Faculty of Engineering

Immobilization of *Thalassospira profundimaris* M02 for Improved Biotransformation of Heterocyclic Hydrocarbon Compounds

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

Heterocyclic hydrocarbon compounds are known as by-products for various industries and has become a threat of contamination in the environment. Bioremediation was applied to solve this problem. However, high toxicity levels tend to inhibit the performance of the bacteria for biodegradation of the compounds. Cell immobilization technique was developed and applied to resolve this issue. This study aimed to develop and investigate the performance of immobilized cell in comparison to free cells in the degradation of heterocyclic hydrocarbon compounds such as carbazole (CAR), dibenzofuran (DBF), dibenzothiophene (DBT) and fluorene (FL). In this study, the optimal cell immobilization conditions of isolated marine bacteria, *T. profundimaris* strain M02 was immobilized by using calcium alginate at 4% (w/v) concentration with cell mass loading of 1.25 g. These developed immobilized cells were also able to be used repetitively although depletion of the cell's performance was observed. On other hand, *T. profundimaris* strain M02 was able to degrade multiple heterocyclic hydrocarbon compounds and showed significant performance improvement when immobilized. Actual performance test was carried out in bioreactor and from the test, both free and immobilized cells showed improved performance in a controlled environment in the bioreactor when compared to the laboratory scale experiment. Comparative study of free and immobilized cell performance in bioreactor showed that immobilized cells exhibited higher concentration of CAR degraded as well as higher specific growth rate. To conclude, the findings of this study discovered the optimum conditions for bacteria immobilization for heterocyclic hydrocarbon compounds degradation as well as revealing that a single bacteria strain are able to degrade multiple compounds. This information is important as it can be utilized for biodegradation of heterocyclic hydrocarbon

compounds in the environment and improved the existing technique that is currently implemented today.

Keywords: Heterocyclic hydrocarbon, bioremediation, cell immobilization, bioreactor

Immobilisasi Sel Thalasosspira profundimaris M02 untuk Peningkatan Biotransformasi Sebatian Hidrokarbon Heterosiklik

ABSTRAK

Sebatian hidrokarbon heterosiklik dikenali sebagai produk sampingan daripada industri dan penggunaan sebatian ini secara meluas menyebabkan peningkatan risiko pencemaran alam sekitar. Masalah ini telah diatasi dengan kaedah bioremediasi. Walau bagaimanapun, tahap ketoksikan yang tinggi telah merencat kemampuan bakteria dalam proses biodegradasi sebatian dan teknik immobilisasi sel telah di gunakan untuk mengatasi masalah ini. Kajian ini bertujuan untuk mengkaji dan membandingkan kemampuan sel yang telah di immobilisasikan dengan sel bebas dalam proses penguraian sebatian hidrokarbon heterosiklik seperti karbazol (CAR), dibenzofuran (DBF), dibenzotiofena (DBT) dan fluorena (FL). Dalam kajian ini, keadaan optimum imobilisasi sel bakteria marin, Thalassosphira profundimaris M02 adalah dengan menggunakan kalsium alginat pada kepekatan 4% (w/v) dan berat sel pada 1.25 g. Keadaan sel yang telah dibangunkan ini mampu digunakan secara berulang kali walaupun terdapat pengurangan dalam kemampuan sel tersebut. Selain itu, M02 juga mampu mengurai pelbagai sebatian hidrokarbon heterosiklik dan menunjukan perkembangan yang signifikan apabila di imobilisasikan. Kemampuan di persekitaran sebenar telah di jalankan dengan mengunakan bioreaktor dan kedua-dua sel ini menunjukkan perkembangan yang baik di dalam bioreaktor. Kajian perbandingan antara sel bebas dan sel imobilisasi dalam bioreaktor menunjukan sel imobilisasi mengurai lebih banyak sebatian dan kadar pertumbuhan spesifik yang lebih tinggi. Kesimpulannya, kajian ini telah berjaya menghasilkan keadaan optimum untuk imobilisasi bakteria dan ianya mampu mengurai pelbagai sebatian menggunakan satu

spesies bakteria. Maklumat ini penting kerana ianya boleh diaplikasi untuk biodegradasi sebatian hidrokarbon heterosiklik dalam alam sekitar dan juga menambahbaik teknik yang sedia ada.

Kata kunci: *Hidrokarbon heterosiklik, bioremediasi, sel imobilisasi, bioreaktor*

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

$CaCl_2$	Calcium chloride
CAR	Carbazole
cm^2/s	Centimetre square per seconds
°C	Degree Celsius
DBF	Dibenzofuran
DBT	Dibenzothiophene
FL	Fluorine
g	Grams
HCl	Hydrochloric acid
L	Litres
μm	Micrometre
mg/L	Milligram per litre
mL	Millilitre
mL/min	Millilitre per minute
mm	Millimetre
M	Molar
rpm	Rotation per minute
NaCl	Sodium chloride
SEM	Scanning Electron Microscope
w/v	Weight over volume

CHAPTER 1

INTRODUCTION

1.1 Introduction

Heterocyclic hydrocarbon compounds exist in the environment due to the incomplete combustions of fuels and biomass. It has 2 or more benzene rings attached to the five membered rings of an aromatic carbon with one carbon replaced by sulphur, oxygen or nitrogen. Most of these compounds are exceedingly recalcitrant to degradation due to their inhibitory nature and low aqueous stability properties, at which become more pronounced as the number of rings increases (Daugulis & Janikowski, 2002).

The process of removing these pollutants from polluted environments varies from physical removal to microbiological removal. Microbiological removal of these compounds is known as bioremediation. Bioremediation was in favour these days as it leaves less harmful by products, effective as well as time efficient. Over time, the knowledge demand and intricacy of bioremediation process increases in order to keep up with the world's increasing drainage of wastes that contributes to the increasing level of contaminants in the environment. Therefore, cell immobilization was identified as the most attractive solution to these problems.

Cell immobilization offers various advantages compared to the conventional method of bioremediation. Immobilization is a term describing the entrapment or attachment of cells or particles in a matrix (López, Lázaro, & Marqués, 1997). It can be applied to catalysts ranging from cellular organisms, animal and plant cells (Martins et al., 2013). It is not only restricted to the field of biotechnology, but is also used in food, environment and other industries Cell immobilization is encountered various bioprocesses

such as wastewater treatment, vinegar production, dairy production and bioleaching of mineral ores (Nedović, Willaert, Leskošek-Čukalović, Obradović, & Bugarski, 2005).

Large scale studies of bioremediation are vital for the understanding of how the process will differs from lab scale studies. In some cases, results from large scale studies differs significantly from the lab scale studies. Hence, bioreactor can be used to mimic the conditions of environment in a large scale. By using bioreactor, the cell's behaviour can be thoroughly observed and analysed. Biochemical process involved can also be studied to study the knowledge behind bioremediation process.

1.2 Problem Statement

Bioremediation is an effective way to treat pollution in the environment. However, over the time, the levels of contaminants are increasing with the uprising of various industries and its factories. This causes conventional bioremediation process unable to sustain its efficiencies. To date, there are no reports on the optimum condition for bacterial immobilization for heterocyclic hydrocarbon compounds degradation. Various reports showed different conditions for the immobilization hence different outcomes were produced each time. Different types of bacteria were also used at each time. Hence, large scale study of bioremediation of heterocyclic compounds were difficult to standardize as there are no standard baseline and identification of a single strain bacteria to degrade multiple compounds.

1.3 Hypothesis

Marine bacteria isolated from contaminated environment can be utilized for heterocyclic hydrocarbon compounds bioremediation and its performance can be improved by immobilization method. A single bacterium strain can be utilized to degrade multiple compounds via immobilization. The cell is expected to show improved performance with immobilization compared to free suspended cells. Scale up study will show better results when compared with small scale studies as developed conditions in bioreactor helps in maintaining the optimal environmental conditions for the immobilized cells to perform biodegradation.

1.4 Objectives

This study aims to achieve the following objectives;

i. To develop and optimize the conditions for immobilization of *Thalassospira profundimaris* strain M02 for degradation of selected heterocyclic hydrocarbon compounds such as carbazole, dibenzofuran, dibenzothiophene and fluorene.
ii. To study the capabilities of *T. profundimaris* M02 in degrading various heterocyclic hydrocarbon compounds and compare the performance of the degradation of these compounds between free and immobilized cell.
iii. To investigate the potential of bioremediation of heterocyclic hydrocarbon compounds using seawater in bioreactor.

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CHAPTER 2

LITERATURE REVIEW

2.1 Heterocyclic Aromatic Hydrocarbon

Heterocyclic hydrocarbons are well known as environmental contaminants resulted from petroleum, timber and textile industries. These compounds may be found in sea water, river sediments and soil (Jha & Bharti, 2002) where the majority originated from anthropogenic sources and has become great threats to environment due to its mutagenic and toxic properties. As the process of industrialization occurs, semisolid tar oil pollutants became ubiquitous, and currently soils and sediments are major sinks of polycyclic aromatic hydrocarbons (PAHs) (Eisentraeger et al., 2008). Heterocyclic substances are present at lower concentrations than their non-substituted analogues (PAHs) in tar oil. However, their increased water solubility leads to higher bioavailability and potential for toxic effects, and their high mobility causes the formation of long plumes of contaminated groundwater at tar oil-polluted sites (Eisentraeger et al., 2008). This enables them to leach into water and contaminate both groundwater and drinking water. There is limited knowledge on these compound occurrence, biological metabolism and toxic effects, thus urging the needs to incorporate heterocyclic hydrocarbons and biotransformation products in toxicological analysis.

Carbazole (CAR), ($C_{12}H_9N$) is a non-basic tricyclic aromatic N-heteroatomic compound and its structure consists of two six-member benzene rings fused on either side of a five-member nitrogen-containing ring. This compound's structure is based on the indole structure, in which the second benzene ring is fused on the five-member ring at 2-3 position of indole. CAR is one of the major N-heterocyclic hydrocarbons in fossil fuels and also found in cigarette smoke, as well as from coal and wood combustion

(Salam et al., 2015). Its released to the environment has become a serious health and environmental concern as it has been classified as a “benign tumorigen” due to its mutagenic and toxic properties (Salam et al., 2015). Isolated bacteria utilizing CAR as their sole carbon and energy source have been shown successfully isolated in several studies. These bacteria have been shown to degrade CAR through the angular deoxygenation and meta-cleavage pathway (Shi, Qu, Zhou, Ma, & Ma, 2015a). As heterocyclic hydrocarbon compounds such as CAR are widespread and usually coexist in polluted environment, cometabolic degradation of these compounds using isolated bacteria growing with some heterocyclic hydrocarbon compounds should be common (Shi et al., 2015a).

