOR.

Notably, not much work has been done where MEOR feasibility investigations are concerned; it is however important to note the success of the few studies reported so far. In 1982, Jenneman *et al.* carried out an in-situ study to investigate the feasibility of the MEOR technique by injecting biosurfactant-producing bacteria into sandstone cores of 170-md permeability. Less than one percent of the influent bacteria culture was recovered in the effluent and a reduction in the permeability of porous rock was observed. In 1992, a study by Behlugil *et al.* investigated the feasibility of MEOR by applying the technique to Turkish heavy oil at a temperature of 38°C.

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The report stated a 12% increase in the flow of the oil from the reservoir due to reduction in viscosity and changes in pH.

The application of MEOR to an oil field using pseudomonas species showed an increase in oil production in 80% of the oil wells to which the technique was applied (Li *et al.* 2002). Soudmand-asli *et al.* (2007) reported an increase in oil recovery efficiency when strains of *Bacillus subtilis* (a biosurfactant-producing bacterium) and *Leuconostoc mesenteroides* (an exopolymer-producing bacterium) were applied to fractured porous media containing oil. A reduction in oil viscosity was recorded during the course of this study. Desouky *et al.* (1996) also reported an increase in oil recovery using *Streptococcus sp.* with molasses as carbon and energy source.

In a different study that focused on the biological treatment from sludge, Joseph and Joseph (2009) reported up to 97% oil recovery from petroleum sludge using biosurfactant-producing bacteria of *Bacillus* species. The bacteria strains were also inoculated into sludge-sand combinations and successful oil recovery was achieved. The surface tension of the system was monitored closely and steady reduction in surface tension was observed with time. With the use of a sand pack column, Suthar *et al.* (2008) were able to prove the feasibility of MEOR by using *Bacillus* species that resulted in an oil recovery increase of over 43%. A similar study by Yakimov *et al.* (1997) achieved successful oil recovery efficiency increase of 22.1 %. Bordoloi and Konwar (2008) also showed with laboratory studies that biosurfactants from *Pseudomonas* species could increase oil recovery from sand pack columns by 15% at 90°C and at 25% with a reduction in temperature.

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Many of these studies have reported good success with MEOR; however, the technique still remains debatable on an industrial scale (Brown 2010). It is important to note that many studies carried out in this area of research have focused on the successful extraction of oil from reservoirs more than the possibilities of applying this technique as a biological waste treatment method to petroleum wastes. This study therefore focused on the feasibility of MEOR as a biological treatment procedure for petroleum sludge.

CHAPTER 3

RESEARCH METHDOLOGY

This chapter describes the methods that were employed for the successful completion of this study. These include the isolation and characterization of biosurfactant-producing microbes from soil samples, simulation of sludge using an appropriate intermediate compound to stabilize oil-water emulsions, oil characterization to determine concentration of selected PAH compounds in sludge, oil recovery from sludge comparing a batch system and a PFR system, the investigation of water-flooding on oil recovery and the analysis of the TOC content of sludge after oil recovery.

3.1 Isolation of Biosurfactant-producing Microbes

The isolation of biosurfactant-producing microbes was crucial to the success of this study. The isolation of these microbes involved acquisition of a potentially suitable source, growth medium preparation, test for biosurfactant activity and identification of positive isolates using 16s rRNA sequencing.

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3.1.1 Acquisition of soil samples

Petroleum-contaminated soil samples were obtained from three different automobile mechanic stalls in the Central Business District (CBD) area of Pretoria South Africa. In addition, uncontaminated soil samples from two home gardens were acquired. These soil samples served as the potential sources of biosurfactant-producing microbes.

3.1.2 Medium preparation

Mineral salts medium was prepared from two stock solutions, i.e., solution A and solution B. Solution A (Basal salts medium) was prepared by dissolving NH_4Cl (0.535), Na_2HPO_4 (4.259), KH_2PO_4 (2.722), Na_2SO_4 (0.114), $MgSO_4$ (0.0493) in grams per litre of distilled water. Solution B (trace elements solution) was prepared by dissolving $FeCl_3.6H_2O$ (0.08), $ZnSO_4.7H_2O$ (0.75), $CoCl_2.6H_2O$ (0.08), $CuSO_4.5H_2O$ (0.075), $MnSO_4.H2O$ (0.75), H_3BO_3 (0.15) Na_2MoO_4 (0.05) in grams per litre of distilled water. 500ml of solution B was prepared and stored in a dark bottle at 4°C. Solution B was added to solution A at a concentration of 0.2%v/v. All the chemicals listed above were purchased from Merck Chemicals (Pty) Ltd.

Carbon source: Glycerol (Merck Chemicals (Pty) Ltd. was added to the mixture of solution A and B at a concentration of 3 g/L as the carbon source.

The medium was autoclaved at 121°C in Erlenmeyer flasks or 2 Litre Schott bottles. Nutrient broth and Nutrient agar (Merck Chemicals (Pty) Ltd. were prepared as required for the isolation of more fastidious bacteria. The nutrient agar plates were stored at 4°C.

3.1.3 Enrichment and isolation of bacteria

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One gram (1g) of each soil sample was added to 250 mL autoclaved nutrient broth in Erlenmeyer flasks; each flask was incubated at ±30°C on a rotary shaker and left for 48 hours in order to allow for adequate microbial growth. With the use of a p1000 pipette (Thermo Corporation), 1000 µL of each culture was transferred to a new set of flasks, each also containing 250 mL autoclaved nutrient broth, and subjected to the same conditions as the first set of flasks. At the end of the second 48-hour incubation period, 100 µL of each culture was transferred to nutrient agar plates. The plates were incubated at 30°c for an additional 72 hours. The resulting colonies were inoculated in autoclaved mineral salts medium with glycerol as carbon source. The flasks were incubated at ±30°C on rotary shakers and growth in the different flasks was monitored. With the use of an inoculation loop, culture from each flask was streaked out on nutrient agar plates. The plates were incubated at ±30°C for 48 hours. The isolated organisms were further tested for biosurfactant activity. The isolation procedure is illustrated in Figure 3-1 below:

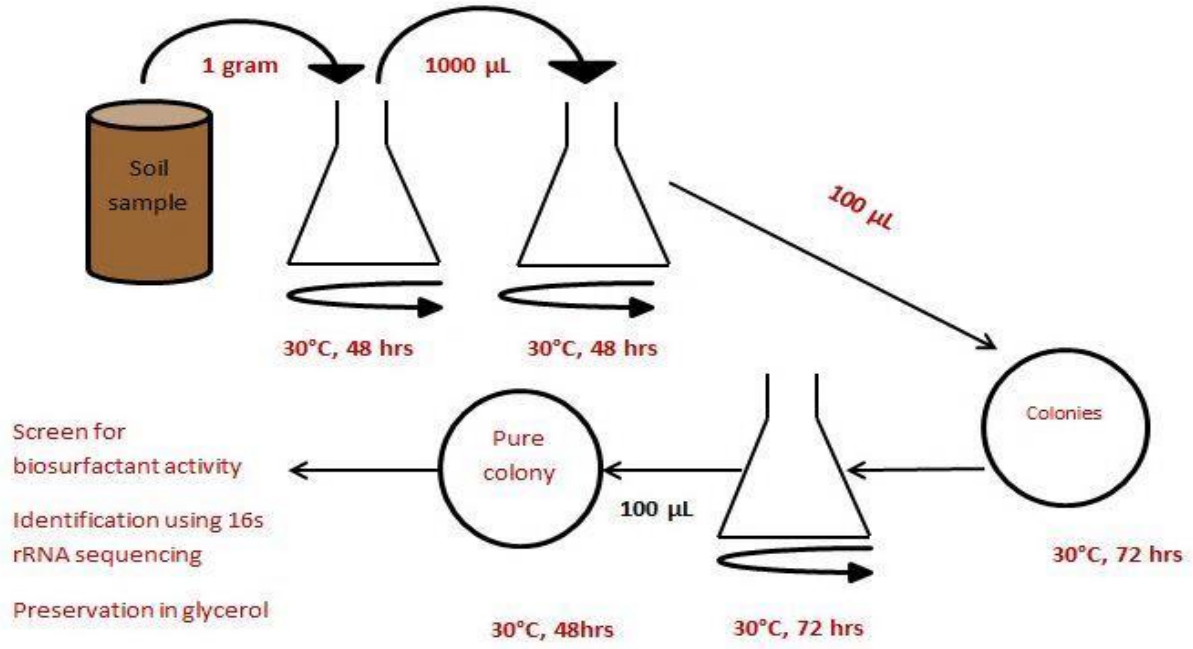

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Figure 3-1: Isolation of biosurfactant-producing microbes from soil samples.

3.1.4 Test for biosurfactant activity

The organisms that were isolated on nutrient agar plates were tested for biosurfactant activity using the drop-collapse technique as described by Bodour and Miller-Maier, 1998. The test was performed in a 96-well micro plate. Each well was lightly coated with oil and left for 12 hours to equilibrate. 5 µL of culture was added to the centre of the well and observed after one minute.

The principle behind the drop collapse technique is based on the structure of biosurfactants. Biosurfactants are amphipathic molecules- they have both hydrophilic and hydrophobic ends. The test is based on the principle that a microbial culture that contains biosurfactants will interact with the oil base in the micro plate. In other words, the culture added to the oil base will collapse if biosurfactants are present or it will form a bead repelling the oil base in the plate in the absence of biosurfactants in the medium. The test is quick to perform, allows for numerous samples to be tested simultaneously, and is considered to be one of the most accurate qualitative tests for biosurfactant activity.

The cultures that showed biosurfactant activity were preserved in glycerol and stored in freezer boxes at -20°C. Sample cultures of the positive isolates were identified at the Microbiology Department of the University of Pretoria, South Africa using 16s rRNA sequencing.

3.1.5 16s rRNA sequencing

The use of 16s ribosomal RNA for the identification and classification of bacteria is a well-accepted and widely used procedure. It is an identification technique based on the irrefutable

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similarities between microbes of the same species in certain regions of the ribosomal RNA known as oligonucleotide signature sequences (Willey *et al.* 2008). These similarities enable the easy identification of a microbe that is closely related to a well-known species.

In order to identify the unknown biosurfactant-producing microbes isolated, pure cultures were streaked out on nutrient agar plates and incubated at 30°C for a period of 48 hours. The first step in the identification procedure involved the isolation of whole cell proteins of these microbes followed by a SDS-PAGE test. This was followed by the isolation of rRNA. Fragments of rRNA genes isolated from each microbe were amplified by PCR using conserved primers pA and pH (sequences shown below)

pA: 5'AGAGTTTGATCCTGGCTGAG 3'

pH: 5' AAGGAGGTGATCCAGCCGCA 3'

The PCR products were purified using a QIAquick PCR purification kit (Qiagen). The subsequent sequencing was carried out using a DNA sequencer with the sequencing primer PD.

PD: 5' CAG CAG CCG CGG TAA TAC 3'.

The resulting sequences were compared using genetic computerized databases such as BLAST (Coenye *et al*. 1999).

3.2 Selection of Carbon Source

Biosurfactants are secondary metabolites that are produced by microorganisms as they grow on suitable carbon sources (Rodrigues *et al.* 2006). Owing to this, it was important to select a

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carbon source that could instigate a high growth density, lead to an increase in biomass and a subsequent increase in the concentration of biosurfactants produced.

The effects of glycerol, glycerol + hexane, hexane and mineral salts medium without any carbon source on each organism were investigated separately.

Erlenmeyer flasks, each containing 250 mL mineral salts medium were prepared and separated into four sets of three each. Set one contained glycerol (3g/L), set two contained hexane (3mL), set three flasks contained both glycerol (3g/L) and hexane (3mL) while set four contained no carbon source and served as control flasks for this study. Each of the biosurfactant-producing microbes isolated was inoculated into flasks containing the various carbon sources to be investigated; it was ensured that each microbe was inoculated in four different flasks (three containing carbon sources and one without a carbon source). The flasks were incubated at ±30°C on a rotary shaker and the growth of the microorganisms was monitored by measuring the OD of the cultures at 600nm.

3.3 Bacteria Incubation Time

Prior to the inception of the oil recovery process, it was important to determine the amount of time required for the bacteria consortium to produce biosurfactants, while growing on an appropriate carbon source. In order to determine the optimum incubation time, the isolates that tested positive for biosurfactant activity were individually inoculated into mineral salts medium containing an appropriate carbon source. Samples were taken from each culture after 8 hours, 12 hours, 18 hours and 24 hours of incubation and tested using the drop-collapse test. A culture

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consortium of all the positive isolates was also tested at the same time intervals for biosurfactant activity using the drop-collapse test.

3.4 Sludge stability test

Petroleum sludge is known for its oily semi-solid, paste-like structure. Although the individual components of oily sludge are unknown, the general components are known to be fine solids in the form of sand, water, oil and a combination of heavy metals. The simulation of sludge, coupled with a crucial stability test involved the investigation of two compounds - fumed silica (SiO_2; Sigma Aldrich, particle size: 0.0007µm) and kaolinite ($Al_2O_7Si_2.2H_2O$; sigma Aldrich).

15g of each chemical was added to separate beakers, each containing 350 mL water and 95 mL oil. The beakers were incubated at ±30°C and monitored for the possible dissociation of the components. The emulsion stability was calculated using the formula below:

$$\%ES = \frac{EV_t}{EV_{t=0}} \times 100 \qquad (3\text{-}1)$$

(Where %ES is the percentage emulsion stability, EV is the emulsion volume, t is the time measured in hours).

The emulsion stability can also be calculated using the emulsion height in the vessel using the formula below:

$$E_{24} = \frac{h_{emulsion}}{h_{total}} \times 100\% \qquad (3\text{-}2)$$

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Where E_{24} is the emulsification index after 24 hours, and h is the height of the emulsion and the height of the total volume.

Furthermore, a mass gradient study was carried out to determine the possible effects of mass of the fine solids component on the stability of oil-water emulsions. Varying masses (5g, 10g, 15g, 20g and 25g) of kaolinite and fumed silica were added to separate beakers, each containing 350 mL of water and 95 mL oil. The beakers were incubated at ±30°C and the emulsion stability of the emulsion formed in each beaker was calculated using equation 3-2.

3.5 Oil Recovery from Oily Sludge

The possibility of recovering oil from petroleum sludge using biosurfactant-producing bacteria was investigated by comparing two systems- a batch system which was synonymous to landfarming and a PFR system

3.5.1 Oil Recovery using Batch System

Oily sludge was prepared in the laboratory by adding a fine solids component (5% m/v) to oil (21% v/v) and water (77% v/v) to produce a total of 1200 mL oily sludge. A consortium (500 mL) of biosurfactant-producing microbes was added to sludge in 2000 mL beakers and stirred at medium speed for a total of 60 minutes. The beakers were incubated at ±30°C with a piece of foil placed over them to reduce the chances of volatilization. The aqueous phase which eluted from the mixture was measured using the millimetre measurements of a ruler (Penflex). The experiment was repeated in three beakers and the results were recorded every 12 hours for a total period of 120 hours. Oil and water recovery was calculated using the formula below:

$$\%R = \frac{h_{aq}}{h_{total}} \times 100 \qquad (3\text{-}3)$$

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Where %R is percentage recovery, h_{aq} is height of aqueous phase and h_{total} is the total height of the mixture in the beaker. An illustration of the batch experiment is shown in Figure 3-2:

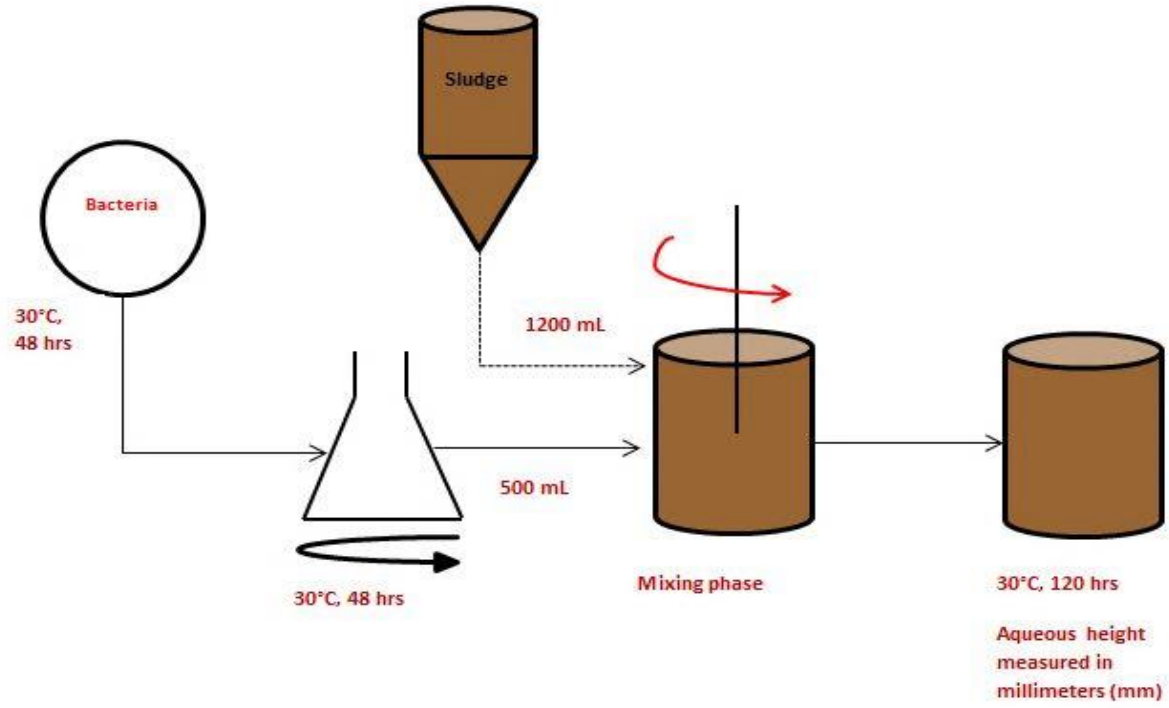

Figure 3-2: Schematic illustration of oil recovery study using batch system.

3.5.2 Oil Recovery using PFR

One of the primary objectives of this study, stated in an earlier chapter, was to design a cost-effective oil recovery system that could facilitate the separation of the components of oily sludge with possible maximum efficiency. In order to achieve this, a simple plug flow reactor system was designed. The reactor was assembled to include a 10 L petroleum sludge tank, a 5 L vessel for biosurfactant-producing bacteria, a 10L mixing vessel with an overhead stirrer denoted as mixing vessel A, a 20 L mixing vessel with an overhead stirrer denoted as mixing vessel B and a 25 L separating vessel built with a moderate steep flow direction and outlets drilled in at the end

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for product collection. The system was operated as a variable velocity plug flow system with the aid of pumps. A comprehensive illustration of the system is shown in Figure 3-3.

The separation vessel of the system was built by meticulously joining measured glass sheets with the aid of glue and a glue gun. The vessel was completely sealed using Pratley clear glue to avoid possible leakage. Three outlet holes were drilled into the separation vessel and glass pipes were glued into these holes; it was expected that at the end of the recovery process, each outlet could serve as a collection point for the three main components that were used to simulate sludge- fine solids, oil and water. The reactor was operated at ±30°C and the amount of oil recovered was recorded at 12 hour intervals for a total period of 120 hours for each experiment.