Dibenzothiophene (DBT), ($C_{12}H_8S$) is a component commonly found in creosote, crude oils and shale oils, which often co-exist with PAHs and other aromatic compounds in the environment. DBT is a sulfur heterocyclic compound and quite persistent in the environment. Usually, sulfur reduction in the environment is achieved by hydro-desulfurization (HDS) where it consists of reduction of sulfur atom into H_2S through hydrogenation on $CoMo/Al_2O_3$ catalyst (Calzada et al., 2009). However, DBT shows resistance to be completely removed by HDS. Therefore, other proposed desulfurization that can overcome conventional HDS is bio-desulfurization (BDS). A research by Calzada et al. (2009) proved that by using BDS, a higher amount of DBT conversion was achieved at shorter time. Some bacterial cultures have been tested for the removal ability of DBT in kerosene as DBT is a major sulphur-containing component in petroleum. A study by Mishra et. al in 2016 discovered that their culture, *Desulfobacterium indolicum* removed 72% of sulphur content from a kerosene sample at 72 hours of incubation. Besides that, a study using native bacteria strain such as *Bacillus cereus* removed 33% of sulphur content

from another sulphur-containing kerosene samples (Mishra, Pradhan, Panda, & Akcil, 2016). The degradation pathway of DBT was studied extensively over the years to understand the most basic mechanism responsible for DBT biodegradation. Such pathways that were mentioned was the Kodoma's pathway and the 4s pathway (Mishra et al., 2016). The Kodoma's pathway was initially named the Oxidative C-C cleavage pathway but was labelled Kodoma's pathway as his team was the initial reporter of the pathway (Mishra et al., 2016). This pathway involves the cleavage of oxidative ring of the DBT forming certain intermediate products and consists of 3 major steps. First, is the hydroxylation, second is the ring cleavage and lastly is the hydrolysis that formed 3-hydroxy-2-formyl benzethiophene (HFBT) as the end product of the pathway. Kilbane was first to propose the 4S pathway of DBT degradation in 1989 (Kilbane, 1989) and was reported by Gallagher and his associates in the bacterium *Rhodococcus rhodochrous* IGTS8 (Gallagher, Olson, & Stanley, 1993). The pathway starts with the transformation of DBT to DBT sulphoxide (DBTO), followed by the sequential conversion of DBTO to DBT sulphone (DBTO2), sulphinate and hydroxybiphenyl. Finally, the pathway ends with the production of 2-hydroxybiphenyl (2-HBP) from DBT sulphite (Mishra et al., 2016).

Dibenzofuran ($C_{12}H_8O$) is a heterocyclic organic compound where two benzene rings were fused to a central furan ring. Dibenzofuran (DBF) are a poorly water soluble polycyclic aromatic hydrocarbons (PAHs) formed by the by-products coal at industrial processes, incineration process and in paper pulp bleaching (Coronado, Roggo, Johnson, Meer, & Roelof, 2012). Due to its toxicity and recalcitrance, the study of microbial degradation of DBF has been widely emerged. DBF and its derivatives are industrially produced as intermediates by multiple processes such as coal combustion and gasification, pesticides manufacturing and pulp bleaching at paper mills. Becher et al.

(2000), states that DBF are able to be degraded by using microorganism that uses it as a sole carbon source. Degradation of DBF starts with an oxygenolytic attack at angular position 4 and 4a adjacent to the ether bridge, which results in the formation of 2,2',3-trihydroxybiphenyl and this intermediate is transformed by meta-cleavage to a 2-hydroxyphenyl hexadienioc acid derivative and salicyclic acid (Becher, Specht, Hammer, Francke, & Schauer, 2000).

Fluorene (FL) is a polycyclic aromatic hydrocarbon which contains three rings that are covalently bonded together. It is a major constituent of fossil fuels and coal derivatives and has become a major environmental concern due to its association with petroleum and oil spills, waste incineration and industrial effluents (Akdogan & Pazarlioglu, 2011). Besides that, FL also have wide application in industry since they are used as base materials for dyes and optical brightening agents (Akdogan & Pazarlioglu, 2011). Although it is known to be toxic, some bacteria are able to use this compound as a sole carbon source and energy such as in the genera of *Arthrobacter*, *Brevibacterium*, *Burkholderia*, *Mycobacterium*, *Pseudomonas* and *Sphingomonas* (Seo, Keum, & Li, 2009). Akdogan & Pazarlioglu (2010) also mentioned that FL characteristics includes light and temperature sensitive, heat resistance and conductivity and corrosion resistance. Hence, making it suitable for use in the areas of thermo and light sensitizers, luminescence chemistry, spectrophotometric analysis and molecular chemistry.

2.2 Marine Bacteria

Microorganism plays an important role in maintenance and sustainability of any ecosystem as they are more capable of rapid adjustment towards environmental changes and detoriation (Dash, Mangwani, Chakraborty, Kumari, & Das, 2013). Marine is well known as the largest habitat on the earth, which includes the habitat for some bacterium.

Besides marine water, marine bacteria also can be isolated from sediments and mangroves associated with the marine habitats, normal flora of the marine organisms and deep-sea hydro thermal vents (Dash et al., 2013). Das et al. (2006) said in their review paper that marine bacteria can be isolated from mangrove and coral reef ecosystem, as well as in deep and inshore waters of all oceans and seas (Das, Lyla, & Khan, 2006). Back in 1944, ZoBell and Upham have characterized 60 species of bacterial diversity in marine environment (ZoBell and Upham, 1994) as cited by Dash et al. (2013).

Marine bacteria have become a potential candidate for bioremediation due to its vast diversity to their functional role they play in the marine environment as well as their fast respond to changing environmental patterns. Such changes include the change in sea surface temperature, environmental pH change, the changing pattern of light and UV light, sea level rise, tropical storm and also terrestrial inputs (Dash et al., 2013). This problem can be overcome by some group of microorganisms by shifting their physical locations beneath sediments or by symbiosis with other organism, which is mostly found in pathogenic microorganisms (Dash et al., 2013).

Application of marine bacteria in bioremediation includes the removal of heavy metals, degradation of polyaromatic hydrocarbon (PAHs) compounds and biodegradation of petroleum and diesel (Dash et al., 2013). Marine microorganism solves the problem of heavy metals because they do not produce any by-products and its efficiency rate is high at low metal concentration (De, Ramaiah, & Vardanyan, 2008). Besides that, Iyer et al. (2005) said that marine bacteria also possesses the properties of chelation of heavy metal, hence removing them from the contaminated environment by the secretion of expolysaccharides which have been evident from the reports of a marine bacterium, *Enterobacter cloaceae* (Iyer, Mody, & Jha, 2005). PAHs are present in nature and are one

of a great environmental concern due to its mutagenicity and toxicity. However, marine bacteria are reported to be potentially effective for bioremediation because these microorganism uses PAH compounds as a carbon source for the process of metabolism to produce carbon dioxide (Dash et al., 2013). Crude oil is one of the most important organic pollutant in the environment as they are released in abundant annually. Using bacterial isolates to observe compounds degradation has been widely studied. The marine environment contains oil-eating microbes which can degrade these organic pollutants and it use these pollutants as their carbon and energy source. Table 2.1 shows the list of potential bacteria and its target substance.

Table 2.1: Potential marine bacteria and target substance

Potential marine bacteria	Target substance	Reference
Pseudomonas aeruginosa	Inorganic mercury	De et al. (2008); Dash & Das (2012)
Cycloclasticus sp.	PAH	Kasai, Kishira, & Harayama (2002)
Pseudomonas sp.	Phenol	Selvaratnam, Schoedel, McFarland, & Kulpa (1997)
Staphylococcus aureus	Chromate	Aguilar-Barajas, Paluscio, Cervantes, & Rensing (2008)
Bacillus subtilis, *Bacillus cereus*	Cobalt–Zinc–Cadmium	Abou-Shanab, van Berkum, & Angle (2007)
Pseudomonas sp., *Bordetella* sp.	Nickel–Cobalt–Cadmium	Abou-Shanab et al. (2003)

In this study, marine bacteria, *T. profundimaris* was used for biotransformation of CAR, DBT, DBF and FL. The bacteria with genus *Thalassospira* were found in ultraligotropic environment of Eastern Mediterranean Sea which has limited phosphate that can limit the growth of bacterioplankton (Hütz, Schubert, & Overmann, 2011). *T. profundimaris* has characteristics such as Gram-negative curved rods that are 0.8-2.3 μm long and 0.3-0.8 μm wide (Liu, Wu, Li, Ma, & Shao, 2007). It is also a non-motile, non-flagellated and non-spore-forming organism (Liu et al., 2007). It is mostly found in a high salinity environment thus making it a suitable candidate for biodegradation in seawater to dispose or discharge of petroleum in the sea water (Liu et al., 2007). The cell grows in optimally 3-4% NaCl (Liu et al., 2007) at between 10 to 37˚C. It actively hydrolyses Tween 40 and Tween 80, but not agar. This cell reduces nitrate to nitrite which is good for CAR denitrification (Zhao, Wang, Li, & Mao, 2010). However, having to use the free-cell into degrading CAR would not benefit the human themselves as it would be lower stability and lower degradability (Elakkiya, Prabhakaran, & Thirumarimurugan, 2016). This would bring about the best solution for higher degradability as well as more stable method which are known as cell immobilization.

2.3 Bioremediation

Bioremediation is a process that uses microorganisms and their aggregates to detoxify or eliminate pollutants to their own capabilities and is usually used to clean up contaminated soil and groundwater. It involves the growth of certain microbes that consume contaminants as a source of food and energy and utilizes the metabolic potential of microorganisms to clean the contaminated environments. This metabolic ability to mineralize or transform organic pollutants into less harmful substance allows it to be integrated into natural biogeochemical cycles.

Bioremediation processes are classified into three categories, following the condition of place and soil handling/conditioning; *in situ, ad situ* and *ex situ* (Robles-González, Fava, & Poggi-Varaldo, 2008). Each category has different elements to remediates. For example, *ad situ* and *ex situ* bioremediation is suitable for the remediation of sludges, soils or sediments polluted with recalcitrant contaminant of high concentrations, diesel, explosives, pesticides and chlorinated organic pollutant and oily sludges from petrochemical industry (Robles-González et al., 2008). The use of bioremediation technology is potentially effective to treat the pollution in the environment in a cost-competitive, eco-friendly as well as sustainably manner by the isolation of microorganisms that has the ability to transform and degrade the polluted compounds (Kuhad, Sood, Tripathi, Singh, & Ward, 2004). For example, DBF and DBD degradation by *S. wittichii* RW1 was observed when applied to inoculated soil microcosms (Halden, Halden, & Dwyer, 1999; Megharaj, Ramakrishnan, Venkateswarlu, Sethunathan, & Naidu, 2011), while Aso et al. reported an increased degradation of DBF when using modified strain of *S. wittichii* RW1 in contaminated soil (Megharaj et al., 2011)

The process of bioremediation is dependent on the metabolic potential of microorganism to detoxify or transform the pollutant molecule which depends on both accessibility and bioavailability. Bioavailability in water and soils may be influenced by aqueous solubility, volatility or re-activity of reactive pollutants. However, on a mass basis, no relationship exists between the chemical pollutant in soil and biological affect. Bioremediation of organic pollutant in soil and aquatic system are affected by factors such as water content, in which it will affect the transport of pollutant and degraded products. While temperature will affect composition of communities and velocity of degradation

and pH affect the rate of microorganism or enzyme performance as the works best at an optimum pH. Besides that, redox potential, organic matter, nutrients, co-contaminants and microbial communities were also some of the factors that can affect the bioremediation rate of inorganic pollutants (Megharaj et al., 2011). A study by (Moscoso, Deive, Longo, & Sanromán, 2015) mentioned that poor availability limits the efficiency of PAH biodegradation under natural condition due to its low aqueous solubility and high hydrophobicity, which together with their high adsorption coefficient and high thermodynamic stability make up inherent features of this kind of pollutants (Moscoso et al., 2015).

2.4 Cell immobilization

High concentration of xenobiotics usually limits the degradation of pollutants and toxic compounds by natural microbial activities as it inhibits the growth of the microorganism (Ahmad et al., 2012). Hence, cell immobilization is found to be an attractive strategy to produce a robust cell as a biocatalyst. Chen, Li, Liu, Sun, & Bao (2017) conducted a study on comparison between free cell bacteria and immobilized cell and he found that immobilized cells produced higher biomass and provide higher cells t ability to promote biodegradation process (Chen et al., 2017). This would overcome the limitation of slow growth rate and low biodegradation activity of the free cells. In addition, immobilized cells are viable to be used in continuous process as they exhibit high recovery at lower cost, high reusability, as well as protecting the cells from environmental stress (Martins et al., 2013). These advantages encourage researches to investigate the applications of immobilized cells in the biodegradation of various toxic compounds (Martins et al., 2013; Wang et al., 2007).

Techniques of immobilization varies from adsorption on surfaces, covalent bonding to carriers, entrapment in polymer gel and self-aggregation. Calcium alginate, glass beads, polyacrylamide gel, silanized magnetite, agarose, polyurethane foam and carrageenan are the most commonly used matrices for cell immobilization. The choice of immobilization technique and mechanical properties of the matrices are vital factors affecting the stability of biocatalysts. Table 2.2 shows the examples of cell immobilization and its support matrix.

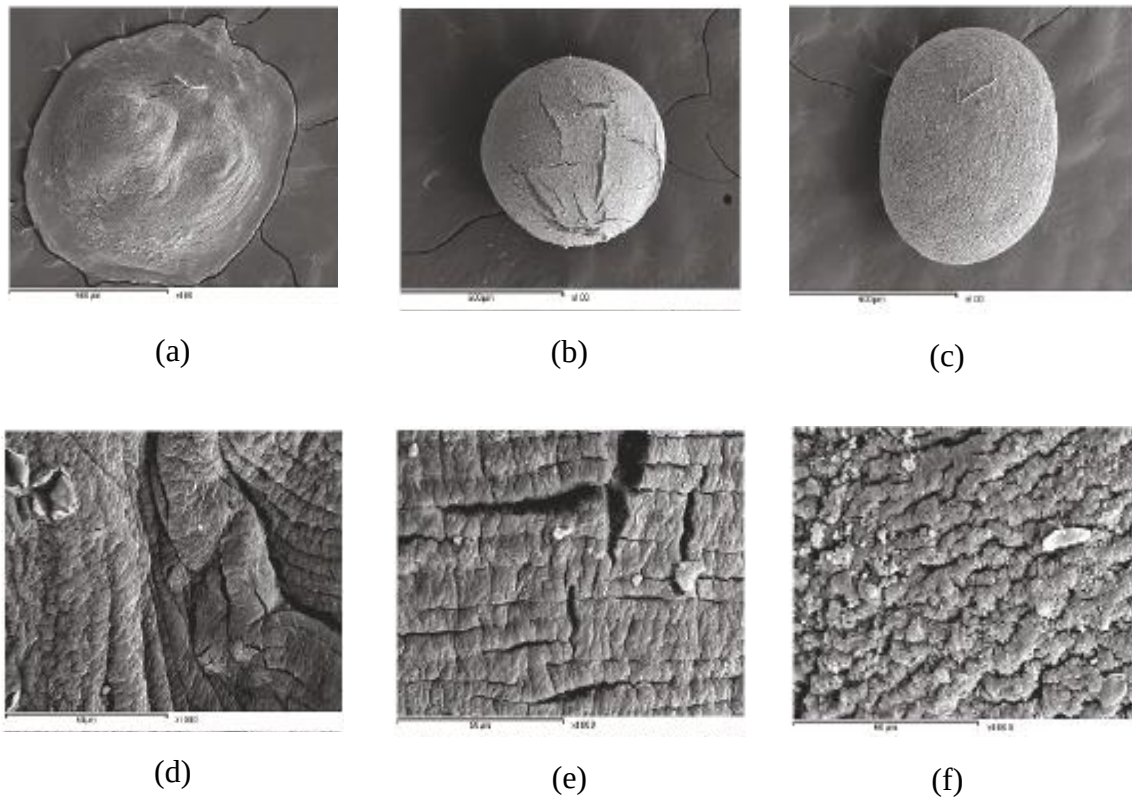
Table 2.2: Examples of Cell Immobilization Using Different Support Materials and its Conversion

Cells	Support matrix	Conversion	Reference
S. cerevisae	k-Carrageenan	Glucose to ethanol	Shuler & Kargi (2002)
Candida tropicalis	Ca-alginate	Phenol degradation	
E. coli	Polyurethane	Penicillin G to G-APA	
Lactobacillus sp.	Gelatin	Glucose to lactic acid	
Streptomyces	Sephadex	Streptomycin	
Solanum aviculare	Polyphenylene oxide-glutaraldehyde	Steroid glycoalkaloids formation	
Acinobacter sp.	Gellan gum	Phenol degradation	Ahmad et al. (2012)

Polymer beads of the matrix should be porous enough for the transport of substrate and products in and out of the beads and it is usually formed in the presence of cells. There are a few ways to prepared polymer beads for immobilization, one of it is polymer gelation. Cell suspensions are mixed with these polymers in high heat and beads are prepared using a template. The decrease in temperature in the template causes solidification of the polymers with the cells entrapped. However, due to diffusional limitations, the inner core of such beads is often not active, so this approach does not necessarily decrease the amount of product made per bead (Shuler and Kargi, 2002). Another method is precipitation of polymers, where cells are dispersed in a polymer solution and by changing the pH or the solvent, the polymer can be precipitated. However, direct contact of cells with this polymer may cause inactivation or even death of cells (Shuler and Kargi, 2002). Example of polymers used in this method are polystyrene, cellulose triacetate and collagen.

In this study, the optimum conditions of immobilization media will be investigated in terms of the type of matrix used, the concentration of the matrix and the cell mass loading into the immobilization media. The different concentration of matrix concentration were studied as there are contradictory reports between journal on the optimum concentration of the matrix for efficient biodegradation of heterocyclic hydrocarbon. (Sathesh Prabu & Thatheyus, 2007) reported of using 3.5% (w/v) of calcium alginate for removal of acrylamide, while Yañez-Ocampo, Sanchez-Salinas, Jimenez-Tobon, Penninckx, & Ortiz-Hernández (2009) uses 4% (w/v) and Jayashree, Nithya, Rajesh, & Krishnaraju (2012) uses 3% (w/v) of calcium alginate concentration for their cell immobilization. In other aspect, selection of optimum concentration of calcium alginate beads plays a role in cell immobilization as it provides stability and protection for

the embedded cells. Figure 2.1 shows the morphology of calcium alginate beads and its surface area at different concentration (Elnashar, Yassin, Moneim, & Abdel Bary, 2010). Hence, this study was made to determine the ideal concentration of calcium alginate for a more robust degradation of compounds.



(a) (b) (c)

(d) (e) (f)

Figure 2.1: SEM image of calcium alginate beads surface at (a) 1.0% (w/v), (b) 2.0% (w/v) concentration and **(c)** 3.0% (w/v) concentration and the surface morphology at (d) 1.0% (w/v) concentration, (e) 2.0% (w/v) concentration and (f) 3.0% (w/v) concentration**.** (M. Elnashar et al., 2010)

However, some drawbacks of immobilized systems explained by Shuler and Kargi (2002) is that the product of interest should be excreted by the cell and some products are not able to escape from the gel. Besides that, this system also leads to diffusional limitations. In such cases, the control of microenvironmental conditions is difficult, owing to the resulting heterogeneity in the systems. The presence of living cells, growth and gas

evolution may cause limitations to this system as it may lead to significant mechanical disruption of the immobilization matrix (Shuler and Kargi, 2002).

2.5 Mechanical Strength Studies of Immobilization Media

The strength of the polymeric beads is recognized by tensile test. The fracture properties of the immobilization matrix are mostly related to the viscoelasticity of the matrix that play crucial roles in immobilization. It is essential to determine the elongation of the matrix and calculated as:

$$\frac{(L-L_0)100}{L_0} \quad (1)$$

Where L_0 is the distance between the markers at initial hour and L is the distance at a given time. The crosshead speed used for the tensile test is 50 mm/min and can be adapted for tensile test. From that, the stress-strain can be defined that can be further used to find tensile modulus (E) (Nakamura, Shinoda, & Tokita, 2001). Strain formula is:

$$\in = \frac{\boldsymbol{L_1-L_0}}{\boldsymbol{L_0}} = \frac{\boldsymbol{\Delta L}}{\boldsymbol{L_0}}$$ (Dandel, Lehmkuhl, Knosalla, Suramelashvili, & Hetzer, 2009) (2)

Engineering stress, σ is defined by the relationship between force applied perpendicularly inversely proportional to the initial area of the matter.

$$\sigma = \frac{F}{A_0} \quad (3)$$

The stress and strain are proportional to each other when being stressed in tension through Hooke's Law where the Young's Modulus is constant (Callister & Rethwisch, 2007).

$$\sigma = E \times \in \quad (4)$$

From the stress-strain graph, the yield strength σ_y can be determined where yielding phenomenon occurs when a structure of component that has experienced a permanent change in shape. The illustration of yield strength σ_y is as shown in Figure 2.2;

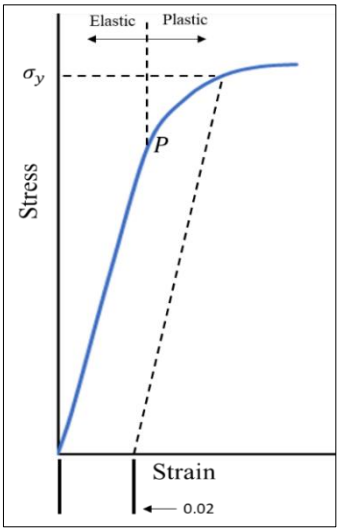


Figure 2.2: The Yield Stress, σ_y of component

After yielding, the stress needed to continue the plastic deformation rises to maximum and eventually fractures (Callister & Rethwisch, 2007). On the engineering curve, the tensile strength, *TS* is the stress at the maximum and this stress corresponds to the maximum stress that can be sustained by the structure. All along to the maximum stress, a phenomenon occurs called "necking" is where small constriction begins to form at some point (Callister & Rethwisch, 2007).

2.6 Diffusivity Studies of Immobilization Media

Cells viability, metabolically active cells retained within a support are the basis of a wide variety of natural and man-made biological systems (Riley, Muzzio, Buettner, & Reyes, 1995). Immobilized cell system behavior is governed by the relationship between nutrient and product diffusion, cell metabolism and cell proliferation. Diffusion is an important part of this system as cells embedded inside do not receive any nutrients by

convective mechanism. Besides that, cells proliferation inside the system increases the total nutrient consumption as well as the diffusional limitations and leads to undesired concentration gradient in the nutrient levels and confine the cellular metabolic activity to the vicinity of the interface between growth media and immobilization media (Riley et al., 1995). The effective diffusivity of a metabolite in immobilized cell is reliant on its diffusivity in both support material and the cells, where the diffusivity of a solute in a gelatinous support can be readily measured using cell-free system (Riley et al., 1995).

According to Hannoun & Stephanopoulos (1986), no film mass transfer resistance is assumed between the bulk fluids in the two chambers and the membrane where the transient diffusion inside the membrane is governed by partial differential equation;

$$\frac{\partial_c}{\partial_t} = D\,\frac{\partial_c^2}{\partial x^2} \tag{5}$$

Where c is concentration in the membrane, D is diffusion coefficient, t is time, and x is distance, subject to the boundary conditions

$$c = c_1 \quad \text{at } x = 0$$
$$c = c_2 \quad \text{at } x = 1$$

Hannoun & Stephanopoulos (1985) also mentioned that the solute diffuses from the chamber where c ≈ c1 through the membrane into the next chamber where c ≈ c2. Thus, the concentration in the two cells are not constant as it should consider the differential equation.

Nevertheless, the diffusion model is changing to approach that describe diffusion data. All the diffusion approaches are a wide-ranging summary based on a solution of Fick's first law. Those models are referred in Table 2.3.