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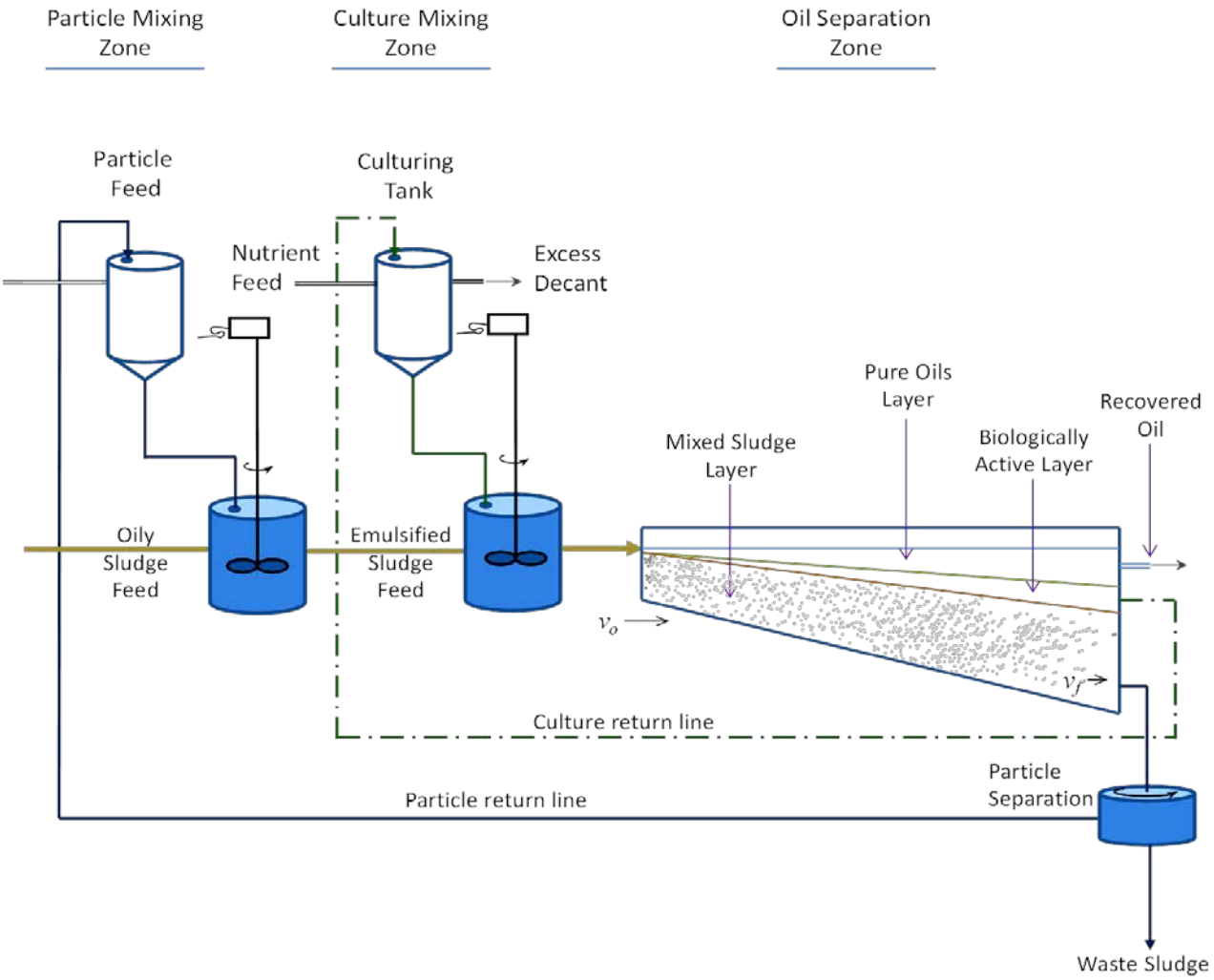

Figure 3-3: Schematic illustration of plug flow reactor system

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The recovery of oil in both batch and plug flow systems was investigated under varying conditions. Firstly, oil recovery was investigated with the omission of biosurfactant producing bacteria. These experiments served as control experiments, and served as a basis against which the other results were compared. Secondly, the recovery of oil with the addition of biosurfactant-producing bacteria in both systems was investigated.

Furthermore, the effect of water on oily sludge, as an emulsifying/demulsifying agent was investigated. The addition of water to oil reservoirs, to aid the recovery of heavy of heavy oil fractions has been in practice since the early 1960s (Gogarty and Tosch 1968). The oil recovery studies in both batch and plug flow systems were investigated with waterflooding as a factor. In the plug flow system, studies were first carried out with 10L of water added to 10L of sludge and the omission of biosurfactant producing bacteria. The studies were repeated with the addition of 5L of distilled water to sludge, followed by the addition of 5L of biosurfactant-producing bacteria. The same process was followed for the batch studies; 500mL of distilled water was added in the first scenario and 250mL distilled water + 250mL biosurfactant-producing bacteria in the second scenario. All the experiments were carried out in duplicate in order to obtain an average of the results.

3.6 Oil Acquisition and Characterization

The oil used in the simulation of oily sludge for this study was obtained from the tribology laboratory at the Chemical Engineering department, University of Pretoria, South Africa. It was waste oil which was to be discarded by the laboratory after experiments and contained a mixture of petrol, diesel, crude oil, and paraffin.

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In order to determine the constituent compounds of the oil mixture, GC-MS analysis was carried out. 500 µL of oil was diluted with 500 µL of dichloromethane (CH_2Cl_2). The resulting mixture was analysed using a Perkin Elmer Clarus Gas Chromatography (GC) system which comprises of a Clarus GC and a Clarus 600T Mass Spectrometer (MS) (Perkin Elmer, South Africa). The GC system injector was operated in a split/splitless mode using a PSI and temperature gradient of 75°C to 300°C for a period of 60 minutes. The injection of the samples was achieved by a multi-mode autosampler which comprises of an 82 vial multi-injection automated rack and uses a 50µL syringe. The chemical separation component used was the Elite 5MS GC system capillary column (length: 30 m, internal diameter: 250 µm, film thickness: 0.5 µm) for Perkin Elmer. The carrier gas used during the course of the analysis was helium (He) gas, 99% purity which was applied at a flow rate of 1 mL/min. The MS interface has an Electron Ioniser (EI), a high performance mass analyser and a detector consisting of a dynode, phosphor plate and a photomultiplier tube. The MS database used for identification of constituent compounds is NIST 11/2011 mass spectral library.

3.6.1 Oil Sample analysis

Oil sample volumes of 0.5µL were injected into the GC system, operating at a fast injection speed, with an injector port temperature set at 250°C. The column oven program was initiated at 75°C (not held) and increased at 5°C per minute until it reached 300°C. The carrier gas split flow rate was set at 3mL/min and of 1 mL/min. The MS method was operated using Electron Ionisation (EI^+) mode centroid data setting. The scan duration was set at 0.3 seconds and an inter-scan delay was set at 0.2 seconds.

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The oil samples were analysed before and after the oil recovery experiments; the peaks obtained from this analysis, in addition to a standard curve created by measuring different concentration of oil diluted with dichloromethane were used to calculate the concentration of individual compounds identified in the oil sample. This was crucial as it later served as a means to compare concentrations before and after recovery and subsequently aided in determining possible degradation that may negatively impact overall oil recovery.

3.7 TOC Analysis

On completion of the oil recovery process using the batch and plug flow systems, the residual sludge from both systems was analysed for its total organic carbon content. Samples from the residual sludge which remained after possible maximum recovery of oil were taken every three days, for a total period of 15 days. 20 mL of each sludge sample was diluted with 20 mL of distilled water. 20 mL of the resulting solution was analysed using Shimadzu Total organic carbon Analyzer, Pretoria, South Africa. The TOC Analyzer works by reacting the compounds in the samples with a persulfate and phosphoric solution (prepared by dissolving 60g of sodium persulfate and 15 mL of 85% phosphoric acid in distilled water to achieve a final volume of 500mL). This reaction results in the release of CO_2 which is then measured as the amount of organic carbon contained in the sample.

The samples were analysed using the NPOC analysis method; this method involved the initial removal of inorganic carbon from the sample by first adding an acid to the sample-phosphoric acid was used in this case and acid addition was automatically handled by the ASI component of the equipment - in order to reduce the pH value to about 2 or 3. This was followed by a purging of the samples with nitrogen gas and subsequent TOC analysis. The carrier gas which was

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nitrogen was set at a carrier gas pressure of 300kPa and a flow rate of 200 mL/min. The TC/IC method was avoided because of the higher chances of errors and background analyses that could adversely affect the results obtained.

The aim of these analyses was to observe any degradation of compounds in the residual sludge matrix, by the bacteria consortium after the oil recovery processes had been completed. A reduction in total organic carbon content of residual sludge could highlight the action of bacteria still active in the system. The use of fumed silica as the fine solids component of oily sludge positively impacts the TOC analysis of hydrocarbons. Fumed silica has no carbon of its own that can interfere with the analysis, therefore creating room for complete monitoring of the hydrocarbons in the sludge matrix.

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CHAPTER 4

BIOSURFACTANT ACTIVITY IN MICROBIAL CULTURES AND SLUDGE SIMULATION

4.1 Isolation of biosurfactant-producing bacteria

Cells cultured from contaminated and uncontaminated soil samples produced approximately seven different colony morphologies on nutrient agar plates, which were tested for biosurfactant activity using the drop-collapse test. Different cultures were re-suspended in nutrient broth for 24 hours till the mid-log phase. Seven wells of a 96 well micro-plate were coated with oil and 5 µL of each culture was added to the wells and observed after less than five minutes. The drop collapse tests revealed only three of the seven isolates were biosurfactant-producing microbes. The identification of these organisms using 16s rRNA sequencing technique showed closest associations with 99% confidence to *Bacillus cereus, Klebsiella oxytoca and Cronobacter dublinensis (*former *Enterobacter sakazakii).*

4.1.1 *Bacillus cereus*

The genus *Bacillus* is one of the most popular for biosurfactant production. *Bacillus subtilis* is known for its ability to produce surfactin- the most effective biosurfactant on the market (Makkar and Cameotra 1997). Although *Bacillus cereus* has only been reported in a couple of studies as a biosurfactant producer (Hsueh *et al.* 2007, Velàzquez-Aradillas *et al.* 2011), it is believed that members of the genus *Bacillus* possess the genetic dexterity for the production of biosurfactants. An example is the presence of the *spf* gene present in Bacillus subtilis known to code for the production of the biosurfactant surfactin. *Bacillus cereus*, like all other *Bacillus* species is a gram positive rod-shaped organism. *Bacillus cereus* is aerobic or facultatively

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anaerobic, suggesting it is able to grow in the presence or absence of oxygen (Drobniewski 1993).

Figure 4-1: Phylogenetic relationship between well-known *Bacillus* species.

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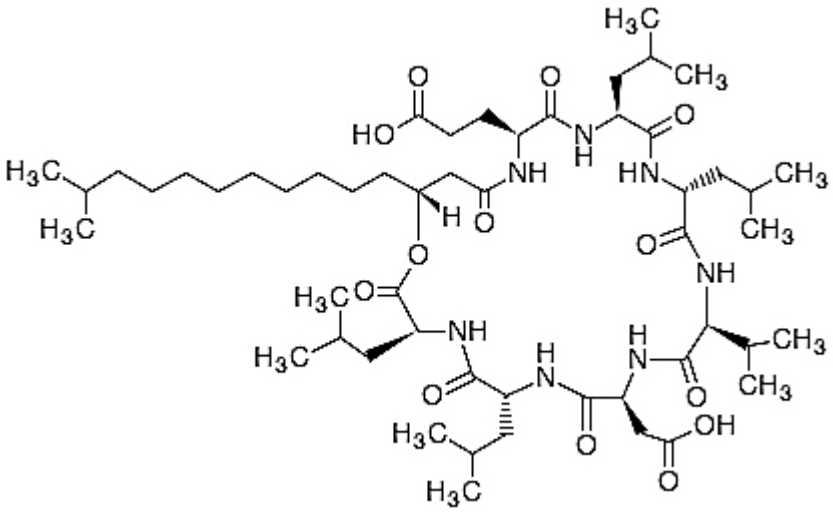

Figure 4-2: Chemical structure of surfactin

4.1.2 *Klebsiella oxytoca* and *Cronobacter dublinensis* (former *Enterobacter sakazakii*)

Klebsiella oxytoca has been sparsely reported as a biosurfactant producer. Although sparse, the studies have confirmed the ability of this organism to produce biosurfactants and also its ability to degrade hydrocarbons and utilize them as carbon and energy sources (Chamkha *et al.* 2011, Kim and Kim 2005). There have been no reports of biosurfactant production by *Cronobacter dublinensis*; however, a study by Jain *et al.* 2012 reported the isolation and characterization of a biosurfactant produced by *Cronobacter sakazakii*, an organism that is believed to be closely related to *Cronobacter dublinensis*.

Klebsiella and *Cronobacter* species both belong to the family Enterobacteriaceae and are very closely related. Members of this family are very well known for their ability to degrade sugars by means of the Embden-Meyerhof pathway (see Appendix) (Willey *et al.* 2008). *Klebsiella*

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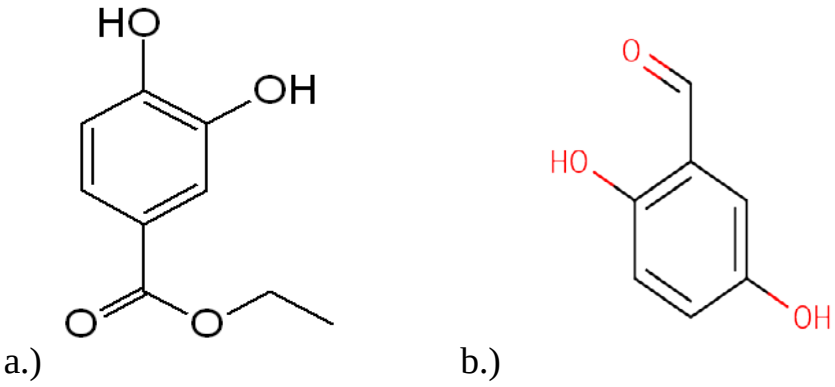
oxytoca and *Cronobacter dublinensis* are not well-studied organisms. For this reason, very little is known about them. A study by Lee *et al.* (2008) showed the biosurfactant production by *Klebsiella* sp. isolated from waste soybean oil. Chamkha *et al.* (2011) focused on the characterization of a *Klebsiella oxytoca* strain that was isolated from a Tunisian off-shore oil field. It was reported during this study that *Klebsiella oxytoca* is able to degrade a wide range of aliphatic hydrocarbons, especially C_{13} to C_{30} compounds. The study also showed that *Klebsiella oxytoca* was able to degrade aromatic compounds gentisate and protocatechuate. This is most likely due to the presence of a mono-oxidase enzyme in *Klebsiella oxytoca*; however, no studies have been conducted showing a possible relationship between this enzyme activity and biosurfactant production. The ability of Klebsiella oxytoca to degrade aromatic hydrocarbons highlights its possible use as a bioremediation organism. Another study by Kim and Kim (2005) focused on the characterization of a novel biosurfactant from *Klebsiella oxytoca*, confirming that this organism is a biosurfactant producer and can be further investigated for use in MEOR.



a.) b.)

Figure 4-3: Chemical structures of protocatechuate (a) and gentisate (b).

It was observed during the course of this study that *Klebsiella* and *Cronobacter* produced a lot of foaming in the culture flasks. Foaming is seen as an attribute of biosurfactant producing organisms (Pagilla *et al.* 2002, Guerro-santos *et al.* 1986, Mulligan 2005); this attribute however

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is believed to cause a reduction in the quantity of biosurfactant produced by bacteria. On the other hand, Mulligan (2005) suggested that the foam produced could be useful in the extraction of oil as it most likely contains secondary metabolites produced by the bacteria and could aid in the dissociation of oil from rocks, facilitating EOR.

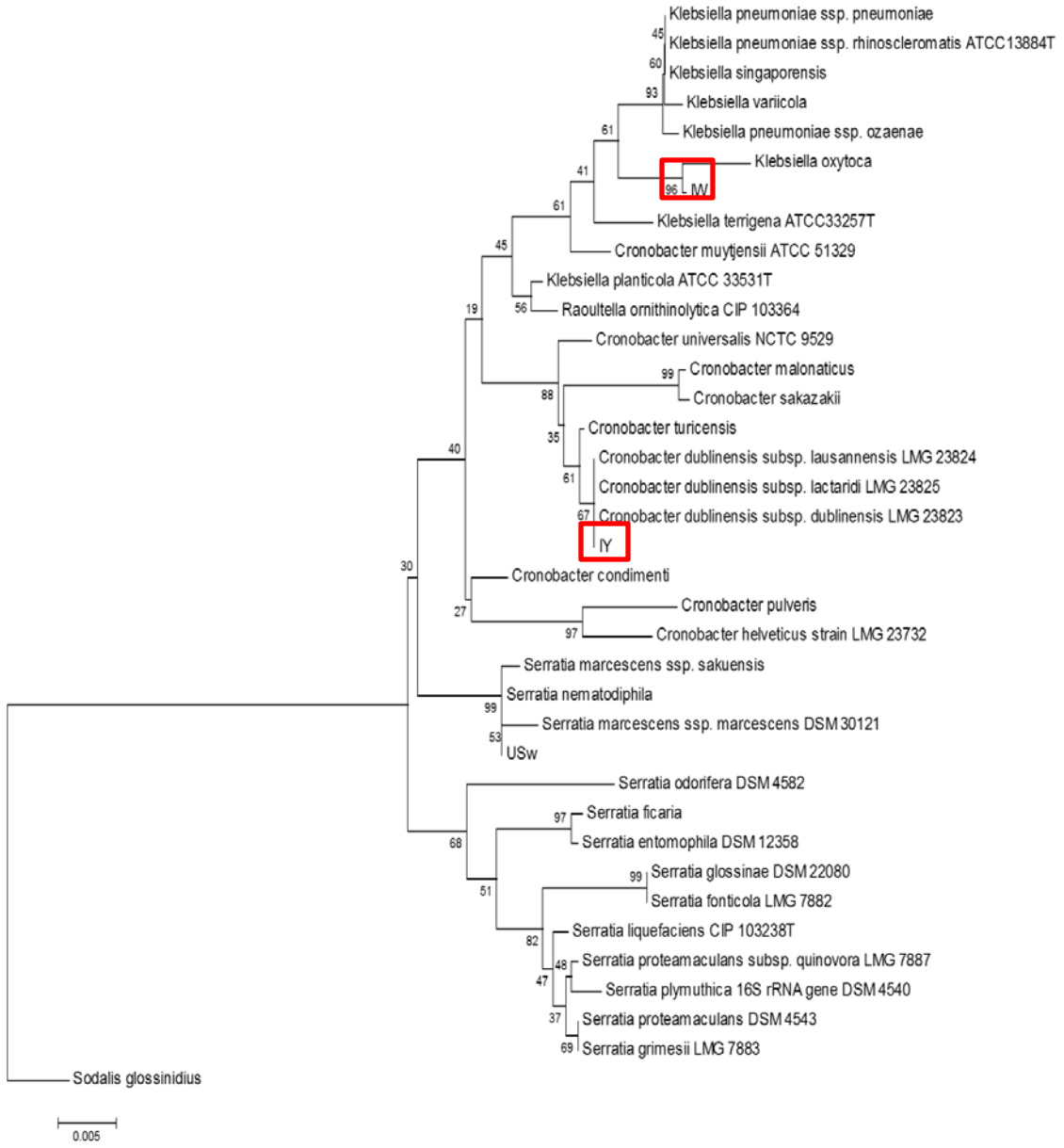

Figure 4-4: Phylogenetic relationships between *Cronobacter* and *Klebsiella* species

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4.2 Selection of Carbon Source