Table 2.3: The Equation Model of Diffusion (Velickova, Kuzmanova, & Winkelhausen, 2011)

Model	Equation	Reference
Steady-state model	$Q = \frac{D_e A C_{LAO}}{l}\ t$	Klein & Schara (1981)
Hydrodynamic scaling model	$\frac{D}{D_0} = e^{-\alpha \emptyset p}$	Philies (1989)
Lustig and Pepas theory	$\frac{D}{D_0} = k\left(1 - \frac{R_A}{R_p}\right) e^{\left(\frac{-\emptyset p}{1-\emptyset p}\right)}$	Lustig & Peppas (1988)
Hydrodynamic model	$\frac{D}{D_0} = e^{-KR_A}$	Cukier (1984)
Fitting Model	$C_s(t) = \frac{\alpha C_{s0}}{1+\alpha}\left(1 + \sum_{n=1} \frac{6(1+\alpha) ex\left(-\frac{D_e q_n^2}{a^2} t\right)}{9 + 9\alpha + q_n^2 \alpha^2}\right)$	Crank (1988)

Calculation of the diffusion is easier when the diffusion time is large by using fitting model (Pu & Yang, 1988). When the concentration of solute is suspended in a well-stirred solution, the external mass transfer resistance surrounding the beads is negligible (Pu & Yang, 1988). Therefore, the transient concentration change of the solute Cs(*t*) is given by Crank (1988);

$$C_s(t) = \frac{\alpha C_{s0}}{1+\alpha}\left(1 + \sum_{n=1} \frac{6(1+\alpha) ex\left(-\frac{D_e q_n^2}{a^2} t\right)}{9+9\alpha+q_n^2 \alpha^2}\right) \quad (6)$$

Where *t* is the diffusion time; a is the diameter of the beads; α is the ratio of the volume of the solution to the volume of the beads; *De* is the effective diffusivity; n is the number of the beads and q_n the positive nonzero root. However, there are two types of fitting model

which are non-linear fitting model and linear fitting model. According to Pu & Yang in 1988, the most suitable fitting model to be used when the diffusion time is considered large is linear fitting model. With the condition where the terms corresponding to n≥ 2 in equation (6) are negligible. Hence, the equation can be converted into the linear form;

$$\ln\left(\frac{C_s(1+\alpha)}{C_{s0}\alpha}-1\right)=\ln\left(\frac{6(1+\alpha)}{9+9\alpha+q_1^2\alpha^2}\right)-\left(\frac{D_e q_1^2}{a^2}\right)t \quad (7)$$

$$\ln\left(1-\frac{C_s(1+\alpha)}{C_{s0}\alpha}\right)=\ln\left(\frac{6(1+\alpha)}{9+9\alpha+q_1^2\alpha^2}\right)-\left(\frac{D_e q_1^2}{a^2}\right)t \quad (8)$$

Equation (7) is used to determine the effective diffusivity into the substrate as equation (8) is mostly used for the study of substrate diffuses out of the media. Once the α is determined, the intercept value can be determined by plotting of the left side of the equation vs t that should yield line with a slope equal to $\left(\frac{D_e q_1^2}{a^2}\right)t$ (9)

2.7 Bioreactor for large scale studies of bioremediation

Bioreactor-based treatment has an advantage over other methods due to its controllable environment for the degradation of hydrocarbon polluted area as well as eliminating most of the rare-limiting or variable factors such as pH level, oxygen supply, nutrients type and concentration and temperature (Chikere, Chikere, & Okpokwasili, 2012). Its efficiency also based on the bacteria ability to attach to inert packing, for instance, granular activated carbon in order to generate higher biomass. Using bioreactor-based for petroleum sludge or slurry treatment also enables the volatile organic compounds (VOCs) to be managed by creating a condition that can accelerate the bioremediation of these VOCs rather than reduction via volatilization as obtained on other open treatment (Chikere et al., 2012). Some basic types of bioreactor include reactors with

internal mechanical agitation, bubble columns, which rely on gas sparging for agitation and loop reactors, where mixing and liquid circulation are induced by the motion of an injected gas, mechanical pump or combination of these two (Shuler & Korgi, 2002). The most traditional fermenter that is still used up to this date is stirred tank bioreactor, which is the prime example of a reactor with internal mechanical agitation. Main advantage of this system that it is highly flexible and able to provide high k_La (volumetric mass-transfer coefficient) values for gas transfer. Gas under pressure is supplied to the sparger and gas dispersion is mainly the function of impeller instead of the sparger. This is because the impeller must provide sufficient rapid agitation to disperse bubbles throughout the tank. Figure 2.3 shows the types of commonly used bioreactors. For *ad situ* and *ex situ* bioremediation, slurry bioreactors are crucial. Treatment of soils and sediments polluted by recalcitrant pollutant in slurry bioreactors are known as the best options for controlled environmental conditions and under slurry conditions, the pollutant depletion rates mainly depends on the activity of microbial degradation and the results obtained normally reflects the definite biological depuration potential of the soil (Robles-González et al., 2008).

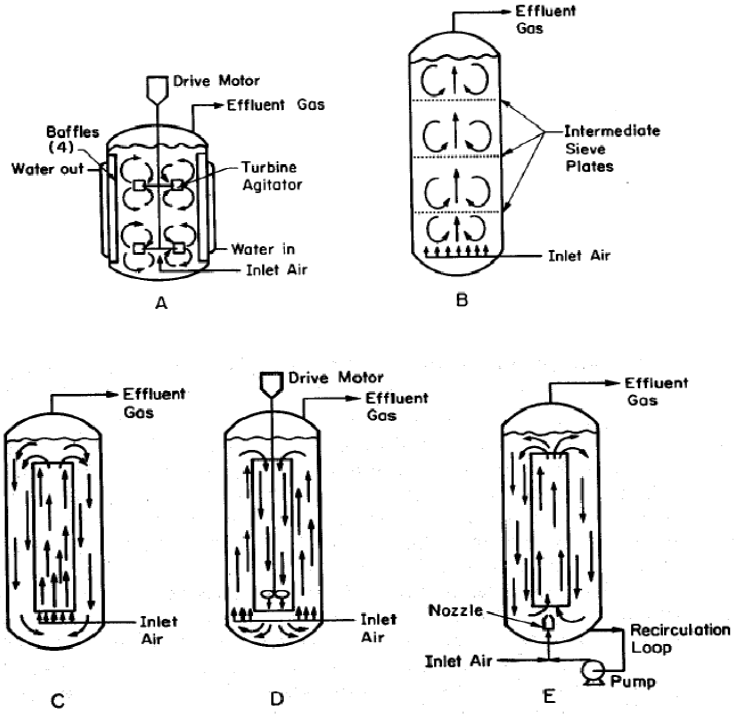


Figure 2.3: Bioreactor types; (A) Stirred tank bioreactors, (B) bubble-column bioreactor, (C) airlift bioreactor with central draft tube, (D) propeller loop reactor and (E) jet loop reactor. The arrows in the figure indicates fluid circulation direction (*Bioprocess Engineering: Basic Concepts*, Shuler & Korgi, 2002)

Problems emerged when an experiment was set up in a large scale due to the difficulty of maintaining homogeneity in large systems, changes in volume ratios and changes in the cultures itself due to the increasing culture time. Another problem emerged in commercial fermentation is foaming. If foam escapes from the fermenter, it may wet filters and lead to the increase of pressure drop and decreasing the gas flow (Shuler & Korgi, 2002). Another concern is that it may provide contaminating cells or agents to enter the fermenter. However, foams can be controlled with a mechanical foam breaker or with the addition of surface-active chemical agents. These scales up problems are all related to transport processes. In particular, the relative time scales for mixing and

reaction are important in determining the degree of heterogeneity of a fermenter (Shuler & Korgi, 2002). Scaling up draws away the microkinetic control of the system response at small scale to one where transport limitations control the systems at large scale (Shuler & Korgi, 2002). When the change takes place, the results of a small-scale experiment becomes unreliable with respect to predicting large-scale performance.

Overcoming the limitations in scale up projects is using characteristic time constants for conversion and transport processes. Shuler & Korgi (2002) explained in their book that processes with time constants that are small compared to the main processes appear to be essentially at equilibrium. In addition, traditional scale-up is highly empirical and makes sense only if there is no change in the controlling regime during scale-up, particularly if the system is only reaction or only transport controlled. In order to maximize the efficiency of the results, the common scale up rules to follow are the maintenance of constant power-to-volume ratios, constant k_La, constant tip speed, a combination of mixing time and Reynolds number and the maintenance of a constant substrate or product level (usually dissolved-oxygen concentration).

CHAPTER 3

INVESTIGATION OF OPTIMUM CONDITON FOR CELL IMMOBILIZATION

3.1 Introduction

Optimum condition for cell immobilization was investigated to maximize the degradation of heterocyclic hydrocarbon for efficient biodegradation. In this study, the conditions that were investigated were types of immobilization matrix used, concentrations of immobilization matrix and cell mass loadings of the bacteria.

Different types of immobilization matrix will exhibit different effects on the degradation of heterocyclic hydrocarbons. This is because the consideration of immobilization matrix with its compatibility with the substrate that it will interact with. There are possibilities that substrates will contribute to the degradation of matrix. Besides that, the cross-linking between polymer and its gelation agent will also be affected by the formation of the beads. Formation of thick or soft membrane affects the diffusion of the substrates into the beads to the cell embedded inside. These factors require consideration as they all will affect the cells performance and the substrate diffusion which will eventually influence biodegradation rate of the substrate.

Immobilization matrix concentration will affect in terms of the membrane size as well as the pore size of the beads. However, no particular studies investigate on what is the best concentration of the matrix for optimum degradation. Some reports indicated that 0.5%-1.0% (w/v) concentration of gellan gum for the immobilization (Moslemy, Neufeld, & Guiot, 2002; Wang et al., 2007). For immobilization in calcium alginate, the concentration used varies from 3% to 5% (w/v) (Ahmad et al., 2012; Usha, Sanjay, Gaddad, & Shivannavar, 2010).

Cell mass loading will factor the most in the degradation of heterocyclic hydrocarbon as the bacteria is the key role for biodegradation. However, there are no specific study on how much cell is optimum for cell immobilization as different journals used different concentration of cell for their immobilization. For example, Usha et al., (2010) used 5% (w/v) of cells concentration for calcium alginate immobilization while Moslemy, Neufeld, & Guiot (2002) and Shi, Qu, Zhou, Ma, & Ma, (2015b) used 3% (w/v) of the cell concentration and Ahmad et al., (2012)used 3.5g/L of cell concentration for immobilization in gellan gum. Therefore, there are no exact concentration of cells that can be used as a base line for cell immobilization, making it difficult to scale up the production of immobilized cell as a start for large scale biodegradation. This study aims to improve the performance of degradation by increasing the cell mass loading of *T. profundimaris*.

3.2 Materials and Method

3.2.1 Microorganism and Cultivation

Pure strain of previously isolated *T. profundimaris* strain M02 were maintained in double layer agar of ONR7a and 0.1% of CAR. *T. profundimaris* strain M02 were cultivated in 10 mL ONR7a medium enriched with 0.1% CAR. Prior to immobilization, cultivation of *T. profundimaris* strain M02 were done in 100 mL ONR7a media with marine broth at 9:1 ratio. After 24 hours of cultivation, the broth was centrifuged at 7000 rpm for 10 minutes to obtain cell pellets.

3.2.2 Preparation of gellan gum and calcium alginate beads for cell immobilization

Cell immobilization in gellan gum was carried out following the method described by Moslemy et al. (2002) and the process is shown in Figure 3.1. To prepare the gellan gum, 0.75% (w/v) was added to 100 mL of distilled water and heated to 75°C to dissolve

the gum completely. Then, 0.1% (w/v) of $CaCl_2$ was added to the mixture and left cool to approximately 50°C. The pH of the solution was adjusted to pH 7.0 and left to slowly cool down again until 45°C before adding the cell pellet. Harvested cell pellets were dispersed into the gum mixture and continuously stirred. Beads were formed using syringe and dropping the gum mixture into oil. The beads were separated from the oil by transferring them into 500 mL of 0.1% (w/v) $CaCl_2$. After 2 hours, the beads were repeatedly rinsed with 0.1% (w/v) Tween80 solution.

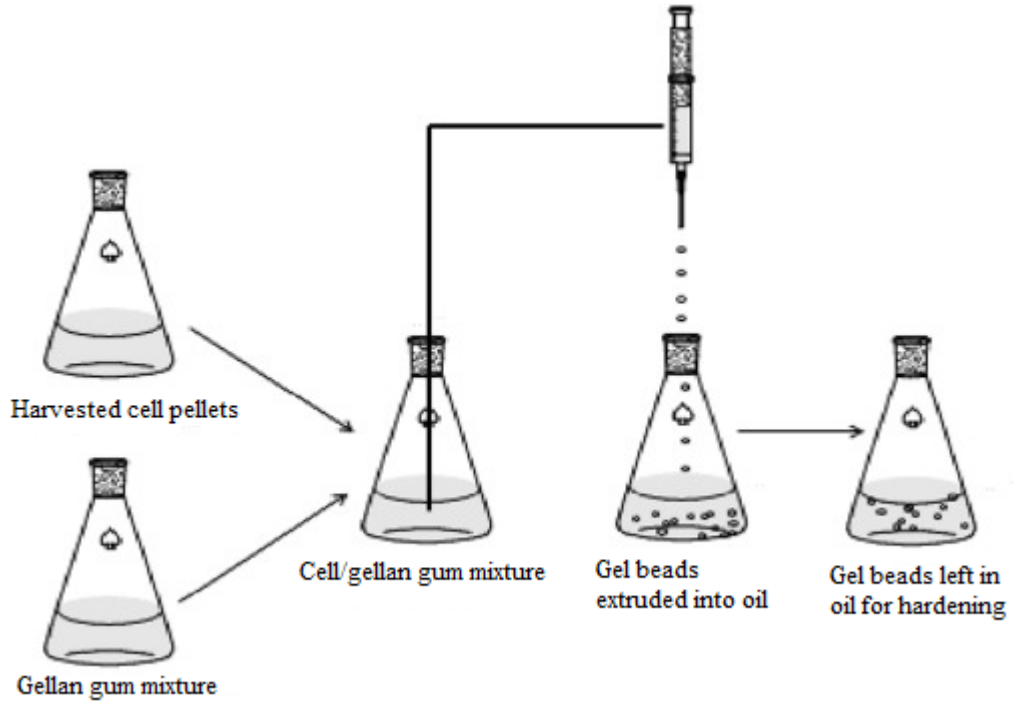


Figure 3.1: Schematic diagram of gellan gum beads production

Immobilization using calcium alginate was done according the method described by Usha et al. (2010) and the process is shown in Figure 3.2. Calcium alginate (4% w/v) was dissolved with 10 mL distilled and autoclaved at 121°C for 15 minutes. After the mixture cooled down, bacterial suspension was added into the mixture and stirred gently.

The alginate/suspension mixture were extruded into cold, sterile 0.2M $CaCl_2$ using a syringe. Beads formed were stored in fresh $CaCl_2$ for 2 hours to harden it. Lastly, the beads were washed with sterile distilled water before using it for experimentation.

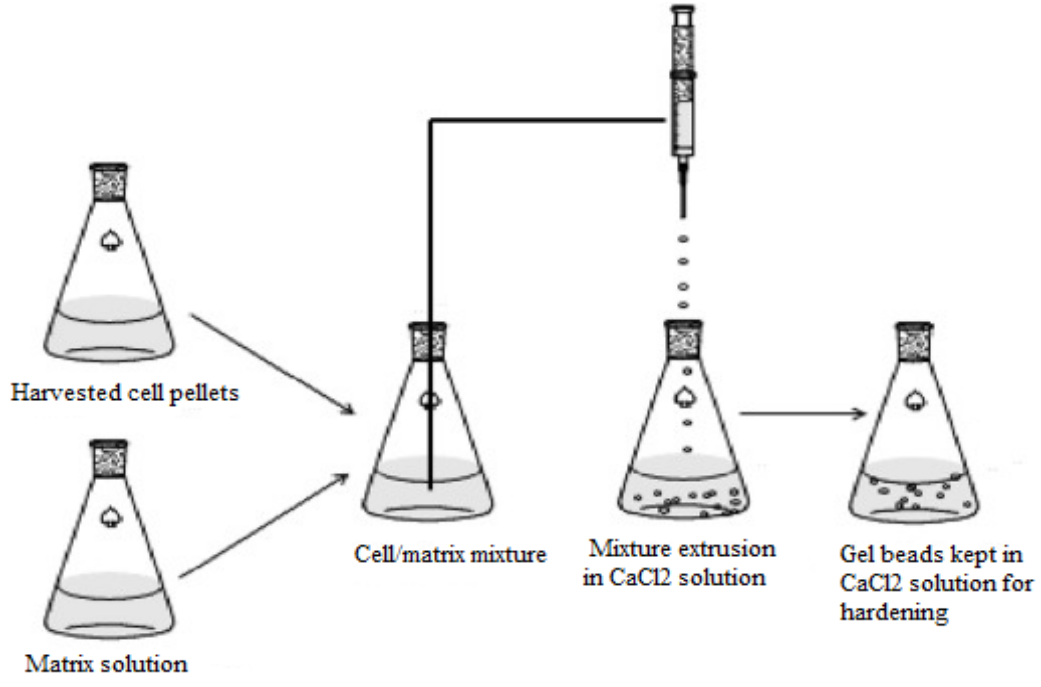


Figure 3.2: Schematic diagram for calcium alginate beads production

3.2.3 Diffusivity studies on different immobilization matrix for cell immobilization

The degradation data obtained from the experiment was used to study the diffusion study. The data analysis of diffusion was mainly conducted by assuming the 6 hours interval of the experiment where the substrates was beginning to obtain its equilibrium concentration. From Section 2.6, the model used was linear fitting model where it involved in diffusion in a large diffusion time, t. The formula used was as follows;

$$\ln\left(\frac{C_s(1+\alpha)}{C_{s0}\alpha}-1\right)=\ln\left(\frac{6(1+\alpha)}{9+9\alpha+q_1^2\alpha^2}\right)-\left(\frac{D_e q_1^2}{\alpha^2}\right)t \quad \text{(Pu \& Yang, 1988)} \qquad (10)$$

Where *t* is the diffusion time; a is the diameter of the beads; α is the ratio of the volume of the solution to the volume of the beads; *De* is the effective diffusivity; n is the number of the beads and q_n is the positive nonzero root; C_s is concentration of solute; C_{s0} is initial substrate concentration. The effective diffusivity, *De* was calculated on the ratio of volume to beads, α (Pu & Yang, 1988).

3.2.4 Preparation of calcium alginate beads at 3%, 4% and 5% (w/v) concentration cell immobilization

Preparation of calcium alginate beads was done according the method described by (Usha et al., 2010). However, in this experiment, 0.3g, 0.4g and 0.5g of sodium alginate was mixed with 10mL of sterile distilled water that made up 3%, 4% and 5% (w/v) concentration of calcium alginate beads. After the sodium alginate mixture was dissolved, 1.25g of harvested *T. profundimaris* strain M02 bacterial cell was added into the mixture. The mixture was gently stirred until both cell suspension and sodium alginate mixture were mixed well. The solution was then extruded into 0.2M $CaCl_2$ using 10 mL syringe. The beads were stored in $CaCl_2$ solution for 30 minutes to 2 hours for the beads to harden. Lastly, the beads were washed with sterile distilled water before using it for experimentation.

3.2.5 Mechanical strength studies on different immobilization matrix concentration

Mechanical strength of samples was tested for its tensile strength. The method in preparing the gel was different to the preparation of cell immobilization. Three different concentrations ranging from 3.0% to 5.0% (w/v) for Ca-alginate was used as in Section 3.2.4 The mixture of gel was poured onto the mold and the dimension of the mold was shown in Figure 3.3 as 4mm x 100mm (W x H). The molded calcium alginate gel was

hardened by curing it in calcium chloride for 2 hours. The samples were then stored in distilled water at room temperature for 2 hours.

The stress-strain measurement was obtained by using Tensile/Universal testing machine (Shimadzu) at 10 mm/min with 15 kN range and gauge length at 30 mm. The Young's modulus (*E*), tensile strength (TS) was then calculated from the stress-strain graph created after the data collected. The test setting was shown in Figures 3.4(a) and 3.4(b) showed the gauge break of the gel.

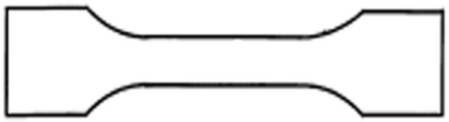
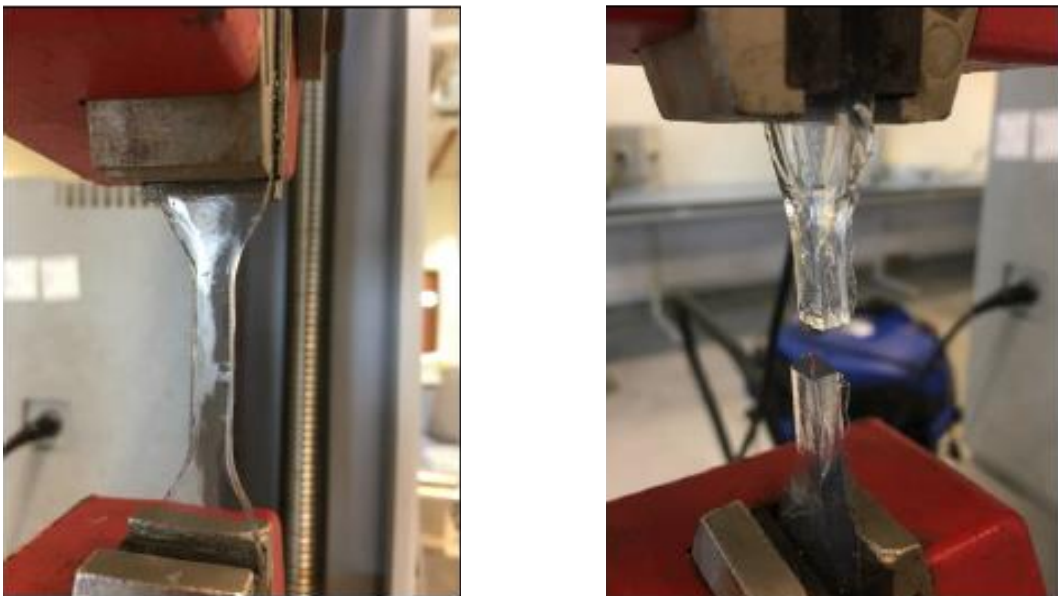


Figure 3.3: The dimension of mold sample set at 4mm x 100mm



(a) **(b)**

Figure 3.4: (a) Placement of gel matrix onto the tensile machine and **(b) C**ondition of the gel matrix at break point

3.2.6 Preparation of calcium alginate beads at 0.6g, 1.25g, 2.5g and 5.0g cell mass loading for cell immobilization

Preparation of calcium alginate beads was done according the method described by (Usha et al., 2010). In this experiment, the concentration of calcium alginate was determined from experiment in Section 3.2.4. After the sodium alginate mixture was dissolved, 0.6g, 1.25g, 2.5g and 5.0g of harvested *T. profundimaris* strain M02 bacterial cell was added into the mixture. Control beads were made with beads with no bacterial cell embedded. The solution was then extruded into 0.2M $CaCl_2$ using 10 mL syringe. The beads were stored in $CaCl_2$ solution for 30 minutes to 2 hours for the beads to harden. Lastly, the beads were washed with sterile distilled water before using it for experiments.