In order to select the appropriate carbon source for bacteria growth, four experiments were carried out. The biosurfactant-positive isolates were incubated on glycerol, glycerol + hexane, hexane, and for the control experiments, carbon sources were omitted. The growth profiles for each microbe on the different carbon sources investigated are presented in figures 4-5 to 4-7:

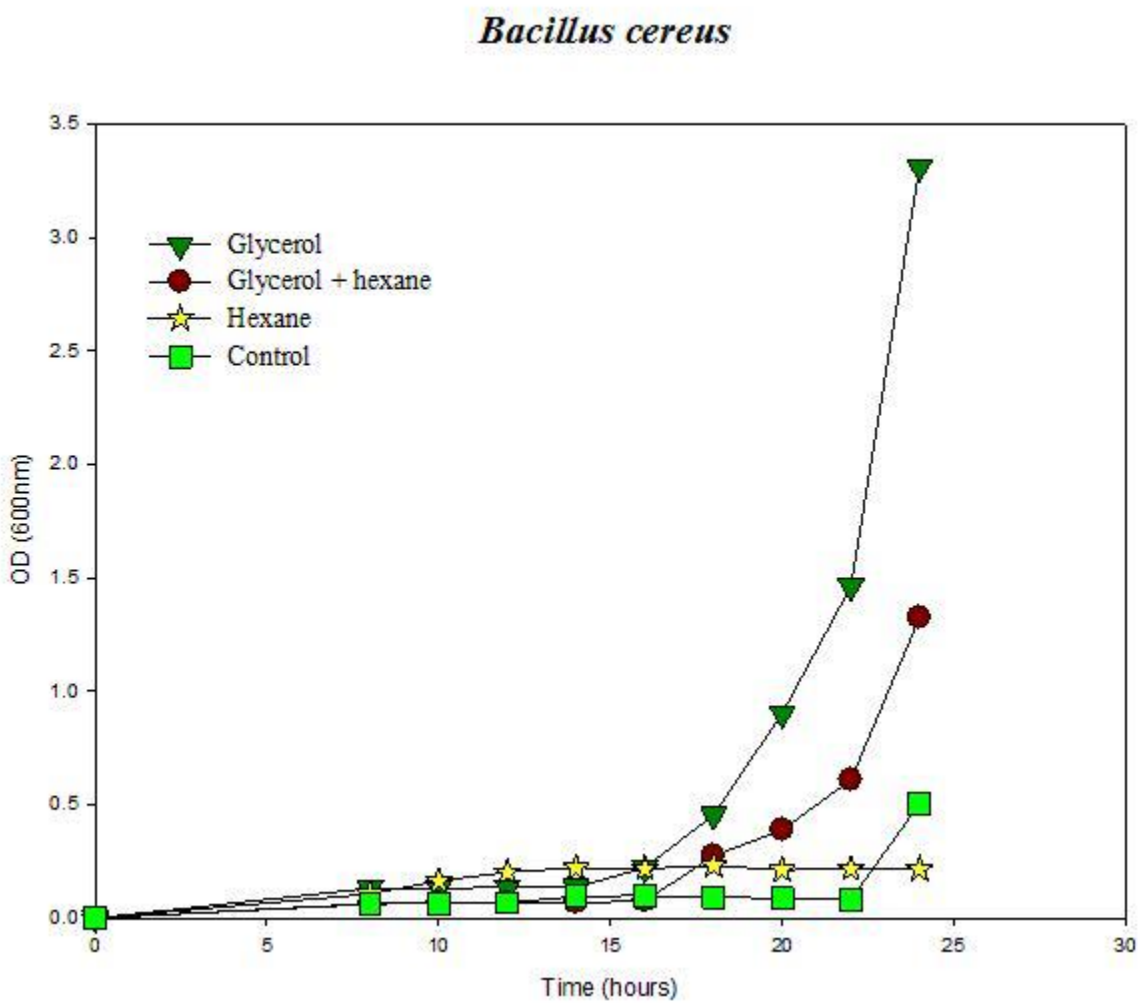

Figure 4-5: Growth profile of *Bacillus cereus* on glycerol, hexane, glycerol + hexane and without any carbon source.

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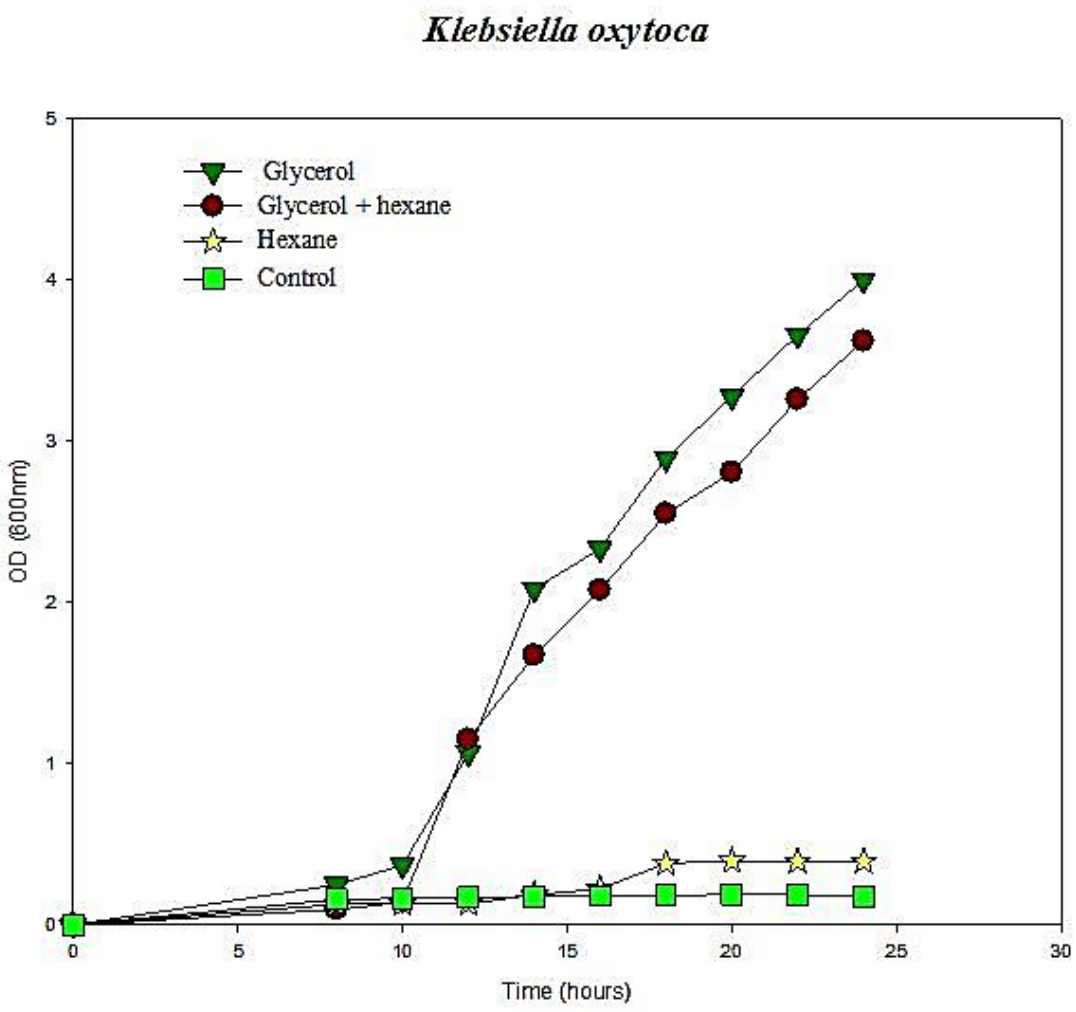

Figure 4-6: Growth profile of *Klebsiella oxytoca* on glycerol, hexane, glycerol +hexane and without any carbon source.

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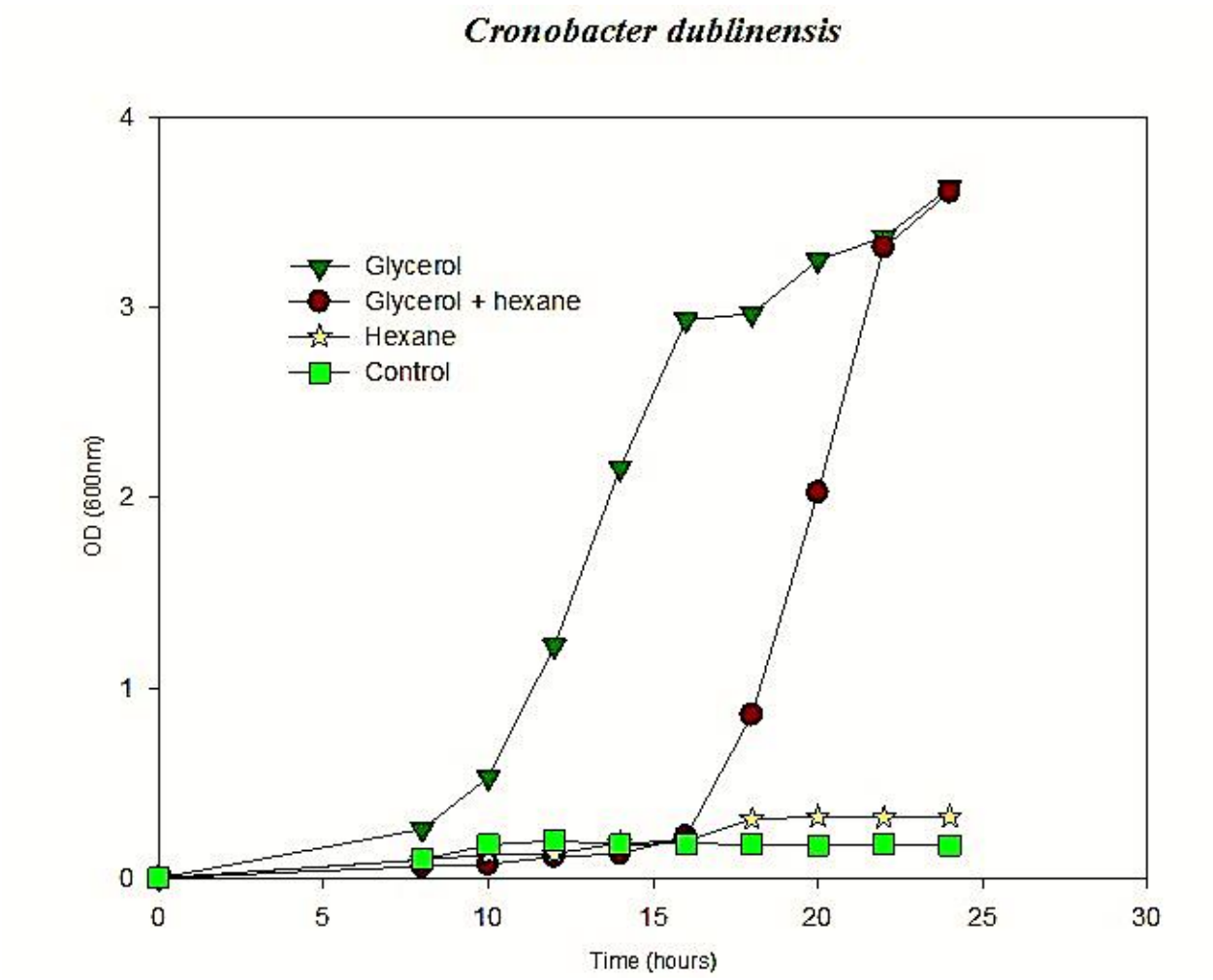

Figure 4-7: Growth profile of *Cronobacter dublinensis* on glycerol, hexane, glycerol + hexane and without any carbon source.

The growth profiles obtained for the three isolates showed conspicuously that all three isolates struggled to grow on hexane. The profiles obtained for hexane as a carbon source was very similar to the profiles obtained for the control experiments where a carbon source was absent from the medium. As can be seen in the graphs above, after 24 hours of incubation on hexane, the biomass of the cells was barely sufficient to initiate biosurfactant production. The inability of the organisms to utilize hexane as a carbon source may lie in the individual genetic makeup of the three organisms as well as the absence of the necessary pathways for degradation. *Klebsiella*

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oxytoca and *Cronobacter dublinensis* are known to degrade sugars using the Embden-Meyerhof pathway to generate two molecules of pyruvic acid (see appendix C) (Willey *et al.* 2008). On the other hand, *Bacillus* species are known to utilize the pentose phosphate pathway (see appendix) or glycolysis followed by the TCA cycle (see appendix C) (Stülke and Hillen 2000). The pathways for the degradation of aromatic and aliphatic hydrocarbons by *Bacillus cereus*, *Cronobacter dublinensis* and *Klebsiella oxytoca* are unknown at this point. However, some studies suggest that the degradation pathways of hydrocarbons may be subjected to repressors and activators, depending on various environmental conditions (Abril *et al.* 1989; Prabu and Phale 2002).

The inability of the three isolates to degrade hexane was considered to be a positive attribute. It was important to achieve little or no degradation of small chain alkanes during oil recovery. Small chain alkanes and alkenes are used in the production of many petroleum compounds, hence their degradation during the process of oil recovery should not be encouraged. Some small chain hydrocarbons also serve as platform compounds in the synthesis of chemicals in other industries.

The profiles for glycerol and the combination of glycerol and hexane were much different from the profiles obtained for hexane. *Klebsiella oxytoca* and *Cronobacter dublinensis* grew to higher densities in both scenarios. Although *Bacillus cereus* struggled, it was able to reach an impressive density by 24 hours. From these growth profiles, it was decided that glycerol was the best carbon source to facilitate the growth of the bacteria consortium which was to be used in the oil recovery process.

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4.3 Bacteria Incubation Time

Bacillus cereus, *Klebsiella oxytoca* and *Cronobacter dublinensis* as well as a consortium of all three bacteria cultures were tested for biosurfactant activity using the drop-collapse test to estimate the minimum amount of time required for the production of biosurfactants. The organisms were separately incubated in MSM at ±30°C. Samples were taken at time intervals of 8 hours, 12 hours, 18 hours and 24 hours after incubation and tested for biosurfactant activity. The results obtained from these series of test are presented in table 4-1.

Table 4-1: Evidence of biosurfactant activity by bacteria cultures at different time intervals.

Time (hours)	***Bacillus cereus***	***Klebsiella oxytoca***	***Cronobacter dublinensis***	**Bacteria consortium**
0	-	-	-	-
8	-	-	-	-
12	-	-	-	-
18	-	+	+	-
24	+	+	+	+

Klebisella oxytoca and *Cronobacter dublinensis* tested positive for biosurfactant activity after 18 hours of incubation on glycerol while *Bacillus cereus* only tested positive after a period of 24 hours. The bacteria consortium also showed positive activity after an incubation period of 24 hours. Bacteria are known for being competitive, especially in their natural environment where resources and space are often limited (Hibbing *et al.* 2010). The biosurfactant consortium culture

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took a longer time to show any biosurfactant activity. This can be explained by possible competition between the different species for available resources. Although the concentration of glycerol was tripled to 9 g/L, competition between the bacteria species cannot be ruled out. Given that the individual growth profiles showed *Bacillus cereus* required a longer lag phase, it was easy to determine that this organism struggled in a more competitive environment. The growth profiles of *Klebsiella oxytoca* and *Cronobacter dublinensis* showed these two bacteria were able to grow at almost the same rate; this most likely led to a more belligerent competition between the two species for available nutrients, subsequently leading to a delay in the production of biosurfactants in the bacteria consortium. Owing to these results, it was decided that the bacteria consortium would be harvested after an incubation period of 36 hours, allowing for a higher concentration of biosurfactants at the time of harvest to ensure a higher efficacy during the recovery process.

4.4 Sludge Simulation and Stability Test

Fumed silica (SiO_2; particle size: 0.0007 µm) and kaolinite ($Al_2O_7Si_2.2H_2O$) were compared as fine solid components of the synthetic sludge used in this study. The beakers were incubated at ±30°C and observed after 24 hours. Complete dissociation of components was observed in the beaker containing kaolinite. The kaolinite particles segregated to the bottom of the flask while oil segregated to the top, resulting in three distinct layers (see figure 4-7). Increasing masses of kaolinite added to the same volume of water and oil and incubated under similar conditions showed no difference in results. Owing to these results, kaolinite was considered to be inappropriate for the simulation of sludge.

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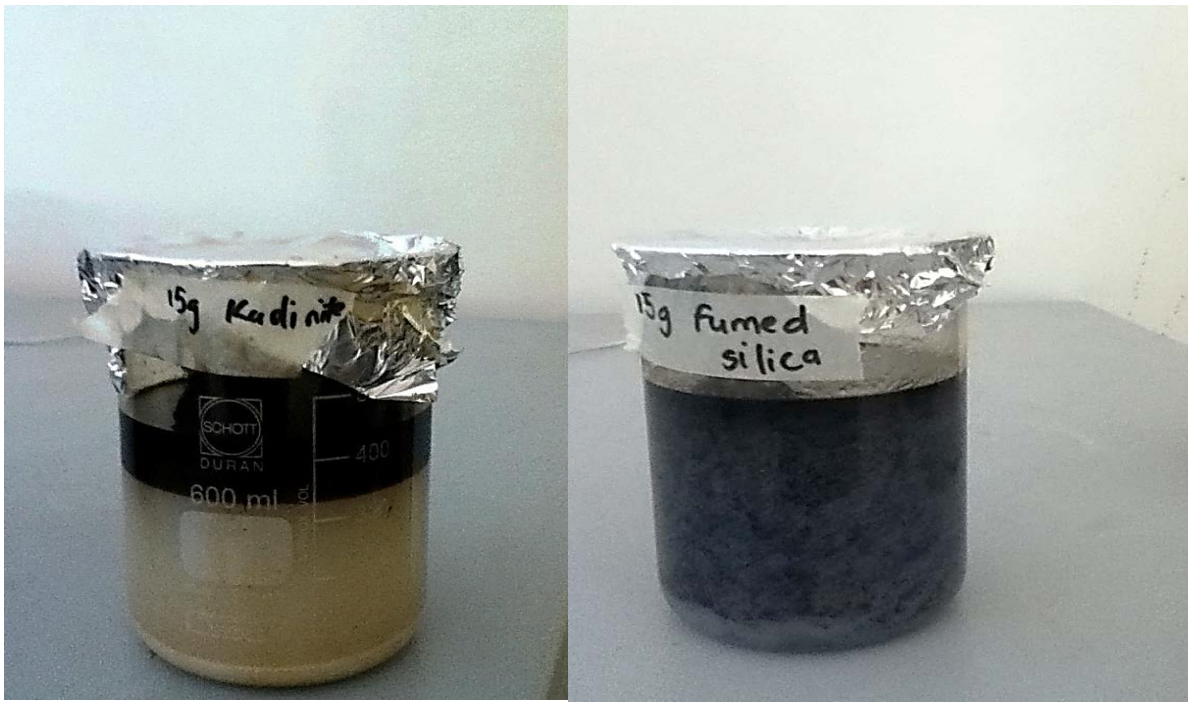

Figure 4-8: Comparison of fumed silica and kaolinite.

On the other hand, the fumed silica emulsion remained stable for more than five days. The stability of the emulsion formed by the fumed silica particles, oil and water was calculated using the following formula:

$$\%ES = \frac{EV_t}{EV_{t=0}} \times 100 \qquad (4\text{-}1)$$

Where %ES is the percentage emulsion stability, EV_t is the emulsion volume at a certain time and $EV_{t=0}$ is the initial emulsion volume.

The effect of varying masses of fumed silica on emulsion stability was investigated. The results obtained, using Equation 4-1, are presented in Table 4-2.