3.2.7 Reusability experiment of immobilized *T. profundimaris* strain M02 in calcium alginate beads

For reusability experiments, the immobilized cells were collected after each biodegradation batch and washed with fresh ONR7a medium once in order to remove remaining substrate and free cells. The washed immobilized cells were then added to fresh ONR7a medium containing the same amount of substrate as the initial cycle. These cycles were repeated five times to investigate the reusability of the immobilized bacteria. The concentration of substrate was made constant at every cycle and the degradation of every batch was compared by measuring the substrate depletion using GC-FID.

3.2.8 Gas chromatography flame ionization detector (GC-FID) analysis

All biodegradation experiment was done in 250 mL Erlenmeyer flasks, with 100mL artificial seawater media ONR7a as the medium and 0.1% (w/v) CAR (CAR) was used as the sole carbon source for the bacteria. The medium was incubated for 36 hours at room temperature at 100 rpm. Sampling was done at 6 hours interval and in triplicate measures.

Quantitative analysis of CAR degradation was determined using gas chromatography with flame ionization detector (SHIMADZU GC 14B, Japan) as shown in Figure 3.5. 1.0 mL aliquot was sampled from each experiment. Sample of known concentrations with no bacterial culture are used as standards for this experiment and a calibration curve was plotted. The samples are extracted using 1.0 mL ethyl acetate and the inorganic layer of the sample, observed as the upper layer shown in Figure 3.6 were acquired for analysing in GC-FID. The liquid was transferred into a 1.0 mL vial tube for processing. Detection of the compound was done by using HP-5 fused silica capillary column (50mm x 0.32 mm x 0.25 μm) with temperature of 250ºC at the injector, 300ºC at the detector, with column heated to 200 - 250ºC at 5ºC per minute and split less column with helium as the carrier gas. The peak graph obtained from the detection was attached in Appendix C.

Figure 3.5: Gas Chromatography with flame ionization detector (FID) (SHIMADZU GC 14B, Japan)

Figure 3.6: Two-layer solution produced during extraction of substrate using ethyl acetate

3.3 Results and discussion

3.3.1 Preparation of gellan gum and calcium alginate beads for cell immobilization

Gellan gum and calcium alginate were prepared using dropwise technique into its respective hardening agent and the shape produced were spherical with an average diameter of 3.0 mm. Texture of the beads were both rigid and exhibited higher density than water. Gellan gum produced colourless beads and calcium alginate beads produced brownish beads that is similar with the material colours in their powder form. Figure 3.7 showed gellan gum beads at 0.75% (w/v) concentration while Figure 3.8 showed calcium alginate beads at 4% (w/v) concentration.

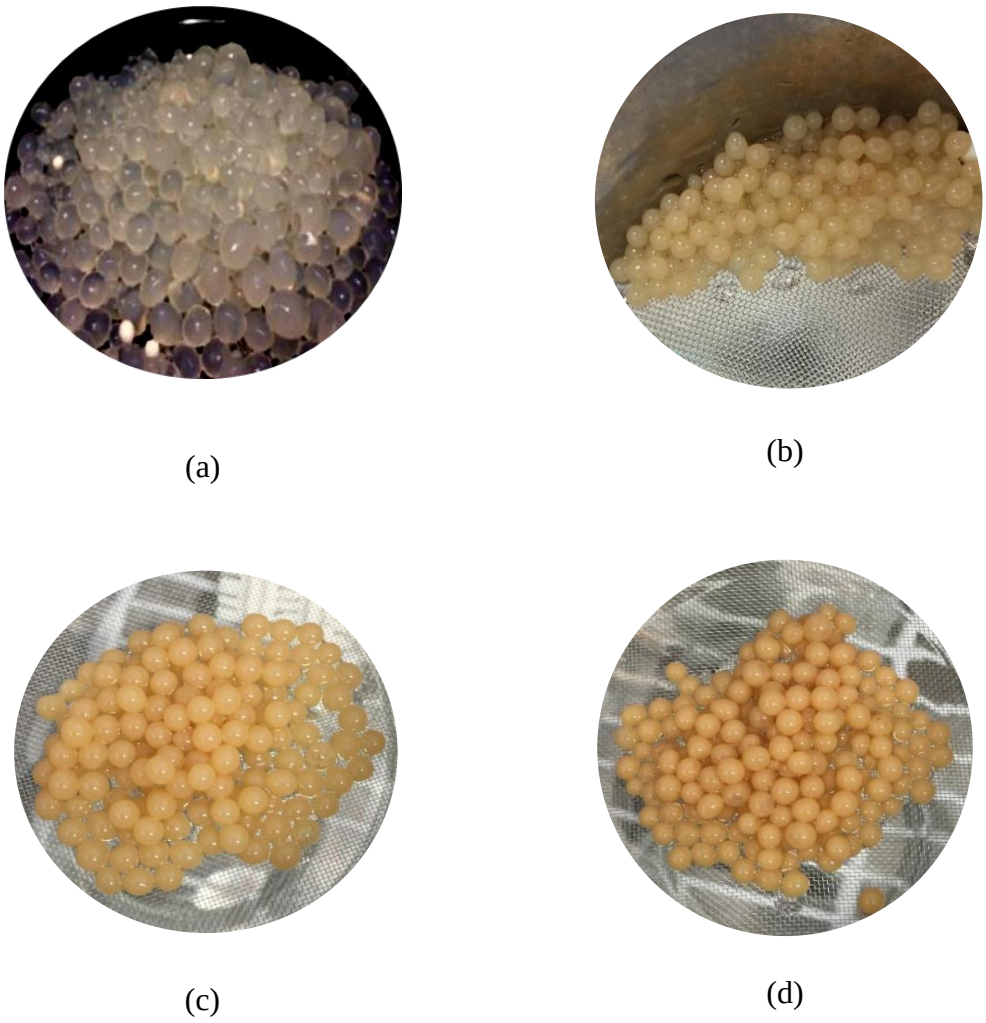
Figure 3.7: 0.75% (w/v) Gellan gum beads

Figure 3.8: 4% (w/v) Calcium Alginate beads

3.3.2 Preparation of calcium alginate beads at 0.6 g, 1.25 g, 2.5 g and 5.0 g cell mass loading for cell immobilization

Calcium alginate preparation was done with different cell mass loadings and from observations, each cell mass loadings exhibited slight differences in terms of the colour of beads. Beads of all cell mass loading had the same rigidity. From observation, colours of

the beads range from light to darker brown with the increase of cell mass loadings. Figure 3.7 shows the product of calcium alginate beads at different cell mass loadings.



(a) (b)

(c) (d)

Figure 3.9: 4% (w/v) Calcium Alginate beads with cell mass loadings of (a) 0.6 g, (b) 1.25 g, (c) 2.5 g and (d) 5.0 g.

3.3.3 Investigation of different matrix type for cell immobilization

This study investigates the optimum condition of immobilized cells condition to maximise the degradation of CAR in artificial seawater ONR7a media. Figure 3.10 showed the concentration of CAR degradation using different immobilization matrix and it was

seen that the concentration of CAR degraded by immobilized *T. profundimaris* strain M02 showed slight difference both calcium alginate and gellan gum. However, at the end of 36th hour, calcium alginate showed higher CAR degradation concentration at 776.98 mg/L while gellan gum showed 674.73 mg/L. Effective diffusivity was investigated between gellan gum and calcium alginate and the result are shown in Table 3.2. From Table 3.2, calcium alginate showed higher effective diffusivity value of 1.502cm^2/s compared to gellan gum which only showed 0.989 cm^2/s. These differences might be due to the different cross-linking activity between both matrices, causing a slight difference in membrane pore size thus affecting the diffusion of the substrate into the beads. Besides, mechanical study of the matrices was also conducted, and the result of the study was tabulated in Table 3.3. From the study, it was observed that calcium alginate had higher tensile strength compared to gellan gum. This would give advantages on the beads when was used in harsh conditions, where it could withhold the environmental stress force. In lab scale study, these properties helped the beads during the reusability experiment where the beads were repeatedly harvested and washed in every batch cycle. However, there were studies that suggested gellan gum was a better matrix as it was more chemically stable and calcium alginate was dissolved in the presence of calcium chelating agent or disintegrate when in contact with medium containing benzene (Moslemy et al., 2002). Though there were arguments on the stability of calcium alginate beads, a simple cell leakage experiment was done at the end of the cycle by spreading 20 uL of the media on to ONR7a with 0.1% substrate. The results showed no sign of cell growth on the media hence there are no cell was leaking out of the beads. This indicated that calcium alginate was a suitable matrix for this experiment. Hence, with consideration of the effectiveness of the substrate diffusion

into the beads as well as its mechanical strength, calcium alginate was chosen to further investigate the optimum condition for cell immobilization of *T. profundimaris* strain M02.

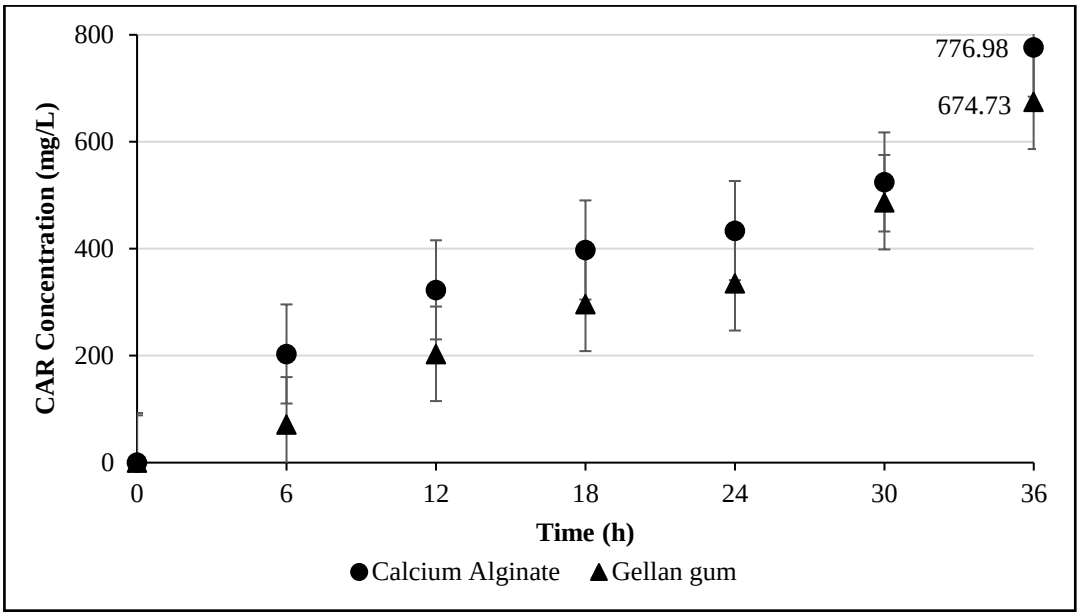


Figure 3.10: Graph of the average concentration of CAR degraded by immobilized *T. profundimaris* strain M02 in calcium alginate (●) and gellan gum (▲)