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Table 4-2: Emulsion stability of varying masses of fumed silica

Mass of fumed silica (g)	Emulsion Volume at 24h ($EV_{t=24}$) (mL)	Initial Emulsion volume ($EV_{t=0}$) (mL)	% Emulsion stability
15	295	445	66.29
20	337	445	75.73
25	386	445	86.74

The results presented in Table 4-2 show that an increase in mass of fumed silica led to a higher stability of the sludge produced. This finding is supported by a study conducted by Frelichowska *et al.* 2010 in which the authors showed that the stability of an oil-in-water emulsion increased with increasing concentration of fumed silica particles. The report also suggested that capillary condensation of oil within silica aggregates probably provided a mechanism that contributed to the stability of emulsions.

On the other hand, fumed silica also did not dissolve in water, but instead formed globular-like structures, suggesting a partial hydrophilic nature. Instead of complete dissolution, the fumed silica formed a paste-like structure suggesting that the micro particles absorbed the water instead of dissolving in it. Continuous addition of fumed silica to water led to more absorption until all water particles were absorbed and discernible agglomerations of fumed silica particles were observed in the mixing vessel.

Given the sharing of electrons between silicon and oxygen, fumed silica (SiO_2) is a non-polar compound while water is a polar compound due to the higher electronegativity oxygen has in comparison to hydrogen. Ideally, no interaction would occur between a polar and a nonpolar

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compound but cohesive and adhesive forces play a role in the ability of fumed silica to stabilize oil-water emulsions. Cohesion is the ability of molecules of the same substance to stick to one another while adhesion is the ability of different molecules or surfaces to cling to each other (Eberhart *et al.* 1991). Water is well known for its cohesive and adhesive properties, making it able to cling to itself or adhere to solid particles such as fumed silica to form Pickering emulsions. Pickering emulsions are formed by using a solid compound to stabilize otherwise immiscible liquid phases (Chirwa *et al.* 2013).

A study by Vakarelski *et al.* 2000 shed more light on the formation of adhesive forces between water and fumed silica particles. According to this study, the adhesive force is generated by van der Waals attraction and some additional force that is most likely due to surface property e.g. the Si-O-Si bridging force. There are some other studies that have supported the theory of adhesion between silica particles and water. Binks and Lumsdon 1999 reported the ability of fumed silica particles to stabilize oil-water emulsions in the absence of salts e.g. NaCl. Perino *et al.* 2013 carried out a similar study during which the authors investigated the effect of fumed silica particles on water-in –crude oil emulsions and reported an increase in stability with the addition of fumed silica particles. The study by Perino *et al.* also suggested that the use of hydrophilic fine solids tend to cause the dissociation of components in oil-water emulsions which suggested that the binding of fumed silica to water was not due to its hydrophilic/hydrophobic nature but could be a result of cohesive and adhesive forces at play. Yan *et al.* 2001 further supported these findings by reporting that the use of hydrophilic particles achieved an emulsion stability that lasted only for short periods. The use of hydrophobic fumed silica particles however led to stable oil-water emulsions that lasted for days. It was suggested that suspension of fumed silica

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particles in the aqueous phase prior to emulsification led to the formation of more stable emulsions as flocculation was unlikely.

The complete dissociation of kaolinite from water and oil can be attributed to its completely hydrophobic nature. During the sludge stability test, kaolinite was first added to water before oil and dissolution of the particles was not observed; instead, the granules remained insoluble in the water. The inability of kaolinite to bind to oil in spite of its hydrophobic nature depends on the density of both compounds. Kaolinite, being a heavier compound, sinks to the bottom of the sludge column in an attempt to segregate away from water while oil segregated at the top.

4.5 Chapter summary

This chapter reports and explains the results obtained from the isolation and characterization of the isolated biosurfactant-producing microbes. The organisms that tested positive for biosurfactant activity showed closest similarities to *Bacillus cereus*, *Klebsiella oxytoca* and *Cronobacter dublinensis* (99% confidence). The selection of an appropriate carbon source prior to oil recovery experiments, as well as the determination of the amount of time required for biosurfactant activity of the bacteria consortium to become evident, was crucial to the recovery process. These tests showed that the bacteria isolates were able to degrade glycerol as their carbon source but failed to grow on hexane. This was considered to be a positive attribute as low chain alkanes are often the most useful in petrochemical industries. The bacteria consortium only showed biosurfactant activity after 24 hours. The ability of fumed silica particles to adhere to water particles and oil, to form pickering emulsions was one of the highlights of this chapter. Kaolinite was tested alongside fumed silica and failed to produce satisfactory results.

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CHAPTER 5

PERFORMANCE OF OIL RECOVERY SYSTEMS

Oil recovery from oil-water emulsions stabilized by fumed silica particles was investigated. Prior to the design of a reactor system to enhance oil recovery, preliminary batch studies involving the comparison of the activity of a chemical surfactant (SDS) with biosurfactant isolated from two separate bacteria cultures were carried out. The results of these studies are presented in sections 5.1 and 5.2. These studies served as the foundation upon which further studies were conducted.

5.1 Preliminary Oil Recovery Studies Using Chemical Surfactant (SDS)

The effectiveness of a well-known chemical surfactant (SDS) as an oil recovery agent was investigated. Oil recovery was achieved in a two-step process. The first step involved the destabilization of the sludge emulsion formed with oil in order to cause the elution of oil and water from the sludge matrix, while the second phase involved the separation of the hydrated oil component. In order to achieve destabilization, the emulsion was dewatered, followed by the addition of SDS to the residual emulsion. The addition of SDS led to the elution of an aqueous phase consisting of both oil and water. The height of the aqueous phase increased with time until equilibrium was achieved. In the second phase of the experiments, the oil layer segregated from residual water molecules, given the immiscibility of both liquids. The height of oil (without any contaminants) served as a performance parameter for this study. The height of oil eluted differed with varying amounts of SDS added to the emulsion. The results obtained are shown in Figure 5-1.

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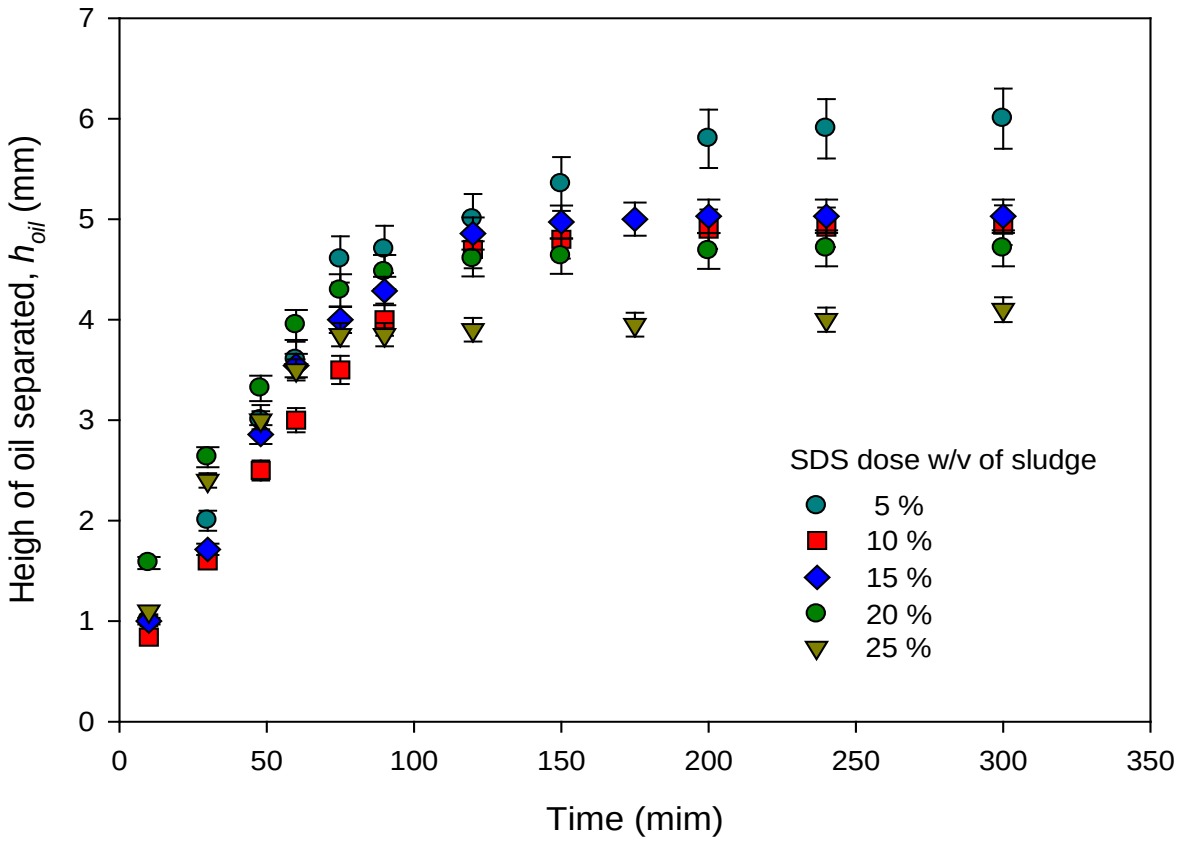

Figure 5-1: Height of eluted oil from sludge emulsions containing varying concentrations of SDS (Chirwa *et al.* 2013).

From Figure 5-1, it can be seen that the addition of 5% SDS achieved the highest oil recovery rate. Increasing concentrations of SDS achieved lower recovery rates, suggesting that the CMC of SDS was approximately at a concentration of 5% SDS. The CMC of a surfactant is defined as the concentration range above which the surfactant molecules begin to form micelles. Hence an increase in concentration of the surfactant leads to a decrease in activity or null change in activity. In other words, the CMC is a saturation point above which molecules of the surfactant start to segregate together to from non-interactive structures, which can be likened to lipid

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bilayers (see Figure 5-2).It has been reported that the CMC of SDS ranges from 0.008M in pure water to 0.0083M in solutions containing hydrocarbons (Khan and Shah, 2008).

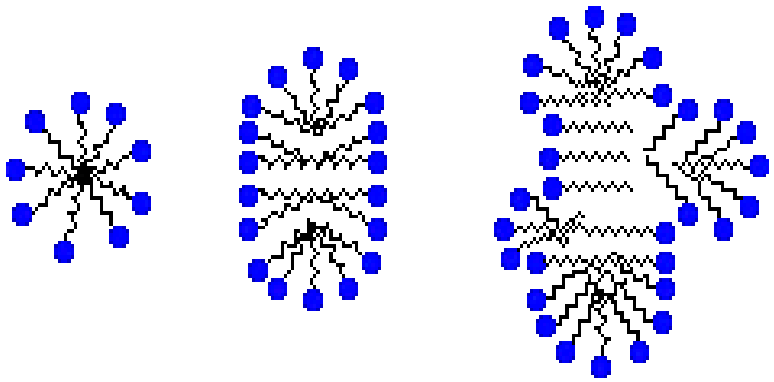

Figure 5-2: Typical structures of micelles formed by amphipathic compounds such as surfactants.

The results obtained from the studies conducted with SDS suggested that low concentrations of biosurfactants produced by isolated bacteria could possibly destabilize recalcitrant sludge emulsions and result in oil recovery (Chirwa *et al.* 2013).

5.2 Preliminary Oil Recovery Studies Using Biosurfactant-Producing Bacteria Cultures

The efficacy of two different biosurfactant-producing bacteria cultures in the recovery of oil from sludge emulsion was investigated. Culture 1 was obtained from a petrochemical contaminated soil sample while culture 2 was obtained from a soil sample taken from an engine oil dump at a car garage. The bacteria were isolated by incubating the soil samples with sterile MSM and an appropriate carbon source. The bacteria cultures were harvested separately after

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varying incubation times and added to prepared sludge emulsions. The recovery of oil from the sludge matrix using the harvested cultures was observed.

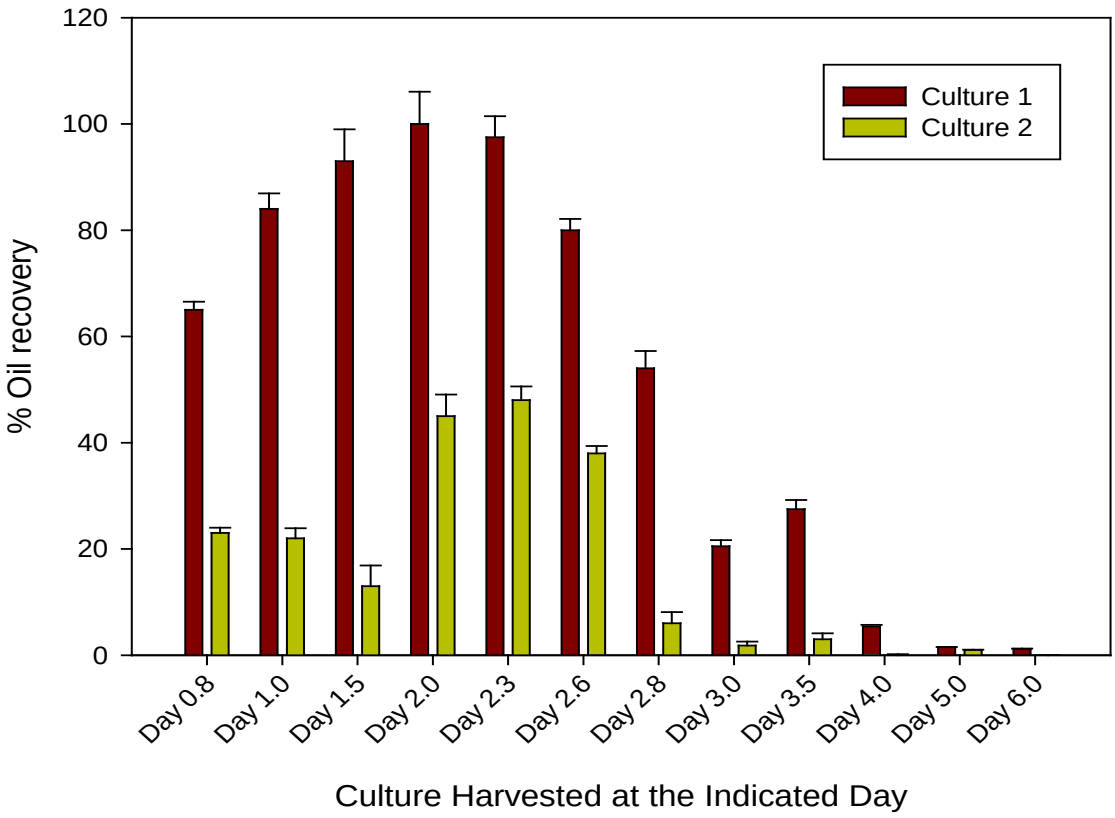

Figure 5-3: Rate of oil recovery with two different bacteria cultures harvested at different phases (one day- early log phase, two days- mid log phase, four days- late log phase) (Chirwa *et al.* 2013).

The highest rates of percentage recovery were observed with culture 1. Generally, both cultures were most effective when harvested after an incubation period of two days (mid-log phase). By the late log phase (three days and above), the cultures were unable to achieve sufficient oil recovery. By the fourth day, it was evident that the bacteria cells were beginning to die, most likely due to a depletion of carbon sources.

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The ability of the bacteria cultures harvested in early or mid-log phase to demulsify sludge and recover oil at faster rates relative to the late log phase cultures can be explained by the production of the biosurfactants in the culture. Biosurfactants are secondary metabolites produced during the growth of bacteria. In other words, the lack of an appropriate carbon source to facilitate growth of the bacteria can lead to the absence of biosurfactants in the culture. By the late log phase, most, if not all of the carbon sources have been consumed, and the cells either start to utilize the metabolites produced during early and mid-log phases or they die via PCD due to lack of nutrients.

5.3 Batch system versus Plug Flow Reactor system

The results from the preliminary studies led to an upscale of oil recovery experiments. A plug flow reactor system was designed and the recovery of oil from sludge emulsion was investigated. As a baseline for comparison, a batch system was investigated alongside the reactor system. Oil recovery was investigated under four different conditions:

I. The sole addition of biosurfactant-producing bacteria consortium culture to the sludge matrix
II. The addition of water to the sludge matrix prior to the addition of the bacteria consortium culture- a process known as waterflooding
III. The sole addition of water to the sludge matrix

The amount of oil recovered under each condition was calculated as a percentage and plotted against time.

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5.3.1: Oil Recovery from Batch System

The results from the batch system are presented in Figure 5-4.

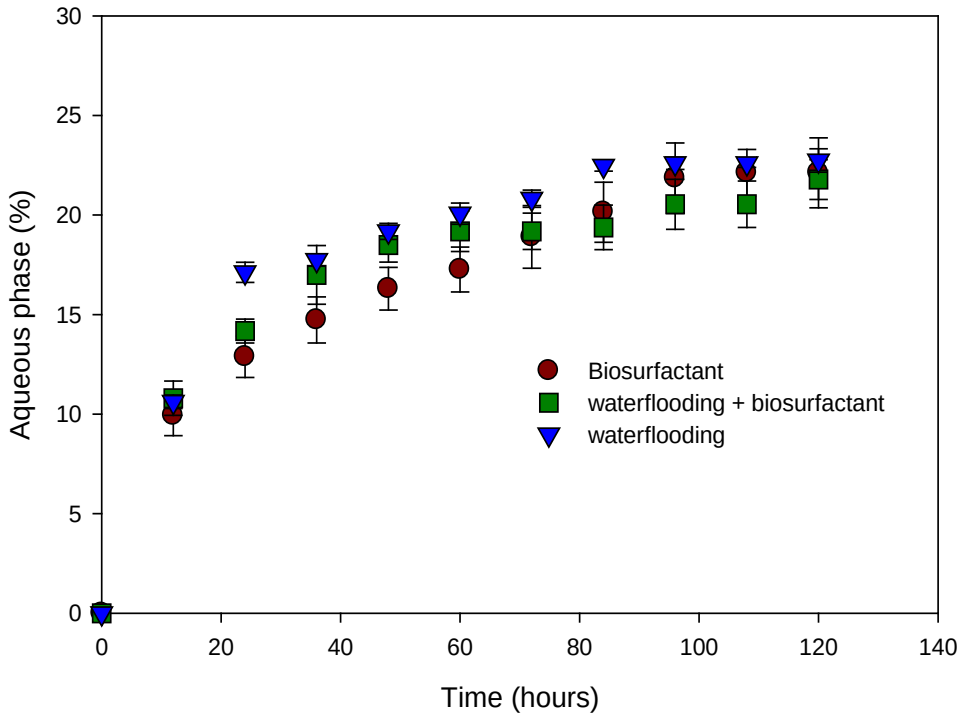

Figure 5-4: Oil recovery from batch reactor.

Oil recovery using the batch system yielded low recovery percentages as expected; the oil layer eluted was rather thin and difficult to measure; hence the aqueous phase (oil + water) eluted was measured. In spite of measuring oil and water elution, the system proved ineffective in the recovery of oil as the percentages obtained remained considerably low. The addition of water to the sludge matrix prior to the addition of biosurfactant did not show any noticeable differences from all the other conditions investigated. The ineptness of the batch system to achieve impressive oil recovery rates under the various conditions investigated can be explained by the lack of a mechanism through which oxygen and nutrients could be effectively distributed. The

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batch system can be likened to landfarming, an ineffective system that takes years to achieve complete bioremediation of oily sludge.

5.3.2 Kinetic Description of Oil Recovery Using Batch System

The rate of oil recovery using biosurfactant-producing bacteria in a batch reactor can be described by the adsorption isotherm kinetics known as the Langmuir kinetics (Liu and Shen 2008). Although there have been some success recorded in the area of MEOR (Behlugil *et al.* 1992; Yakimov *et al.* 1997; Bordoloi and Konwar 2008), none of these studies have attempted to describe the process using an appropriate kinetic model.

The data obtained from the batch reactor was converted to give the concentration of oil (%v/v), remaining in the sludge matrix as the recovery process went underway. The equation used to describe the kinetics of the batch recovery system is described by Liu and Shen (2008) as a modification of the Langmuir isotherm to give a second order rate law and is given below:

$$R = K([C_0 - C_t])^2 \qquad (5\text{-}1)$$

Where:

R= Rate of oil recovery from sludge

K= recovery rate coefficient (h^{-1})

C_t= Oil concentration (%v/v) at time t (hours)

C_0= Initial oil concentration (%v/v) at time (t=0)

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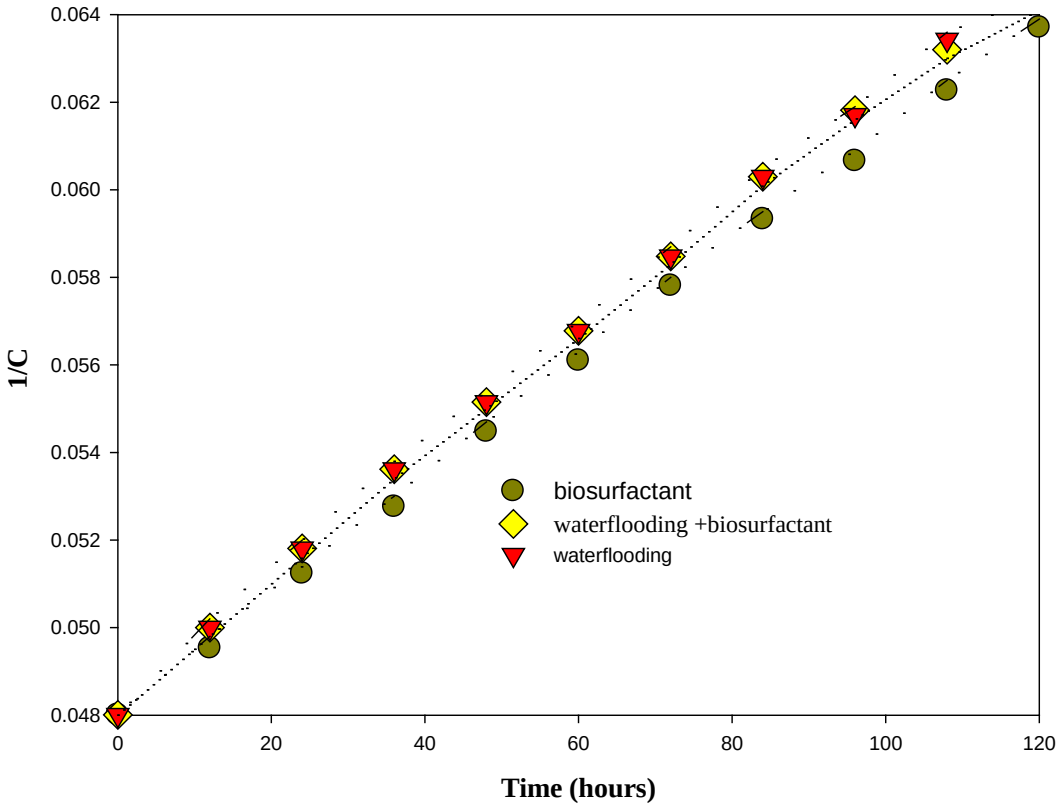

Figure 5-5: Second order linear transformations of 1/C versus time for oil recovery under varying conditions using the batch system.

Straight line graphs of 1/[C] versus time with slope K showed that the reaction followed a second rate order. The kinetic parameters estimated are presented in Table 5-1. The reasonably good fit of the data points as well as the high r^2 value obtained for the varying conditions under which oil recovery was carried out suggests the Langmuir model was appropriate for this data set. The concentration of oil recovered from the batch system under varying conditions, relative to the initial concentration of oil added to the sludge matrix was almost negligible.

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Table 5-1: Estimated kinetic parameters for oil recovery using batch system.

Parameters estimated	Biosurfactants	Waterflooding + Biosurfactants	Waterflooding only
K	0.034	0.036	0.032
$_{\chi}2$	516.557	516.492	607.003
R^2	0.998	0.996	0.997

5.3.3 Oil Recovery Using Plug Flow Reactor System

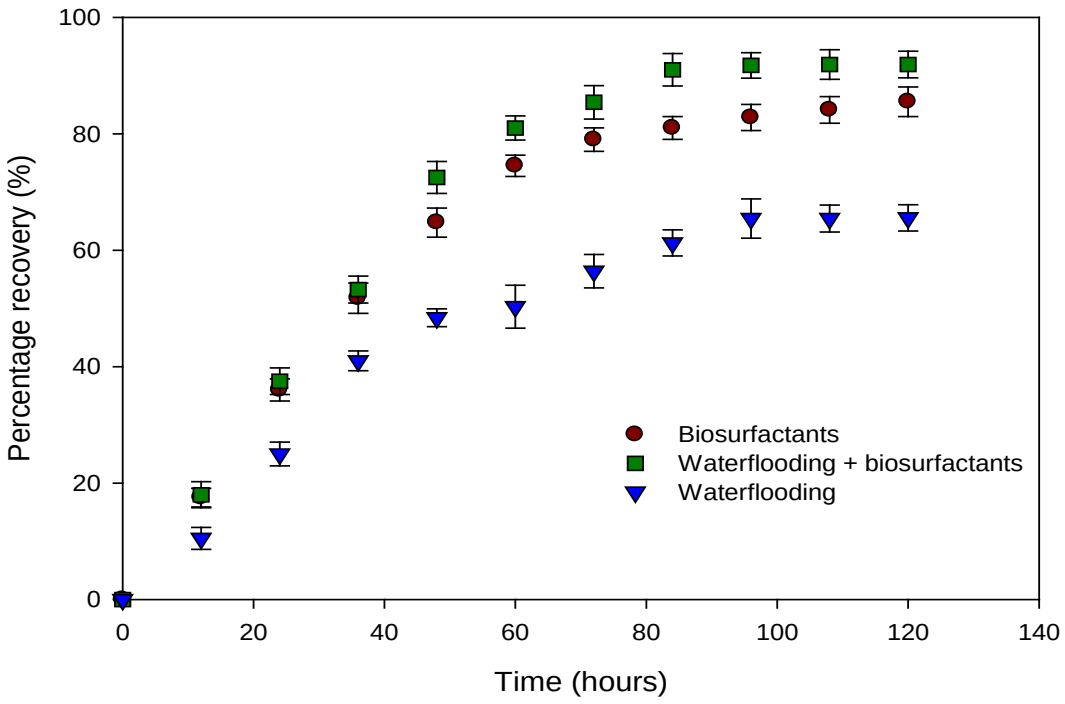

Figure 5-6: Oil recovery from plug flow reactor.

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The percentage recovery of oil from sludge using the PFR was higher than the percentage recovery in the batch reactor. This higher percentage recovery can be attributed partly to the mechanical force of the overhead stirrer in the mixing vessel. Before the addition of water or biosurfactant-producing bacteria or a combination of both to the stable sludge emulsion, the sludge was subjected to mixing at a high speed which must have resulted in a partial breakdown of its recalcitrant structure. The addition of waterflooding and/or biosurfactants to sludge after the initial mixing phase facilitated much better separation. The second mixing phase allowed for an even distribution of the biosurfactant-producing bacteria consortium through the sludge, facilitating better bonding between surfactant molecules and sludge particles.

The separation tank which was built to have a moderate steep flow direction also played a crucial role in the recovery of oil. In the batch system, it was expected that the separated oil particles will automatically segregate to the top phase of the demulsified sludge matrix; however, given the thickness of the matrix, this must have been almost impossible. The plug flow system on the other hand had a separation tank that allowed for the different components to separate as they flowed down its moderate steep slope. As the different particles of the demulsified sludge traveled down the slope of the separation tank, fumed silica particles travelled at the slowest pace due to loss in energy, which in turn resulted in reduction of velocity, while oil and water traveled at almost the same speed. Given the immiscibility of oil and water, separation of these two components was inevitable at the end of the tank. Flow rate from mixing vessel B into the separation vessel was found to play a major role in the percentage recovery of oil and is explained in section 5.4.

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5.3.4 Kinetics of Oil Recovery Using Plug Flow Reactor

The Langmuir adsorption isotherm was used to describe the reaction rate in the plug flow system. The adsorption principle was considered as an appropriate model based on the interactions that supposedly occurred between oily sludge and the biosurfactant-producing bacteria culture. The equation used to describe the data obtained from the plug flow system is shown below:

$$V = \frac{k_a K C_t}{1 + K C_t} \qquad (5\text{-}2)$$

Where k_a is the adsorption coefficient (%v/v^{-1}), K is the recovery rate coefficient, and C_t is the concentration (%v/v) of oil remaining in the sludge matrix at time t.

In the case of studies carried out with only waterflooding, Equation 5-2 did not apply as these experiments did not have any addition of bacteria culture. Instead, only water was added and the equation used to describe the waterflooding experiments is given below:

$$V = \frac{K C_t C_w}{K + 3C_w + 3C_t} \qquad (5\text{-}3)$$

Where K is the recovery rate coefficient (h^{-1}), C_t is the concentration of oil remaining in the sludge matrix at time t and C_w is the concentration of water added to the sludge matrix (%v/v).

Aquasim 2.0 (AQUASIM ^{TM} EAWAG, Dübenhorf Switzerland) was used to achieve numerical solutions for the parameters to be estimated. The resulting model for the varying conditions under which the oil recovery process was carried out was deemed to be a good fit with non-linear regression coefficients ranging from 0. 99- 0.98.

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The Langmuir model effectively described the data obtained for the oil recovery process. The use of waterflooding in addition to biosurfactants to facilitate oil recovery as well as the sole addition of water to the sludge matrix to recover oil did not affect the suitability of the model, suggesting that the process depended mainly on the surface properties of the biosurfactants contained in the bacteria consortium culture. Biosurfactants are known to be able to reduce surface tension in liquids and are also known to be able to reduce interfacial tensions leading to demulsification of otherwise stable emulsions (Banat 1995). Hence, it was clear from the good fit of the Langmuir model that the process was dependent on the ability of biosurfactants to adsorb at the interface of the sludge components, leading to demulsification. The reasonably good fit of the model under varying conditions is shown in Figure 5-7 below and the parameters estimated for the plug flow process are presented in Table 5-2.

Table 5-2: Estimated parameters for oil recovery using plug flow reactor

Parameters estimated	Biosurfactants	Waterflooding + Biosurfactants	Waterflooding only
K	0.017	0.102	0.118
k_a	1.358	0.498	-
$\chi 2$	106.528	155.307	192.562
R^2	0.9999	0.9974	0.9999

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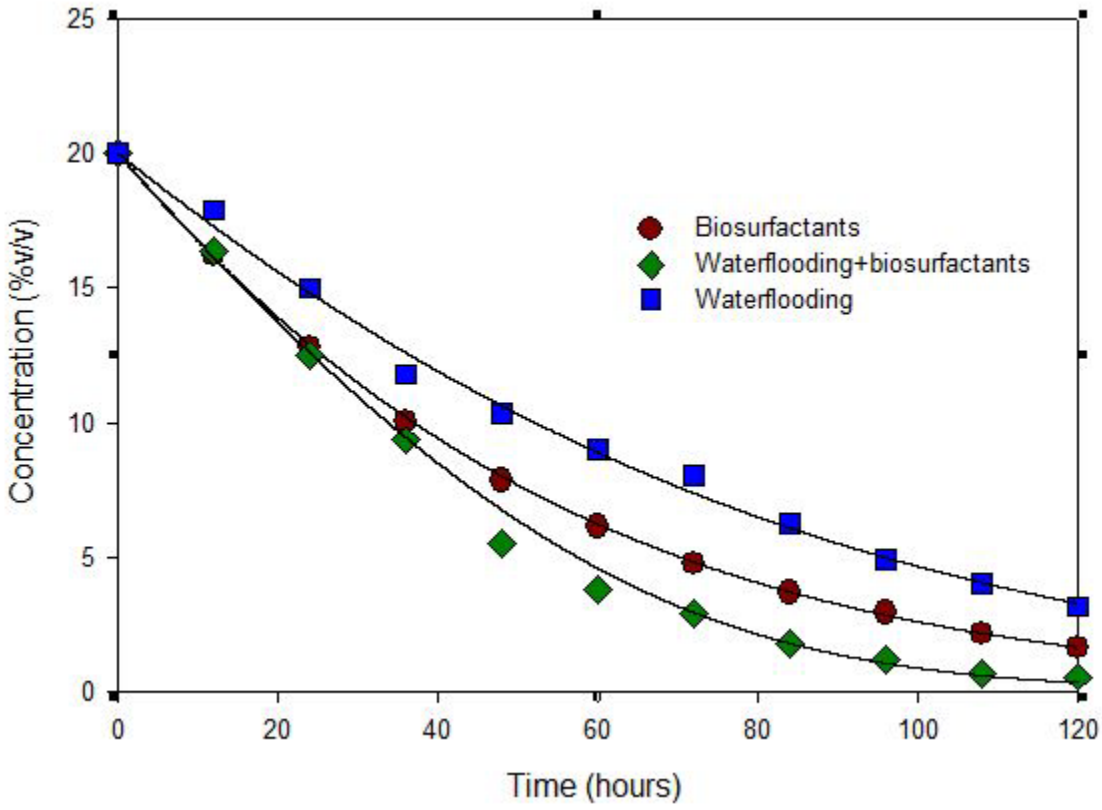

Figure 5-7: Kinetic models obtained using Langmuir adsorption kinetics.

The amount of oil recovered per kilogram of sludge as well as the rate of oil recovery of both systems was estimated. In the batch system, the amount of sludge analyzed each time was approximately 2kg (actual value: 1,986.96g). After being able to successfully decant the layer of oil obtained in the beakers, the amount of oil recovered per kg of sludge in the batch system was calculated using the formula:

$$Y = \frac{V}{m} \times 1000g \qquad (5\text{-}4)$$

Where Y represents yield per kg of oily sludge, V is the volume of oil recovered measured in mL and m is the mass of sludge put in the reactor.

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Using equation 4-2, the average total of oil recovered from one kg of sludge in the batch system can be calculated to be

$$Y_b = \frac{86}{1986.96} \times 1000 = 43.28\, mL$$

While the plug flow reactor yield is:

$$Y_{sb} = \frac{3308.8}{16558} \times 1000 = 199.83\, mL$$

The yield of oil per kg of sludge using the plug flow reactor is approximately five times higher than that of the batch reactor. The average rate of oil recovery was calculated by deducting the percentage recovery end value from the initial value and dividing the resulting value by the total period of the process. The average rate of oil recovery for the plug flow system was calculated to be 0.723 mL per hour while the average rate of oil recovery for the batch system was calculated to be 0.102mL per hour.

5.4 Effect of waterflooding on oil recovery

Waterflooding is a technique that has been employed in the process of oil recovery as far back as the late 1950s (Jadhunandan and Morrow 1995); it is especially known to be used to recover oil trapped in porous sand rocks (Amott 1959). The effectiveness of waterflooding is known to be affected by the wettability of the surface to which it is applied; wettability is the ability of a solid surface to maintain contact with a liquid and is enhanced by forces of cohesion and adhesion (Cassie and Baxter 1944).

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During the course of the oil recovery experiments, waterflooding was investigated as a technique that could possibly enhance the recovery of oil, given its popularity since the early 90s. In the batch system, the effect of waterflooding is negligible as can be seen in Figure 5-5. The explanation for this may be the fact that fumed silica particles tend to absorb water and further stabilize the emulsion formed between oil and water as explained in section 4.4. In the plug flow reactor system, the recovery rate was higher with waterflooding but only by less than 10%. The use of waterflooding as a technique to increase oil recovery may be frowned upon in current times, given the environmental battle with trying to provide sufficient water to accommodate increasing needs. Given that the difference in recovery rate was less than 10%, it was decided that oil recovery with the sole use of biosurfactant-producing bacteria is sufficient.

5.5 Effect of Flow rate on Oil Recovery

The effect of flow rate on the recovery of oil was investigated. During the course of recovering oil from the sludge matrix using the plug flow reactor, it was observed that the flow rate of the pump from mixing vessel B to the separation tank played an important role in the separation of components. Figure 5-8 shows the difference in recovery rate as flow rate was changed.

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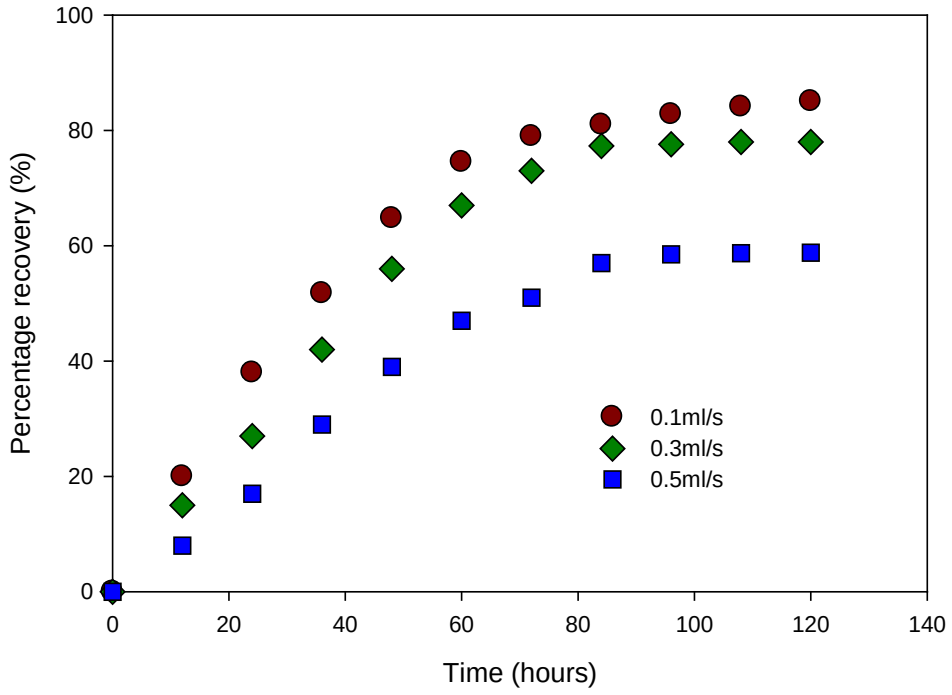

Figure 5-8: Differences in percentage oil recovery from sludge with varying flow rates.

Figure 5-8 shows a trend- the lower the flow rate, the higher the percentage oil recovery. This can be explained by the fact that a higher flow rate does not encourage sufficient loss of energy of the fumed silica particles before they reach the end of the slope of the separation tank. It was observed that the higher flow rate caused the silica particles to flow in sync with the oil and water particles, allowing for little separation relative to a lower flow rate. The lower flow rate prevented accumulation during the course of the flow and allowed for sufficient loss of energy before the sludge particles reached the end of the slope. In order for separation to occur at a higher flow rate, it was necessary to leave the emulsified mix in the separation vessel for up to 6 hours, causing a delay in the process.

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The flow rate of sludge from the second mixing vessel into the separation tank is not the only factor that directly affects the velocity of sludge. The design of the separation vessel ensures that the velocity of the sludge components change at different points in the tank. This can be explained by the illustration below:

Although the width of the tank is constant, the cross sectional area of the tank changes as the gradient increases. The velocity of the sludge components can be calculated using the formula:

$$V = \frac{Q}{A} \tag{5-5}$$

Where V is the velocity of the sludge components (m/s), Q is the flow rate (mL/s) from the mixing vessel to the separation tank and A is the cross-sectional area (m^2) of the tank at different sections.