Table 3.1: Average concentration of CAR consumed by immobilized cell in calcium alginate and gellan gum at 6 hours interval for 36 hours

	Concentration of CAR (mg/L)	
Hour	**Calcium alginate**	**Gellan gum**
0	0.00	0.00
6	203.17	71.54
12	323.10	203.48
18	397.64	296.86
24	433.92	335.34
30	524.87	487.03
36	776.98	674.73

Table 3.2: The effective diffusivity of gellan gum (GG) and calcium alginate (Ca-Al)

Matrix Type	Diameter, D (cm)	q1	Intercept	Slope	α	Gradient, m	Effective diffusivity, De (cm2/s)
GG	0.3	3.47	0.73	-0.04	2.300	-0.036	0.989
Ca-Al	0.3	3.47	0.62	-0.054	2.300	-0.054	1.502

Table 3.3: The elongation, strain, force, engineering stress and Young's modulus of calcium alginate and gellan gum

Matrix Type	Average Elongation	Std Dev.	Strain, $\in$ (Dimensionless)	Average Force, F (N)	Standard Deviation	Engineering Stress, (N)	Young's Modulus, E (N/mm2)
Gellan gum	19.51	3.20	0.65	0.87	0.09	0.03	0.04
Ca-Al	42.38	6.40	1.31	3.88	0.64	0.121	0.09

3.3.4 Investigation of different matrix concentration for cell immobilization

CAR degradation by immobilized *T. profundimaris* M02 was investigated using different concentration of calcium alginate to study at which concentration the degradation was more efficient. Initial concentration of CAR in the media was kept constant in every experiment. Concentration ranging at 3% (w/v), 4% (w/v) and 5% (w/v) were used in this experiment and the degradation profiles were shown in Figure 3.11. From Figure 3.11, it was observed that at the 36th hour of incubation, calcium alginate at 3% showed the lowest CAR consumption form the beginning while 4% and 5% calcium alginate concentration starts off at the same rate but at the end, 4% (w/v) concentration showed the most CAR reduction. According to a study by Moslemy et al., (2002), they found out that at 3% calcium alginate concentration showed an optimum condition for the degradation of phenol. However, it conflicted in this study as the degradation of CAR was the lowest at 3% concentration. This might be due to the different characteristics of CAR and phenol. Phenol structure only has one benzene ring, whereas CAR contained 2 benzene rings , which caused it to have mutagenic and toxic properties (Salam et al., 2015) towards the cells. At lower calcium alginate concentration, the stability of the beads was reduced due to the reduced stability of the beads at lower alginate concentrations (Banerjee & Ghoshal, 2011) and the ruptured beads directly exposed the embedded cells inside to the toxic environment which in turn depleted the growth of the cells and causing the degradation rate to be lower (Chung, Loh, & Tay, 1998). On the contrary, at higher calcium alginate concentrations, the matrix become more rigid and in turn it affected the diffusion of CAR into the beads thus affecting the degradation of CAR (Banerjee & Ghoshal, 2011). Diffusivity of calcium alginate was investigated where it showed highest effective diffusivity at 4% (w/v) concentration. Table 3.5 shows effective diffusivity of calcium

alginate at different concentrations and it could be observed that at 4% (w/v) concentration, diffusivity of the substrate toward the beads were highest at 1.65 cm^2/s while at 3% (w/v) concentration showed the lowest at $0.65cm^2/s$. At low concentration, soft beads were easily damaged during the shaking process, causing the broken beads to reduce its diffusivity and bacteria leakage occurred thus reduced the CAR degradation. The decreasing diffusion rate was due to the degree of crosslinking, where the diffusion coefficient decreased as cross-linking density increased (Shoichet, Li, White, & Winn, 1996). On the other hand, higher concentration of alginate would cause the diffusivity to decreased as pore size of the beads are getting smaller and hinder the substrate to enter the beads (Sinha & Khare, 2012). Blandino, Macías, & Cantero (1999) mentioned that as the concentration of sodium alginate increased, the thickness of the membrane pores decreased at a given gelation time due to the increasing number of binding sites for Ca^{2+} (Blandino et al., 1999). These results in a more densely cross-linked gel structure thus forming smaller pore thickness and Table 3.6 showed the results of mechanical study on different concentrations of calcium alginate beads. Although the results of the studies showed that the matrix were stronger at 5% (w/v) concentration, it does not help in terms of the substrate diffusivity into the beads (Blandino et al., 1999). Hence, the best condition for calcium alginate concentration for the degradation of CAR was at 4% as it showed the highest amount of CAR reduction as well as best effective diffusivity and therefore, calcium alginate at 4% (w/v) was chosen to further investigate the best condition for cell immobilization.

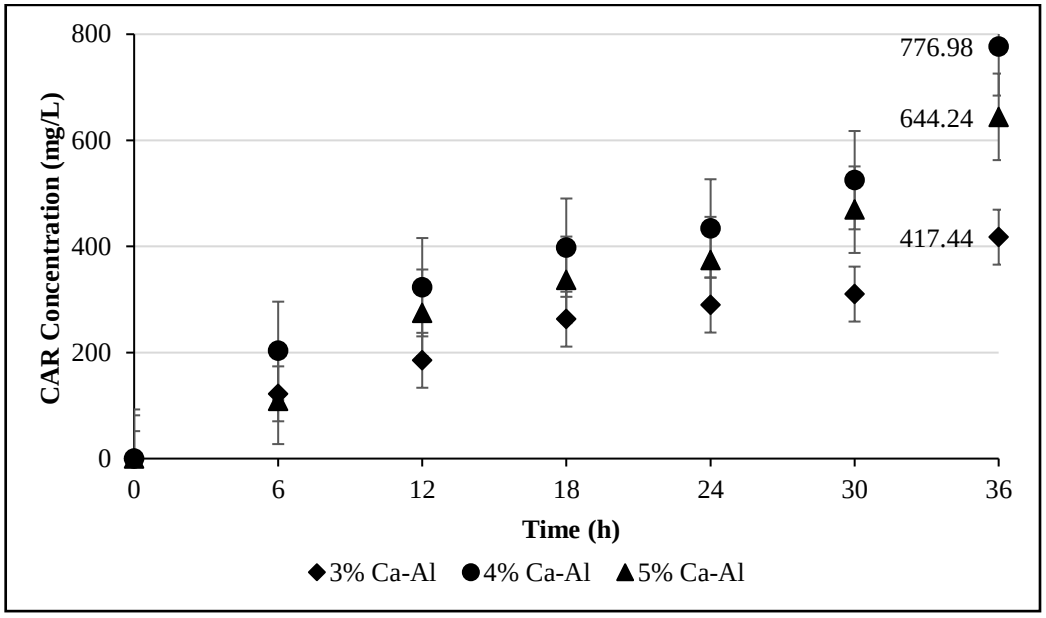


Figure 3.11: Degradation profiles of CAR degraded by immobilized *T. profundimaris* strain M02 in 3% (♦), 4% (●) and 5% (▲) (w/v) calcium alginate concentration

Table 3.4: Average concentration of CAR degraded by immobilized cell in calcium alginate at 3%. 4% and 5% (w/v) concentration

	Concentration of CAR (mg/L)		
Hour	**3% Ca-Al**	**4% Ca-Al**	**5% Ca-Al**
0	0.00	0.00	0.00
6	122.10	203.17	108.88
12	185.32	323.10	274.89
18	262.87	397.64	336.96
24	289.40	433.92	374.15
30	310.12	524.87	469.25
36	417.44	776.98	644.22

Table 3.5: The effective diffusivity of calcium alginate at 3%, 4% and 5% (w/v) concentration

Concentration (% w/v)	Diameter, D (cm)	q_1	Intercept	Slope	Effective Diffusivity (D_e) (cm^2/s)
3%	0.3	3.47	0.63	-0.03	0.65
4%	0.3	3.47	0.69	-0.06	1.65
5%	0.3	3.47	0.77	-0.06	1.62

Table 3.6: The elongation, strain, force, engineering stress and Young's modulus of various concentration of calcium alginate

Concentration	Average Elongation (mm)	Std Dev.	Strain, ∈	Average Force, F (N)	Std Dev.	Engineering Stress, (N)	Young's Modulus, E (N/mm2)
Ca-Al 3%	41.93	6.66	1.4	2.53	0.51	0.08	0.06
Ca-Al 4%	42.38	6.41	1.31	3.88	0.64	0.12	0.09
Ca-Al 5%	39.59	7.04	1.32	4.83	0.67	0.15	0.11

3.3.5 Investigation of different cell mass loading effect on cell immobilization

The effect of cell mass loading towards the degradation CAR was investigated by varying the cell mass loading inside the cell. Results of the experiment showed that as the cell mass increased, the degradation of CAR decreased. It could be seen in Figure 3.12 that at 1.25 g of cell, the amount of CAR degraded was significantly high which was at 776.98 mg/L indicating that 84.71% of CAR has been degraded at the end of 36^{th} hour. On the contrary, the highest cell mass used in this experiment was at 0.6g and it showed the lowest CAR degradation at 333.31 mg/L. As less cell was available in the system, the degradation of CAR was becoming less active as the number of cells could not accommodate the large

amount of substrate available in the system. Some cells in the bead may experience death due to the high level of CAR diffused into the beads thus increasing the toxicity inside the bead environment. This might explain the decrease in degradation of CAR after 18th hour mark. At 5.0 g of cell mass loading also showed less efficient degradation profiles. The higher mass of cell immobilized in the beads caused higher competition among the cells thus limiting the CAR degradation (Moslemy et al., 2002). High concentration of cells immobilized in the beads has also affected the diffusion of substrate into the beads. This could be seen when 5.0 g of cell took longer time to diffuse the substrate into the beads compared with the controlled beads. Although increasing cell mass loading was expected to enhance biodegradation, geometrical and biological constraints should be considered. As such, the limited capacity of the microbead (< 50 μm) to encapsulate more cells (Moslemy et al., 2002). Besides that, a highly dense microbead with cells may lose its efficiency as most cells encapsulated at the surface of the beads tend to get all the nutrients from the media, leaving most of the cells embedded at the centre of the beads to starve and eventually die. This would end up causing only half of the cells inside the beads are functioning thus lowering the biodegradation rate (Moslemy et al., 2002). The control experiment was done to investigate the behaviour of CAR in the media and it was observed that CAR was adsorbed into the beads during the incubation thus explaining the decrease of CAR concentration in the media even without the presence of cells. After the 18th hour of incubation, the concentration of CAR reduced was seen almost stationary indicating CAR has fully occupied most of the beads in the media. This also explains that at the almost stationary trend in the graph shown by 5.0 g cell mass loading, indicating the beads already on its full capacity hence the limiting the diffusion of CAR. It was concluded that at higher cell mass loading, the biodegradation was limited by the substrate concentration

while at lower cell mass loading, the degradation was rather limited by the cell mass loading itself. Therefore, best cell mass loading for cell immobilization was at 1.25 g.