The formula for the cross-sectional area of this reactor is the same as that of a rectangle and is given by:

$$A = b \times h \tag{5-6}$$

Where b is the breadth of the reactor and h is the height of the sides

Based on the measurements taken of the separation tank, the area can be calculated for six different sections of the separation tank with the first height being 0.12m and the last measurement being 0.30m. Therefore, V_1 to V_6 can be calculated using Equation 5-4. The values obtained for the changes in velocity at different points in the separation tank are presented in Table 5-3.

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Table 5-3: Different velocities of sludge components, at different flow rates, based on differences in cross-sectional area, at different points in the separation tank.

Distance from starting point (m)	Cross sectional Area (m^2)	Velocity (m/s)		
		0.1mL/s	0.3mL/s	0.5mL/s
0.00	0.0192	5.208	15.625	26.042
0.12	0.0240	4.167	12.5	20.833
0.32	0.0304	3.289	9.868	16.447
0.54	0.0384	2.604	7.813	13.021
0.62	0.0416	2.404	7.212	12.019
0.80	0.0480	2.083	6.25	10.417

Table 5-3 shows the calculated velocity values of the sludge components at different flow rates. The design of the tank was specified to encourage varying values for the cross-sectional area as this could directly impact the velocity of the sludge components. At a high flow rate of 0.5ml/s, the velocity of the sludge components is still considerably high at a value of 10.417m/s. The velocity at the beginning is a high value of more than 25m/s. This supports the notion that a higher flow rate caused the sludge components to flow in sync, reducing the chances of separation before the components reached the collection ports.

At a flow rate of 0.1m/s, the velocity of the sludge components is considerably lower. At the beginning of the separation tank, the velocity is just over 5m/s and by the time the components reach the collection port, the velocity is at a low value of approximately 2.1m/s. At such a low velocity, the sludge components cannot flow in sync and the differences in density begin to play a role in the separation process, causing the heavier compound (fumed silica) to flow at a much slower velocity relative to oil and water. In addition, it was observed that fumed silica particles

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automatically segregated to the bottom of the tank when it eventually reached the collection ports. This can also be attributed to the differences in density. Water is known to have a density of 1000kg/m^3 while fumed silica has a higher density value of approximately 2300kg/m^3 (Sigma Aldrich).

5.6 GC-MS Analysis of Oil Samples

The characterization of oil using GC-MS analysis revealed the presence of aliphatic and aromatic hydrocarbons such as hexadecane, eicosane, 2, 2-dimethyl propane, sulfinyl sulfone, celesticetin, toluene, pentane-1-butoxy, 2-hexyl-1-octanol, 6-tetradecane sulfonic acid butyl ester, amongst many others. Due to the wide range of peaks obtained, five prominent peaks (denoted as C1, C2, C3, C4 and C5) were designated as the reference peaks and were the peaks observed for possible degradation after the recovery processes was completed. The concentration of each reference compound was calculated using standard curves that had been prepared before analysis commenced. The original concentrations of these compounds as well as their residual concentrations after the recovery processes were completed are shown Figure 5-9.

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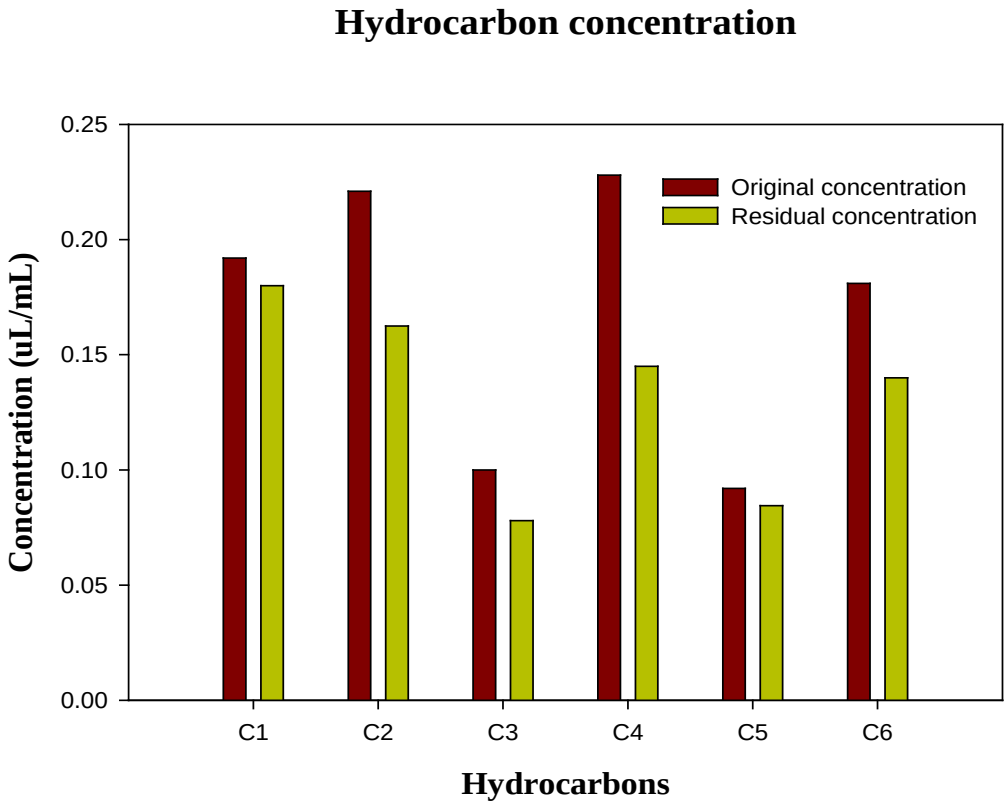

Figure 5-9: Graph showing original and residual concentrations of PAH compounds in oil used to simulate sludge. C1 to C6 are chromatogram peaks used as reference peaks to check for chances of degradation during the recovery process. C1 represents chrysene, C2 represents 3-heptanone-4-methyl, C3 represents toluene, C4 represents celesticetin, C5 represents 1, 2-benzene-diiso-octyl ester and C6 represents Dibenzothiophene. These results are from the plug flow reactor.

The difference between the original and residual concentrations of some of C1 (Chrysene) and C5 (1, 2-benzene-diiso-octyl ester) was negligible. These compounds were not degraded by the bacteria during the course of the recovery process as much as the other three reference compounds. There were conspicuous differences between the original concentrations and residual concentrations of C2 (3-hepatanone-4-methyl), C3 (toluene), C4 (celesticetin) and C6 (dibenzothiopene). These results highlight the fact that some degradation occurred during the recovery process; *Klebsiella oxytoca*, one of the isolated biosurfactant-producing bacteria has been proven in earlier studies to be able to degrade poly aromatic hydrocarbons and utilize them as carbon sources; however, it is able to degrade aliphatic C_{13} to C_{30} compounds more

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effectively. Given that there is not much known about *Cronobacter dublinensis*, it is difficult to ascertain its ability to degrade recalcitrant compounds such as toluene, celesticetin etc. The degradation pathways for *Bacillus cereus* for such compounds also remain unknown; hence it is difficult to attribute the differences in concentrations of these compounds to degradation action by this bacterium. There are proposed pathways for the degradation of some PAHs; however, the genetic dexterity of many organisms with regards to possession of these pathways remains to be confirmed. Some of these degradation pathways are attached in Appendix C.

A plausible explanation for the differences in concentrations is volatility of the compounds; Some PAH compounds are known to have adverse respiratory effects on onsite workers and other forms of life residing in close proximity to petroleum waste disposal sites due to their volatility, which makes them present in the atmosphere and available for inhalation. Toluene has been identified as one of such compounds (Greenberg 1997); Chiarenzelli *et al.* (1998) also suggested that some of the hydrocarbons found in petroleum wastes are semi-volatile and may cause health problems for onsite workers.

5.7 Total Organic Carbon analysis

After completion of the recovery process, the residual sludge was pumped out of the reactors and incubated at 30°C in separate containers; the residual sludge from the batch reactor was kept in a separate container from that of the plug flow reactor. Samples were taken from these containers every 3 days for a total period of fifteen days; these samples were analyzed for TOC content. The results obtained over the fifteen day period are presented in Figure 5-10:

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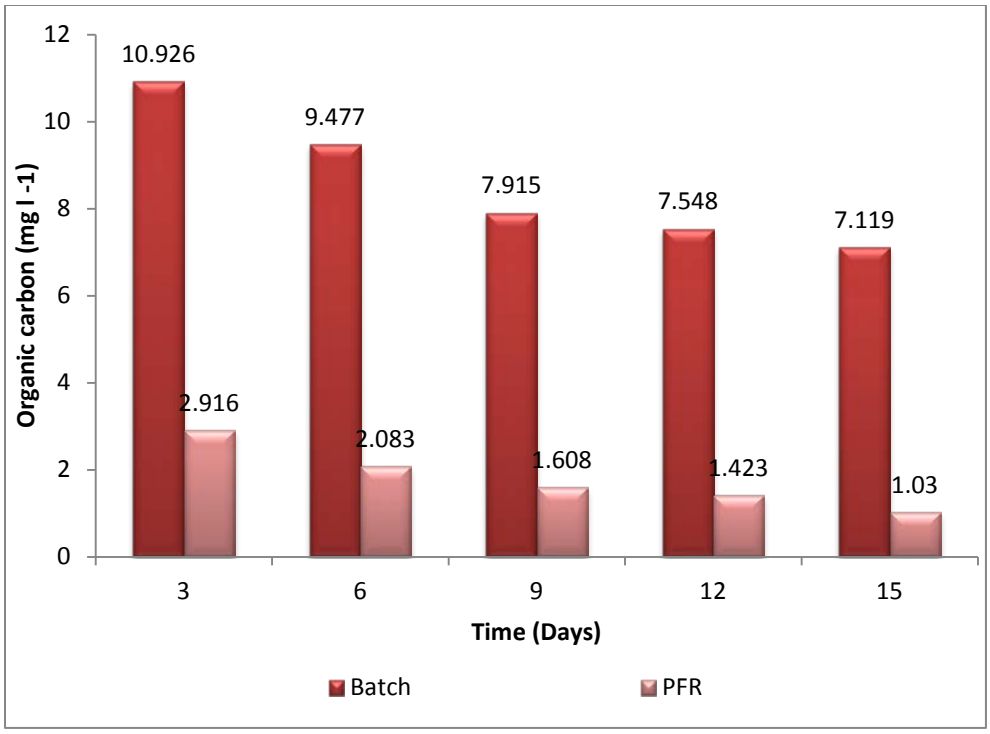

Figure 5-10: TOC content of sludge matrix after completion of oil recovery process.

There was a conspicuous difference in the TOC content of sludge recovered from both systems. The sludge from the full batch system showed a relatively high level of organic carbon content highlighting the high content of oil still trapped in the emulsion. This also showed that the bacteria were least effective the batch system in the process of MEOR. The full batch system investigated in this study can be likened to landfarming- a form of biotreatment where static conditions are experienced by bacteria. This system does not in any way allow for even distribution of nutrients, nor does it allow for sufficient distribution of oxygen as one would have a dynamic or semi-dynamic system. This was highlighted by Lazar *et al.* 1997 as a problem with landfarming; it was reported that only 16.85% success rate was achieved with landfarming under static conditions. On the other hand, the total organic carbon content from the semi-continuous

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system showed a steady decrease over the period of fifteen days. On the fifteenth day, the concentration of organic carbon left in the sludge matrix was 1.03mg/L.

The lower organic carbon concentration observed in the sludge obtained from the plug flow system highlighted the efficiency of the plug flow reactor with regards to the oil recovery process. The plug flow system showed a higher concentration of total organic carbon, also highlighting the inefficiency of the system. As can be seen in Figure 5-10, there was a decrease in total organic carbon content as the days went by; this can be attributed to degradation action by the bacteria still trapped in the sludge matrix. The degradation in the residual sludge obtained from the plug flow reactor was much higher. This can be explained by the fact that the plug flow reactor system not only exposed the bacteria to compounds that can be easily utilized as carbon and energy sources, it subsequently led to an increase in biomass, causing a demand for more carbon source. Even after the completion of the oil recovery process, the bacteria in the sludge matrix from the plug flow reactor remained active and could still access some compounds, leading to their degradation and hence reduction in total organic carbon.

A limitation with this aspect of the study is that fumed silica was used to stabilize oil-in-water emulsion. In real sludge, it is possible that the total organic concentration will much higher even after an impressive recovery percentage due to heavy hydrocarbon fractions that may remain trapped in the residual sludge matrix. The conspicuously lower organic carbon concentrations however still highlight the effectiveness of the plug flow system in oil recovery. In the case of sludge that has been found to contain metals from the refinery tanks and transportation pipelines, bioremediation can be carried out using bacteria that are able to utilize such metals as carbon and energy sources.

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5.8 Chapter Summary

The performance of oil recovery systems were reported in this chapter. The inefficiency of a batch system compared with a plug flow reactor. Under different conditions, the rate of oil recovery using the PFR differed. The sole addition of water achieved a percentage recovery of 65.55% while the sole addition of a biosurfactant-producing bacteria consortium yielded a percentage recovery rate of more than 85% within a period of 120 hours. The initial destabilization of the sludge emulsion using water followed by the addition of bacteria achieved a percentage recovery that was only slightly higher at approximately 91%.

The oil recovery process was described using the Langmuir adsorption kinetics. The reasonably good fit of the data, coupled with high r2 values showed this model was an appropriate description for the data set. Prior to this study, there have been no reports describing the process of MEOR, using an appropriate model, making it one of the highlights of this study.

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CHAPTER 6

CONCLUSION

6.1 Conclusion

The use of MEOR as a biological waste treatment method for petroleum sludge was investigated. Biosurfactant producing bacteria (*Klebsiella oxytoca*, *Cronobacter dublinensis* and *Bacillus cereus*) were isolated from petroleum-contaminated and uncontaminated soil samples and used as a consortium to treat oily sludge. Oily sludge was simulated using a combination of waste oil (which consisted of paraffin, petrol, and diesel and lubricating oil), water and fumed silica as an intermediate compound to simulate the fine solids component of oily sludge and also to stabilize the oil-water emulsion.

A plug flow reactor system was designed and used to enhance the recovery of oil from oily sludge. This system consisted of mixing vessels and a separation tank that enhanced the separation of sludge components based on their varying velocities as they travelled down the length of the tank. The recovery of oil was investigated under varying conditions which included the use of waterflooding as a technique to achieve demulsification, the use of a combination of waterflooding and a bacteria consortium and the sole use of a bacteria consortium. The plug flow reactor was able to achieve an average recovery of approximately 85% while a batch system investigated as a baseline for comparison only achieved a maximum of 30% aqueous phase elution. The recovery of oil from sludge using the PFR system resulted in a noticeable reduction of TOC content of residual sludge. The TOC concentration of residual sludge was found to be 1.03mg/mL after an incubation period of fifteen days; this highlighted the efficacy of this system to treat sludge and ensure its disposal into the environment, with the level of organics being

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considerably lower and safer. In addition, the activity of bacteria after the recovery process was completed suggested possible reuse of bacteria consortium and also suggested the possibility of incorporating this recovery system with landfarming to ensure a higher efficacy of oily sludge treatment.

The results obtained from the oil recovery experiments were described using the Langmuir adsorption isotherm as well as an adaptation of the model to fit second order rate reactions. The reasonably good fit of the model, coupled with high r^2 values suggested the Langmuir model was an appropriate description for the process of oil recovery, as it took into consideration the surface binding of the different components involved.

6.2 Recommendations for Future Studies

This study was an experimental study carried out under laboratory conditions which do not entirely mimic the exact conditions of a petroleum refinery. It is therefore recommended that further studies be conducted possibly at refinery sites; this is to determine the ability of the isolated microbes to survive under the harsh environmental conditions of a refinery site. Furthermore, it is recommended that real sludge obtained from a refinery be used for further studies to determine if the laboratory recovery rates obtained with oil-water emulsions stabilized by fumed silica can be reproduced using real sludge.

In addition, the characterization of the biosurfactants isolated from the bacteria would be helpful during future studies; this would require the use of a tensiometer to measure the reduction of surface tensions in bacteria cultures with time and will also aid the determination of the critical

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micelle concentration (CMC) of the biosurfactants. The monitoring of the surface tension can also be a more accurate determination of when biosurfactant production occurs and when it stops, depending on the amount of carbon source available (hence there will be a need to monitor the reduction in glycerol levels alongside the reduction of surface tension). Further characterization of the biosurfactants will give an insight into their exact structure and help to classify them according to the various structural classifications currently available. Such studies will be considered novel for *Bacillus cereus* and *Cronobacter dublinensis* as the biosurfactant structures of these organisms have not been reported at the time this study was conducted.

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APPENDICES

APPENDIX A: TABLES AND FIGURES

Table A1: Typical concentrations of PAHs in sludge and slop oil.

Constituent	Concentration in Sludge (mg/L)	Concentration in slop oil
*Toluene	2.72	5.78
Trimethylbenzene	0.06	0.31
Tetramethylbenzene	0.04	0.29
Methyl dihydroindene	1.00	0.04
Naphthalene	0.15	1.61
Methyl naphthalene	1.20	9.83
Dimethyl naphthalene	3.19	15.98
Methyl (acenaphthene/biphenyl	0.58	5.11
Fluorene	0.14	1.25
Methyl fluorene	0.76	1.48
Naphthol thianaphthene	0.84	-
Dibenzothiophene	0.50	0.56
Anthracene/Phenanthrene	3.57	5.14
Methyl (anthracene/Phenanthrene)	13.85	11.64
Pyrene	9.43	1.65
C2- (pyrene/fluoranthene	6.04	0.51
C3- pyrene/fluoranthene	16.86	-
*Chrysene/benzanthracene	14.90	1.51
*Benzopyrene/benzofluoranthene	22.71	2.86

Asterisk depicts known carcinogenic compounds.

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Table A2: Some types of biosurfactants produced by bacteria

Bacteria	**Biosurfactant Type**
Aeromonas sp.	Glycolipid
Bacillus subtilis	Lipopeptide
Bacillus sp.	Lipopolysaccharide
Klebsiella oxitoca	Lipopolysaccharide
Pantoea sp.	Glycolipid
Pseudomonas aeruginosa	Rhamnolipid
Pseudomonas aeruginosa HR	Glycolipid
Pseudomonas XD-1	Lipopeptide, Glycopeptide
Streptococcus thermophilus A	Glycolipid

Table A3: Commonly used biosurfactant recovery processes

Process	**Type (Example) of biosurfactant**
Ammonium sulfate precipitation	Emulsan, bioemulsifier
Acetone precipitation	Bioemulsifier
Acid precipitation	Surfactin, lichenysin
Organic solvent extraction	Trehalolipids, sophorolipids, liposan
Crystallization	Cellibiolipids, glycolipids
Centrifugation	Glycolipids
Adsorption	Rhamnolipids, glycolipids
Foam fractionation	Surfactin
Tangential flow filtration	Mixed biosurfactant
Diafiltration and precipitation	Glycolipids
Membrane ultrafiltration	Glycolipids, surfactin

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Table A4: Studies done in the last decade involving the use of biosurfactants for environmental remediation

Biosurfactant	Pollutant	Remediation medium	Year of study
Rhamnolipid	Phenanthrene	Soil slurries	1995
Rhamnolipid	Metals, phenanthrene and PCBs	Soil	1995
Rhamnolipid and oleophilic fertilizer	Mixture of alkanes and naphthalene	Soil	1995
Rhamnolipid	4,4-dichlorobiphenyl	Soil	1996
SDS and Rhamnolipid	Phenanthrene, pyrene B(a)P	Soil	1996
Rhamnolipid	Naphthalene	Soil	1996
Glycolipid and Tween 80	Naphthalene and methyl naphthalene	Liquid	1997
EKoil	Oil polluted waters	Liquid	1997
Crude surfactin	Hexadecane, kerosene	Soil	1997
Glycolipids (GL-K12)	Arochlor	Soil	1997
Surfactin	Heavy metals	Soil	1999
Alasan	Phenanthrene, fluoranthene and pyrene	Liquid	1999
Rhamnolipids mixture + Triton X100	Trifluralin, coumaphos, atrazine	Liquid	2000, 2001
Saponin	Metal rememdiation	Soil	2002
Rhamnolipids + bacteria consortium + nutrients	n-alkanes	Petroleum sludge	2003
Biosurfactant	PAHs	Soil	2004
Rhamnolipid + SDS	Crude oil	Stirred tank reactor	2005

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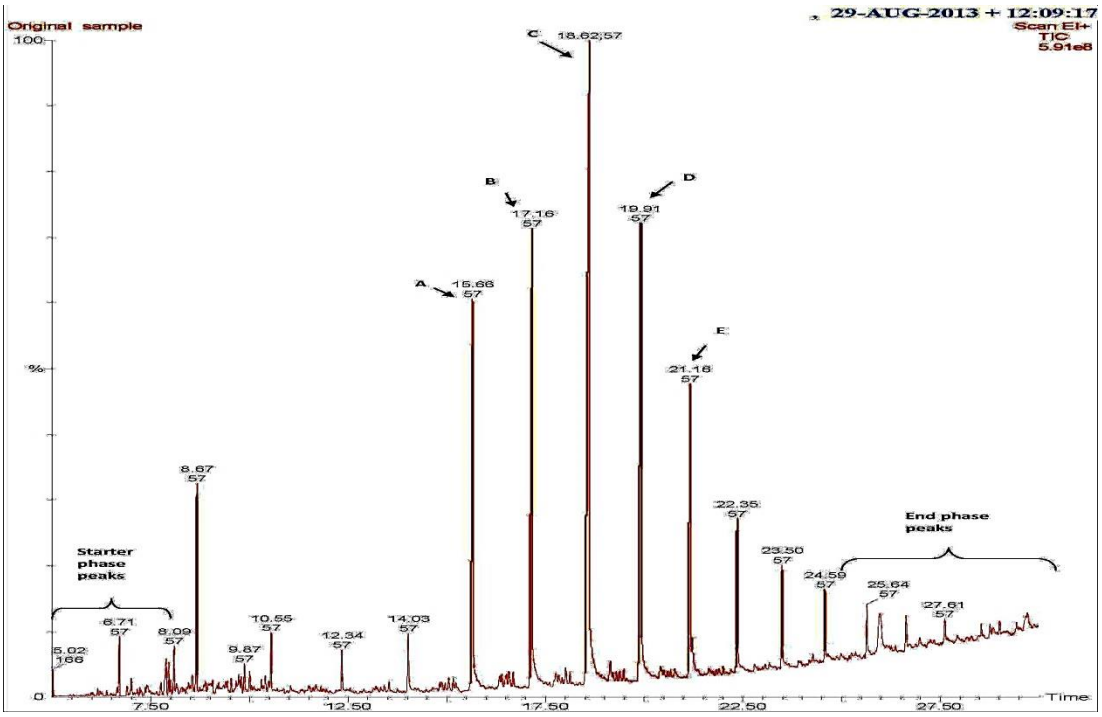

Figure A-1: GC chromatogram of original oil sample prior to recovery process.

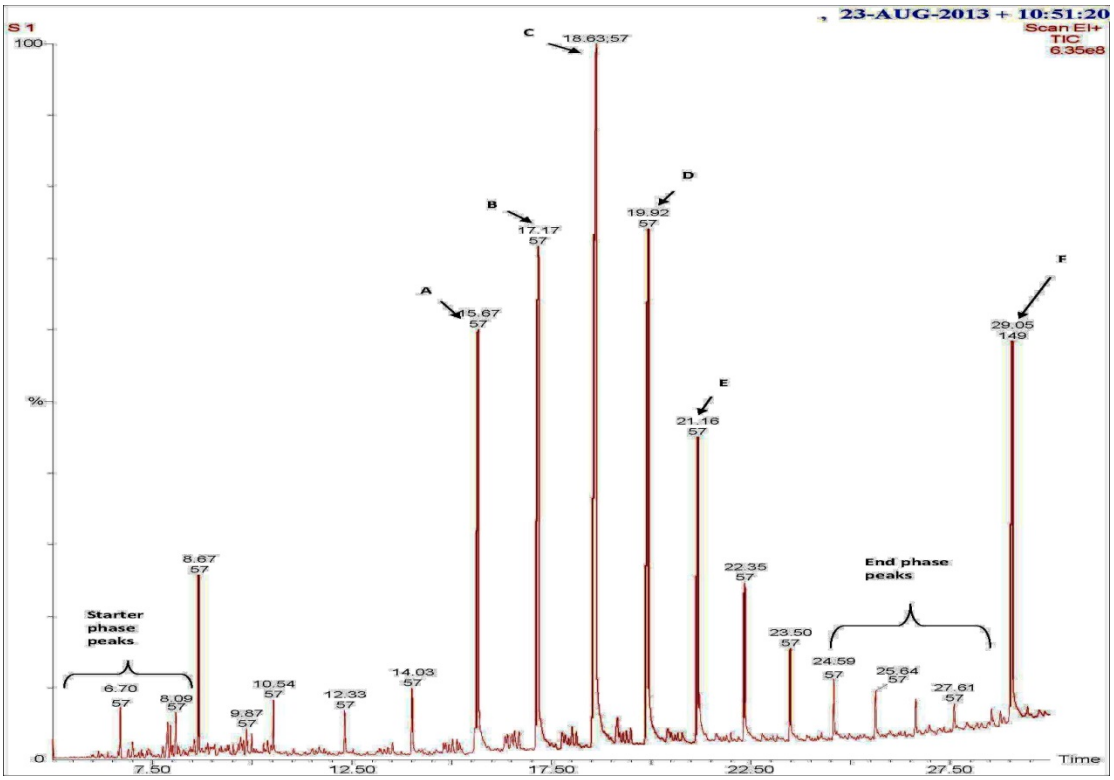

Figure A-2: GC chromatogram of oil recovered from sludge matrix.

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APPENDIX B: AQUASIM SIMULATIONS AND STATISTICAL ANALYSES

B1: Simulation for oil recovery using a combination of waterflooding and biosurfactant

```
AQUASIM Version 2.0 (win/mfc) - Listing of System Definition. Date and
time of listing:  04/17/2014  08:15:00

Variables
*************************************************************************
C:              Description:          concentration
                Type:                 Dyn. Volume State Var.
                Unit:                 %v/v
                Relative Accuracy:    1e-006
                Absolute Accuracy:    1e-006
-------------------------------------------------------------------------
Cmeas:          Description:          Oil concentration
                Type:                 Real List Variable
                Unit:                 %v/v
                Argument:             t
                Standard Deviations:  global
                Rel. Stand. Deviat.:  0
                Abs. Stand. Deviat.:  1
                Minimum:              0
                Maximum:              1e+009
                Interpolation Method: linear interpolation
                Sensitivity Analysis: inactive
                Real Data Pairs (11 pairs):
                   0              20
                   12             16.4
                   24             12.5
                   36             9.35
                   48             5.5
                   60             3.8
                   72             2.92
                   84             1.8
                   96             1.201
                   108            0.655
                   120            0.546
-------------------------------------------------------------------------
Co:             Description:          initial concentration
                Type:                 Formula Variable
                Unit:                 %v/v
                Expression:           20
-------------------------------------------------------------------------
K:              Description:          rate coefficient
                Type:                 Constant Variable
                Unit:                 1/h
                Value:                0.10214712
                Standard Deviation:   1
                Minimum:              0
                Maximum:              10
```


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```
                Sensitivity Analysis: active
                Parameter Estimation: active
-------------------------------------------------------------------------
ka:             Description:          adsorption coefficient
                Type:                 Constant Variable
                Unit:                 1/(%v/v)
                Value:                0.49756432
                Standard Deviation:   1
                Minimum:              0
                Maximum:              10
                Sensitivity Analysis: active
                Parameter Estimation: active
-------------------------------------------------------------------------
t:              Description:          time
                Type:                 Program Variable
                Unit:                 h
                Reference to:         Time
*************************************************************************




*************************************************************************
Processes
*************************************************************************
reduction:      Description:          oil recovery
                Type:                 Dynamic Process
                Rate:                 (ka*K*C)/(1+K*C)
                Stoichiometry:
                  Variable : Stoichiometric Coefficient
                  C : -1
*************************************************************************

Compartments
*************************************************************************
reactor:        Description:
                Type:                 Mixed Reactor Compartment
                Compartment Index:    0
                Active Variables:     C
                Active Processes:     reduction
                Initial Conditions:
                  Variable(Zone) : Initial Condition
                  C(Bulk Volume) : Co
                Inflow:               0
                Loadings:
                Volume:               1
                Accuracies:
                  Rel. Acc. Q:        0.001
                  Abs. Acc. Q:        0.001
                  Rel. Acc. V:        0.001
                  Abs. Acc. V:        0.001
*************************************************************************
```


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```
Definitions of Calculations
**************************************************************************
calc1:          Description:
                Calculation Number:   0
                Initial Time:         0
                Initial State:        given, made consistent
                Step Size:            0.001
                Num. Steps:           120000
                Status:               active for simulation
                                      inactive for sensitivity analysis
**************************************************************************

Definitions of Parameter Estimation Calculations
**************************************************************************
fit1:           Description:
                Calculation Number:   0
                Initial Time:         0
                Initial State:        given, made consistent
                Status:               active
                Fit Targets:
                  Data : Variable (Compartment,Zone,Time/Space)
                  Cmeas : C (reactor,Bulk Volume,0)
**************************************************************************

Plot Definitions
**************************************************************************
plot1:          Description:
                Abscissa:             Time
                Title:                Oil Recovery
                Abscissa Label:       time,h
                Ordinate Label:       Oil concentration (%v/v)
                Curves:
                  Type : Variable [CalcNum,Comp.,Zone,Time/Space]
                  Value : Cmeas [0,reactor,Bulk Volume,0]
                  Value : C [0,reactor,Bulk Volume,0,
                  rel.space]
**************************************************************************

Calculation Parameters
**************************************************************************
Numerical Parameters:    Maximum Int. Step Size:  1
                         Maximum Integrat. Order: 5
                         Number of Codiagonals:   1000
                         Maximum Number of Steps: 1000
--------------------------------------------------------------------------
                         Fit Method:              secant
                         Max. Number of Iterat.:  100
**************************************************************************

Calculated States
**************************************************************************
Calc. Num.  Num. States  Comments
0           120001       Range of Times: 0 - 120
```


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B2: Simulation for oil recovery using biosurfactant-producing bacteria consortium.

```
AQUASIM Version 2.0 (win/mfc) - Listing of System Definition. Date and
time of listing:  04/17/2014 12:47:27

Variables
**********************************************************************
C:              Description:          concentration
                Type:                 Dyn. Volume State Var.
                Unit:                 %v/v
                Relative Accuracy:    1e-006
                Absolute Accuracy:    1e-006
----------------------------------------------------------------------
Cmeas:          Description:          measured oil concentrations
                Type:                 Real List Variable
                Unit:                 %v/v
                Argument:             t
                Standard Deviations:  global
                Rel. Stand. Deviat.:  0
                Abs. Stand. Deviat.:  1
                Minimum:              0
                Maximum:              1e+009
                Interpolation Method: linear interpolation
                Sensitivity Analysis: inactive
                Real Data Pairs (11 pairs):
                   0              20
                   12             16.2
                   24             12.8
                   36             10.06
                   48             7.847
                   60             6.137
                   72             4.779
                   84             3.672
                   96             2.941
                   108            2.147
                   120            1.662
----------------------------------------------------------------------
Co:             Description:          initial conc
                Type:                 Formula Variable
                Unit:                 %v/v
                Expression:           20
----------------------------------------------------------------------
Cs:             Description:          surfactant conc
                Type:                 Formula Variable
                Unit:                 %v/v
                Expression:           20
----------------------------------------------------------------------
K:              Description:          rate coefficient
                Type:                 Constant Variable
                Unit:
                Value:                0.017232265
                Standard Deviation:   1
```


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```
                Minimum:              0
                Maximum:              10
                Sensitivity Analysis: active
                Parameter Estimation: active
--------------------------------------------------------------------------
ka:             Description:          affinity coefficient
                Type:                 Constant Variable
                Unit:                 1/(%v/v)
                Value:                1.3582754
                Standard Deviation:   1
                Minimum:              0
                Maximum:              10
                Sensitivity Analysis: active
                Parameter Estimation: active
--------------------------------------------------------------------------
t:              Description:          time
                Type:                 Program Variable
                Unit:                 h
                Reference to:         Time
**************************************************************************

Processes
**************************************************************************
reduction:      Description:          oil recovery
                Type:                 Dynamic Process
                Rate:                 (ka*K*C)/(1+K*C)
                Stoichiometry:
                  Variable : Stoichiometric Coefficient
                  C : -1
**************************************************************************


Compartments
**************************************************************************
reactor:        Description:
                Type:                 Mixed Reactor Compartment
                Compartment Index:    0
                Active Variables:     C
                Active Processes:     reduction
                Initial Conditions:
                  Variable(Zone) : Initial Condition
                  C(Bulk Volume) : Co
                Inflow:               0
                Loadings:
                Volume:               1
                Accuracies:
                  Rel. Acc. Q:        0.001
                  Abs. Acc. Q:        0.001
                  Rel. Acc. V:        0.001
                  Abs. Acc. V:        0.001
**************************************************************************
```


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```
Definitions of Calculations
**************************************************************************
calc1:          Description:
                Calculation Number:   0
                Initial Time:         0
                Initial State:        given, made consistent
                Step Size:            0.001
                Num. Steps:           120000
                Status:               active for simulation
                                      inactive for sensitivity analysis
**************************************************************************

Definitions of Parameter Estimation Calculations
**************************************************************************
fit1:           Description:
                Calculation Number:   0
                Initial Time:         0
                Initial State:        given, made consistent
                Status:               active
                Fit Targets:
                  Data : Variable (Compartment,Zone,Time/Space)
                  Cmeas : C (reactor,Bulk Volume,0)
**************************************************************************

Plot Definitions
**************************************************************************
plot1:          Description:
                Abscissa:             Time
                Title:                Oil recovery with biosurfactant
                Abscissa Label:       time, h
                Ordinate Label:       Oil concentration (%v/v)
                Curves:
                  Type : Variable [CalcNum,Comp.,Zone,Time/Space]
                  Value : Cmeas [0,reactor,Bulk Volume,0]
                  Value : C [0,reactor,Bulk Volume,0,
                  rel.space]
**************************************************************************

Calculation Parameters
**************************************************************************
Numerical Parameters:    Maximum Int. Step Size:  1
                         Maximum Integrat. Order: 5
                         Number of Codiagonals:   1000
                         Maximum Number of Steps: 1000
--------------------------------------------------------------------------
                         Fit Method:              simplex
                         Max. Number of Iterat.:  100
**************************************************************************

Calculated States
**************************************************************************
Calc. Num.  Num. States  Comments
0           120001       Range of Times: 0 - 120
**************************************************************************
```


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B3: Statistical analysis of Batch data (Biosurfactants only)

Linear Regression Tuesday, April 08, 2014, 11:56:15 AM

Data source: Batch kinetics

Biosurfactant model = 0.0482 + (0.000133 * Time)

N = 11

R = 0.998 Rsqr = 0.998 Adj Rsqr = 0.999

Standard Error of Estimate = 0.000

	Coefficient	Std. Error	t	P
Constant	0.0482	0.0000860	560.999	<0.001
Time	0.000133	0.00000121	109.556	<0.001

Analysis of Variance:

	DF	SS	MS	F	P
Regression	1	0.000279	0.000279	12002.614	<0.001
Residual	9	0.000000209	0.0000000232		
Total	10	0.000279	0.0000279		

Normality Test (Shapiro-Wilk) Passed (P = 0.544); Constant Variance Test: Passed (P = 0.245)

B4: Statistical analysis of batch data (Waterflooding + Biosurfactants)

Linear Regression Tuesday, April 08, 2014, 4:31:39 PM

Data source: Batch kinetics

Waterflooding + Biosurfactant model = 0.0485 + (0.000136 * Time)

N = 11

R = 0.998 Rsqr = 0.996 Adj Rsqr = 0.996

Standard Error of Estimate = 0.000

	Coefficient	Std. Error	t	P
Constant	0.0485	0.000198	245.347	<0.001
Time	0.000136	0.00000278	48.988	<0.001

Analysis of Variance:

	DF	SS	MS	F	P
Regression	1	0.000294	0.000294	2399.835	<0.001
Residual	9	0.00000110	0.000000123		
Total	10	0.000296	0.0000296		

Normality Test (Shapiro-Wilk) Failed (P = 0.047); Constant Variance Test: Passed (P = 0.575)

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B5: Statistical analysis of Batch data (Waterflooding)

Linear Regression Tuesday, April 08, 2014, 4:33:28 PM

Data source: Batch kinetics

Waterflooding model = 0.0485 + (0.000137 * Time)

N = 11

R = 0.998 Rsqr = 0.997 Adj Rsqr = 0.996

Standard Error of Estimate = 0.000

	Coefficient	Std. Error	t	P
Constant	0.0485	0.000184	263.242	<0.001
Time	0.000137	0.00000259	52.912	<0.001

Analysis of Variance:

	DF	SS	MS	F	P
Regression	1	0.000298	0.000298	2799.650	<0.001
Residual	9	0.000000958	0.000000106		
Total	10	0.000299	0.0000299		

Normality Test (Shapiro-Wilk) Failed (P = 0.011); Constant Variance Test: Passed (P = 0.450)

B6: Statistical analysis of PFR data (Biosurfactants)

Nonlinear Regression Thursday, April 17, 2014, 12:07:16 PM

Data Source: PFR Biosurfactant model
Equation: Exponential Decay, Single, 3 Parameter
f=y0+a*exp(-b*x)

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.9999	0.9999	0.9999	0.0603

	Coefficient	Std. Error	t	P
y0	-0.9152	0.0011	-808.8545	<0.0001
a	21.1114	0.0009	22639.3697	<0.0001
b	0.0179	2.3975E-006	7484.7512	<0.0001

Analysis of Variance:

Analysis of Variance:

	DF	SS	MS
Regression	3	10448224.5406	3482741.5135
Residual	119998	436.7228	0.0036
Total	120001	10448661.2635	87.0715

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Corrected for the mean of the observations:

	DF	SS	MS	F	P
Regression	2	3239811.2864	1619905.6432	445100264.2852	<0.0001
Residual	119998	436.7228	0.0036		
Total	120000	3240248.0092	27.0021		

Statistical Tests:

Normality Test (Kolmogorov-Smirnov) Failed (P = <0.0001)

K-S Statistic = 0.0866 Significance Level = 0.0500; **Constant Variance Test** Failed (P = <0.0001)

B7: Statistical analysis of PFR data (Waterflooding + Biosurfactants)

Nonlinear Regression Thursday, April 17, 2014, 12:18:20 PM

Data Source: PFR Waterflooding + Biosurfactant
Equation: Exponential Decay, Single, 3 Parameter
f=y0+a*exp(-b*x)

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.9987	0.9974	0.9974	0.2973

	Coefficient	Std. Error	t	P
y0	-2.6708	0.0050	-529.0222	<0.0001
a	23.4749	0.0042	5561.8317	<0.0001
b	0.0190	1.0628E-005	1787.5129	<0.0001

Analysis of Variance:

Analysis of Variance:

	DF	SS	MS
Regression	3	9283044.3832	3094348.1277
Residual	119998	10609.0141	0.0884
Total	120001	9293653.3973	77.4465

Corrected for the mean of the observations:

	DF	SS	MS	F	P
Regression	2	4098283.9246	2049141.9623	23177736.8739	<0.0001
Residual	119998	10609.0141	0.0884		
Total	120000	4108892.9387	34.2408		

Statistical Tests:

Normality Test (Kolmogorov-Smirnov) Failed (P = <0.0001)

K-S Statistic = 0.0810 Significance Level = 0.0500; **Constant Variance Test** Failed (P = <0.0001)

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B8: Statistical analysis of PFR data (Waterflooding)

Nonlinear Regression Friday, April 18, 2014, 10:17:34 AM

Data Source: PFR Waterflooding model
Equation: Exponential Decay, Single, 3 Parameter
f=y0+a*exp(-b*x)

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.9999	0.9999	0.9999	0.0563

	Coefficient	Std. Error	t	P
y0	-2.8911	0.0025	-1137.9581	<0.0001
a	23.0520	0.0022	10352.0720	<0.0001
b	0.0112	2.2580E-006	4939.9677	<0.0001

Analysis of Variance:

Analysis of Variance:

	DF	SS	MS
Regression	3	14368950.4448	4789650.1483
Residual	119998	379.7630	0.0032
Total	120001	14369330.2078	119.7434

Corrected for the mean of the observations:

	DF	SS	MS	F	P
Regression	2	2809701.1089	1404850.5544	443906470.9279	<0.0001
Residual	119998	379.7630	0.0032		
Total	120000	2810080.8719	23.4173		

Statistical Tests:

Normality Test (Kolmogorov-Smirnov) Failed (P = <0.0001)

K-S Statistic = 0.0765 Significance Level = 0.0500

Constant Variance Test Failed (P = <0.0001)

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APPENDIX C: PATHWAYS

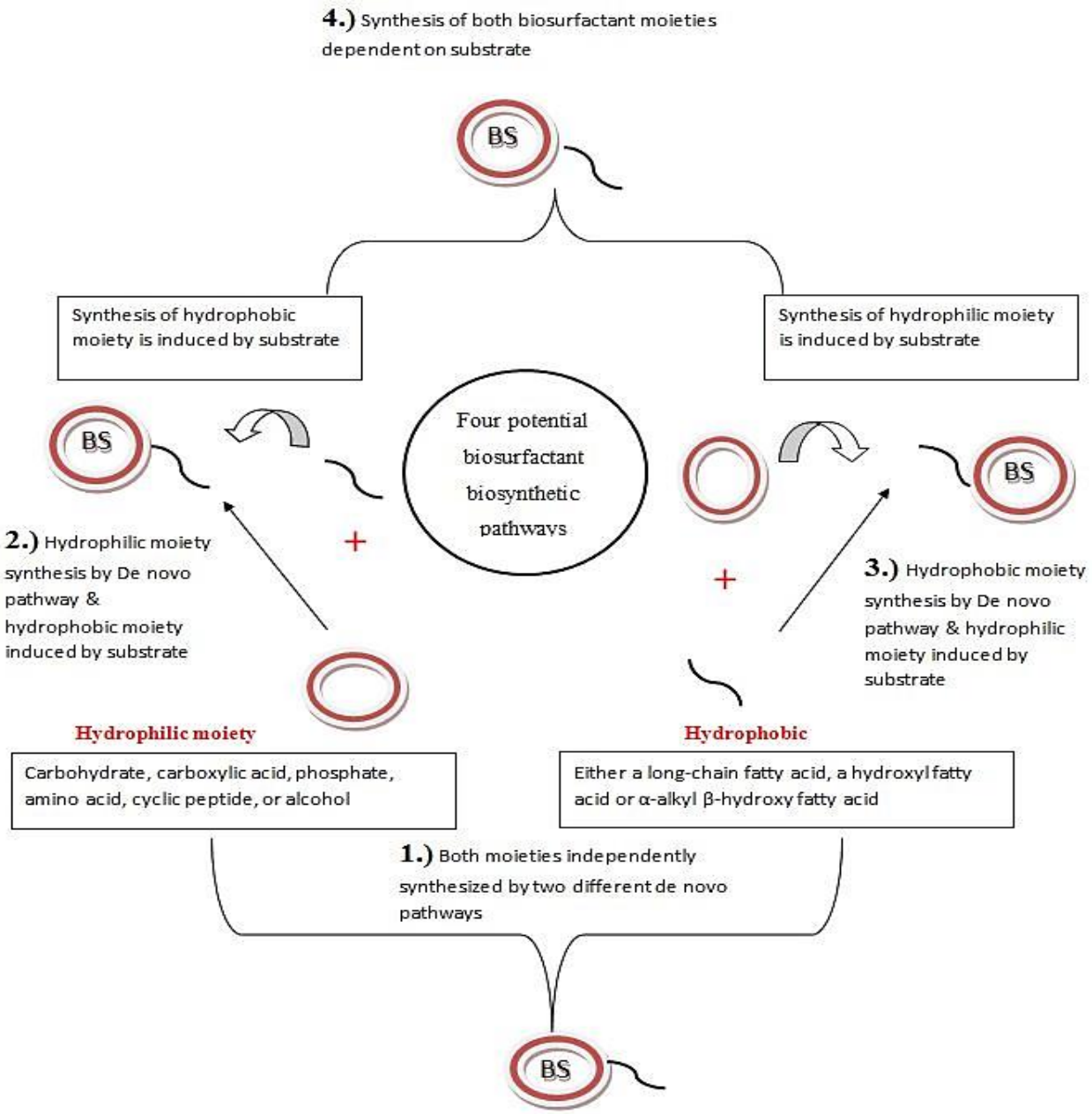

Figure C-1: Potential biosurfactant biosynthetic pathways in microorganisms. BS: Biosurfactant molecule. Diagram based on four assumptions by Syldatk and Wagner (1987).

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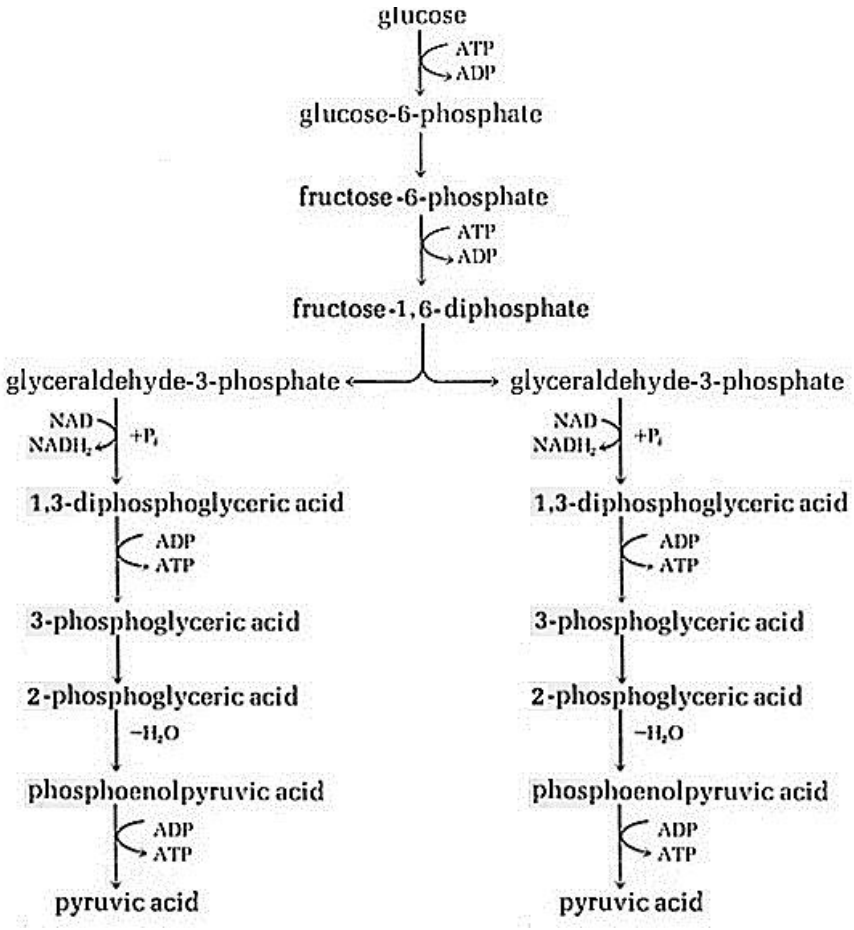

Figure C-2: Embden-Meyerhof pathway for sugar utilization (http://textbookofbacteriology.net/metabolism_3.html)

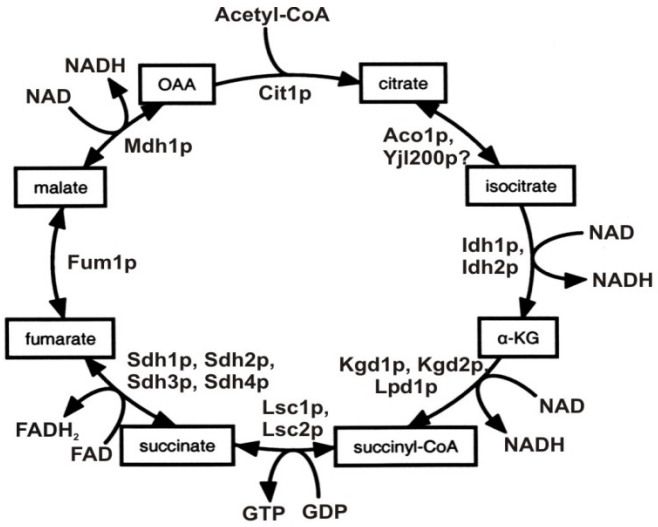

Figure C-3: TCA Cycle. (Steinmetz et al. 2002)

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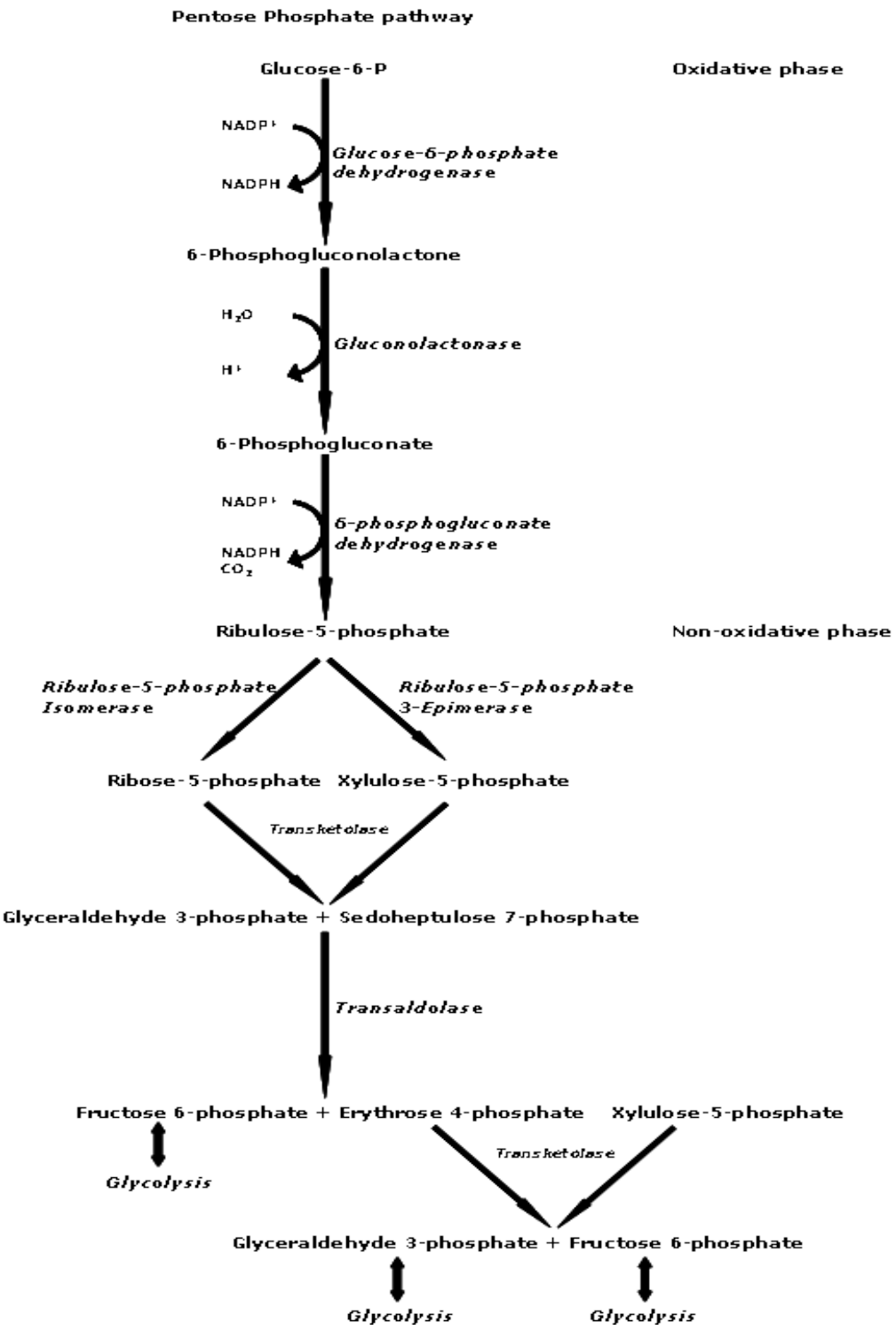

Figure C-4: Pentose phosphate pathway (Kruger & vonSchaewen 2003)

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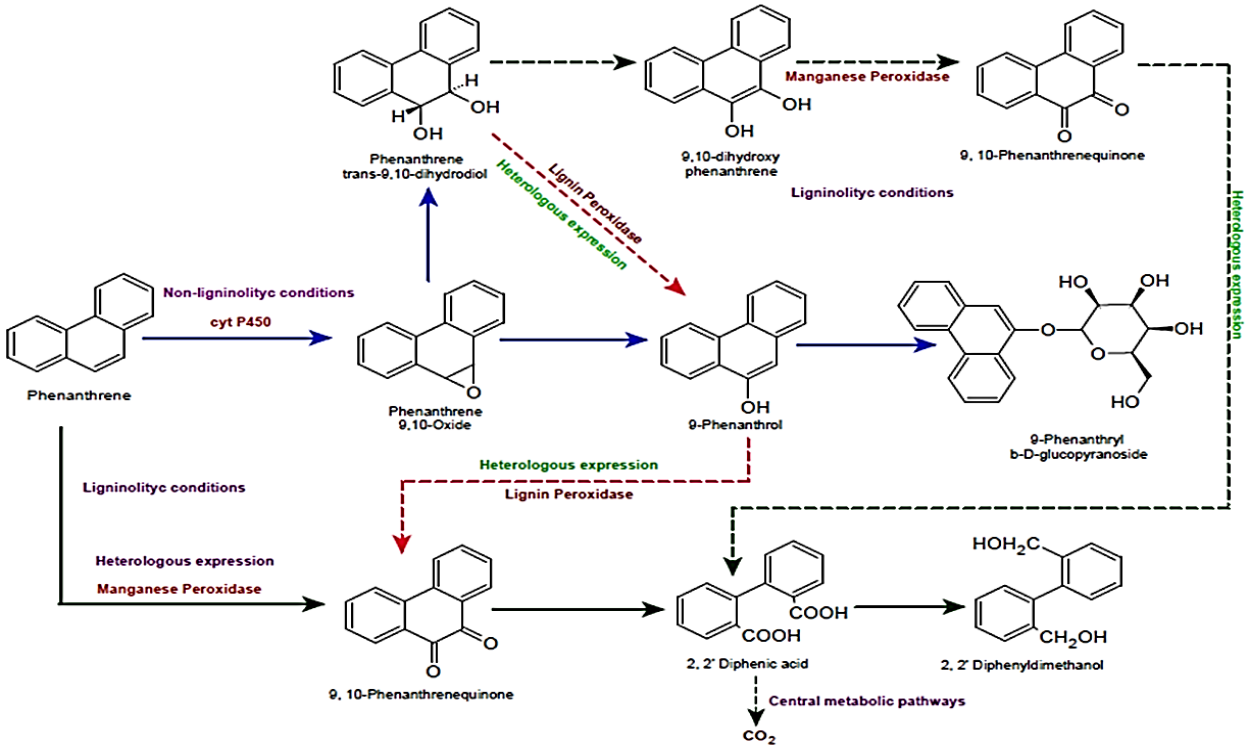

Figure C-5: Pathway for phenanthrene utilization by microorganisms (Cortés-Espinosa & Absalón 2013).

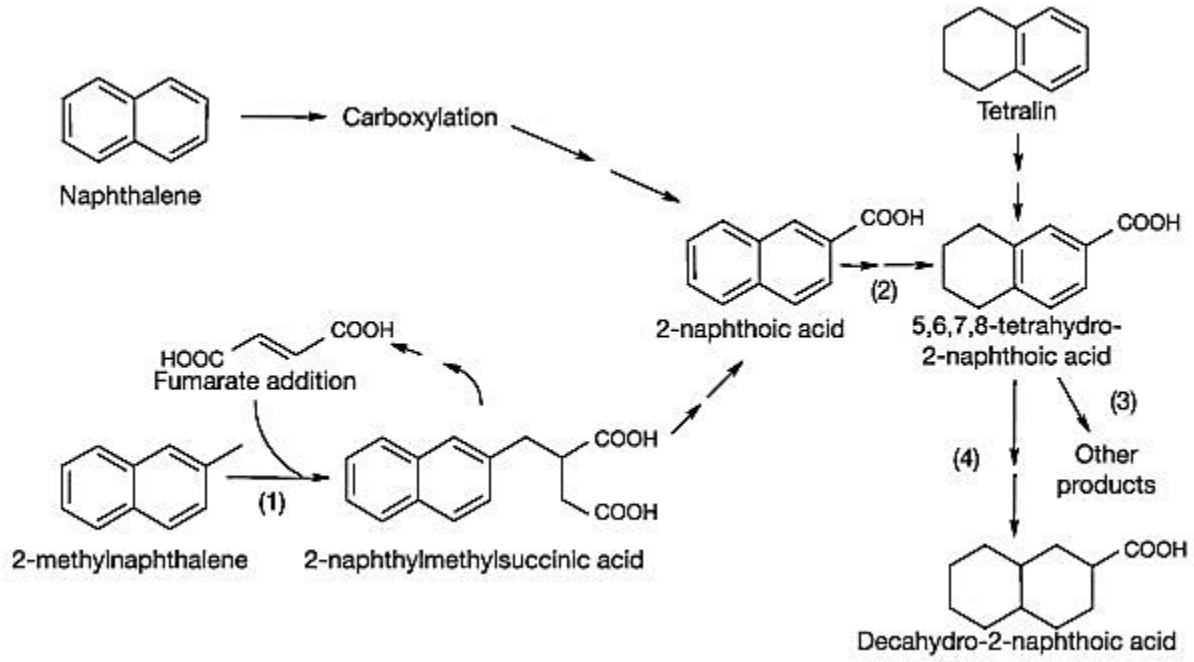

Figure C-6: Pathway for naphthalene utilization by microorganisms (Aitken et al. 2004)

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