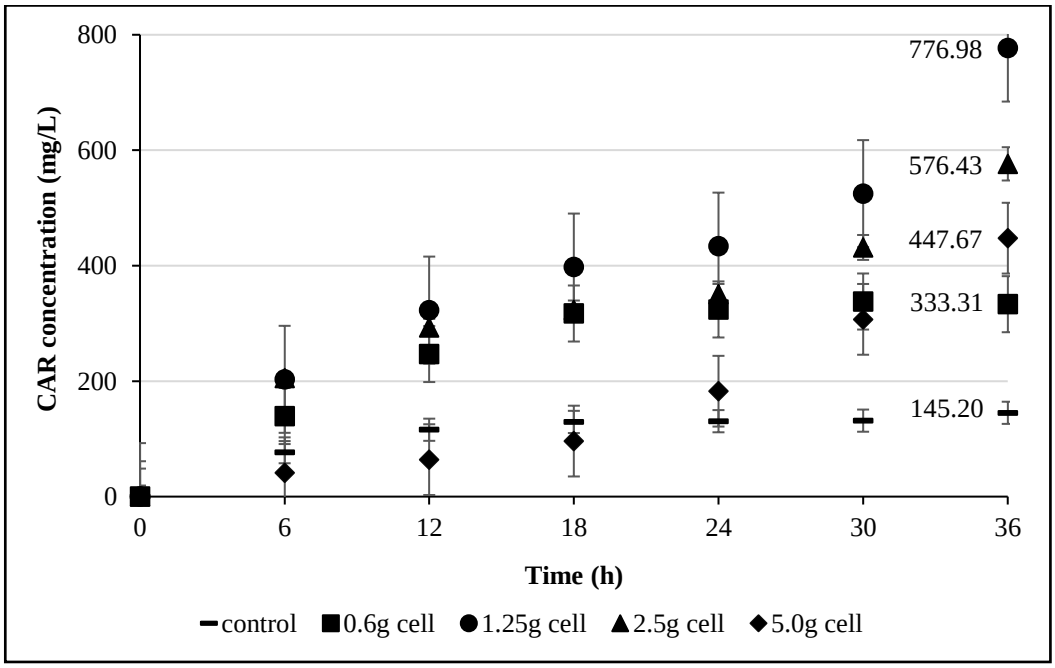


Figure 3.12: Graph of the average concentration of CAR degraded by immobilized *T. profundimaris* strain M02 at 0.6g (■), 1.25g (●), 2,5g (▲) and 5.0g (♦) of cell mass loading

Table 3.7: Average concentration of CAR degraded by immobilized cell in different cell mass loading of *T. profundimaris* strain M02

	Concentration of CAR (mg/L)				
Hour	**Control**	**0.6g**	**1.25g**	**2.5g**	**5.0g**
0	0.00	0.00	0.00	0.00	0.00
6	76.99	139.61	203.17	205.14	41.38
12	115.85	247.02	323.10	293.22	64.03
18	129.37	317.18	397.64	323.49	96.21
24	130.65	324.23	433.92	350.81	182.60
30	131.61	337.97	524.87	431.63	307.05
36	145.20	333.31	776.98	576.43	447.67

3.3.6 Reusability study on immobilized *T. profundimaris* strain M02 in calcium alginate beads

Reusability of immobilized cells were tested in repetitive batch mode. The results were illustrated in Figure 3.13 and significance decrease was observed between the first and second cycle at 42.34%, whereas at third cycles and onwards, the CAR concentration decreased was no more than 10% after each cycle. It was clearly observed that the cell has maximised its capability on consuming CAR at the first cycle after that the CAR depletion was seen almost stationary although some degradation was observed. The efficiency decreased of immobilized cells was due to the loss of cell viability embedded in the cells, as well as the progressive saturation of adsorption sites (Sinha & Khare, 2012). Cells leakage from the alginate beads or the deformation and weakening of the alginate matrix might also had been the reason of the efficiency decreased (Banerjee & Ghoshal, 2011). Cell leakage is a condition where the cells in the beads were not embedded strong enough with the matrix and leak into the medium. This will expose the cells to the toxic environment of the medium hence inhibiting its growth and performance. Aside from that,

some mishandling of the beads might occur causing some of the beads to be lost. This affects the degradation as this decreased the cell concentration in the media from the previous cycles. Besides that, from the investigation of cell mass loading experiment, it was concluded that the beads adsorbed CAR and the adsorbed CAR might not diffuse out of the beads. This causes the beads to be denser after the first cycle causing difficulty for CAR at the next cycle to diffused into the beads, hence causing the poor degradation. At the 2nd cycle, the efficiency of the cells has been depleted to more than half from the initial cycle. Hence, by repeating the experiment for up to 10 cycles might give the possible time track on how long these reused beads were viable before all the cell perished. Therefore, it can be concluded that immobilized cells are not suitable to be used any longer after the first cycle as it had lost most of its degradation capability.

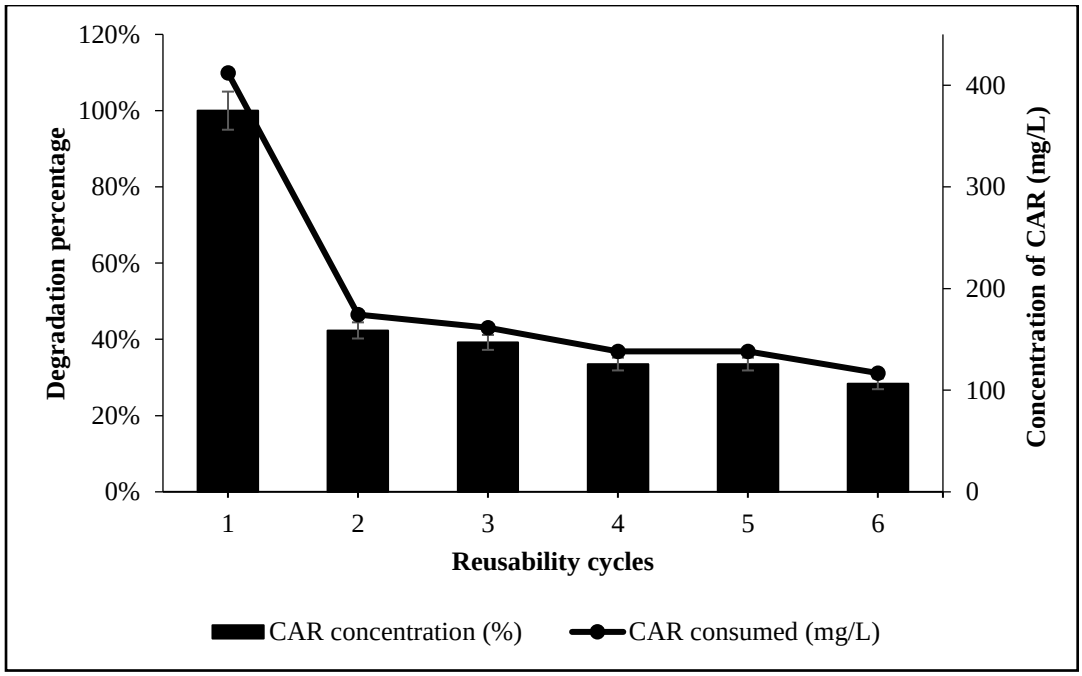


Figure 3.13: Line graph shows the average degradation of CAR using reused immobilized *T. profundimaris* M02 in calcium alginate beads while bar graph shows the percentage of the degradation through every cycle. (Note: At first cycle of the degradation, CAR degradation was expressed as 100% degradation)

Table 3.8: Concentration of CAR degraded by immobilized *T. profundimaris* M02 when it is reused up to 6th cycle and its expression in percentage to show its efficiency on each cycle. Efficiency depletion is the comparison of the cell's degradation from the first cycle of reusability experiment

Cycle	CAR consumed (mg/L)	CAR degradation (%)
1	412.19	100
2	174.54	42.34
3	161.61	39.21
4	138.21	33.53
5	138.19	33.53
6	116.84	28.35

3.4 Conclusion

In the investigation of immobilization matrix type, it was concluded that calcium alginate was a better matrix for cell immobilization. It showed higher concentration of CAR degraded at 777.68 mg/L as well as showing more promising diffusivity at 1.502 cm^2/s. Its mechanical strength was also better than gellan gum at 0.09 N/mm^2. With all these factors combined, calcium alginate was decided as a suitable matrix for immobilization of *T. profundimaris* M02 to degrade heterocyclic hydrocarbon compounds.

Once the immobilization matrix was chosen, the next factor that was investigated was the concentration of the immobilization matrix. In this study 3%, 4% and 5% (w/v) of calcium alginate concentration were used respectively. From the study, it was observed that the optimum condition was at 4% (w/v) concentration with the highest CAR concentration, followed by 3% and lastly 5% (w/v) concentration. 3% (w/v) calcium alginate concentration showed the lowest mechanical strength and diffusivity amongst all

the concentrations. Hence, calcium alginate with 4% (w/v) concentration was used to proceed with the next experiment.

Lastly, the optimum condition that was studied was the cell mass loading. In this experiment, 0 g, 0.6 g, 1.25 g, 2.5 g, and 5.0 g of cells were used to study the effect of cell mass loading on biodegradation. From the experiment, 1.25 g of cells showed the highest degradation of CAR at 777.68 mg/L while 0.6 g shows the lowest. This did not follow the previous hypothesis of higher cell mass would give a higher CAR degradation. Degradation of CAR using higher cell mass loading was limited by the cell mass itself as more cells occupied the beads thus limiting the diffusion of CAR into the beads.

The optimal immobilized cell was tested on its efficiency by reusing the beads for repetitive CAR degradation. From this test, the cells efficiency dropped significantly after the first cycle where at the end of the cycle, the CAR degradation was only at 42.34%. by the end of the 6^{th} cycle, the immobilized beads inefficiency reached 72% showing that the cells were no longer capable of degrading more substrates. Therefore, it was concluded that immobilized cells were not suitable for repetitive use, however these cells could be used in a long run with better efficiency.

CHAPTER 4

BIODEGRADATION OF CARBAZOLE (CAR), DIBENZOFURAN (DBF), DIBENZOTHIOPHENE (DBT) AND FLOURINE (FL) USING FREE AND IMMOBILIZED *Thalassospira profundimaris* STRAIN M02

4.1 Introduction

4.1.1 Heterocyclic Hydrocarbon Compounds

CAR is identified as a white crystalline solid with a distinctive odour and slightly soluble in water (Rannug & Rannug, 2018). It is formed as a product of incomplete combustion of nitrogen-containing organic matter and mainly occurs in petroleum, coal, peat, crude oil and coal tar. CAR release to the environment may result from its production and uses as an intermediate in manufacturing dyes, lubricants and rubber antioxidants and as UV sensitizer for photographic plate while its direct release to the environment are a result from emissions of water incineration and tobacco smoke (Peng et al., 2018). CAR's release to soil will show high to no motility and is not expected to volatilize from dry soil surface. Screening tests indicate that there are adapted organism that uses CAR as source for metabolic activity hence the knowledge of these CAR degrading bacteria is important for enhanced degradation of CAR in the environment (Rannug & Rannug, 2018)

DBT is an organosulfur compound found in crude oil and petroleum as well as also used as chemical intermediate in pharmaceuticals and cosmetics (Ji et al., 2017). The use of DBT as these constituents' results in their release to the environment through various waste streams. DBT is also a component of fossil fuels and accidental spills of petroleum may release large amount of DBT into the environment (Ji et al., 2017). The release of DBT to soil are expected to have to motility but its presence is relatively persistent in the environment. Natural biodegradation may occur after long lag periods or in soils that are

acclimated to petroleum products (Richter-Brockmann & Achten, 2018). DBT release into water are expected to adsorb to suspended solids and sediment. Therefore, it is important to further investigate how the degrade DBT from the environment as its acute exposure to human might cause irritation and photosensitivity to the eyes, respiratory problems, skin problems and even cancer.

DBF is also known as 1-methylpyrene as identified as a brown powder or plate-like solid. This compound is insoluble in water and usually occurs as a result of incomplete burning of fossil fuels, wood, diesel oils and gasoline oils (Oramas-Royo et al., 2017). Besides that, this compound was also found in tobacco smoke, charcoal smoke, gasoline and fossil fuels. Its release to the environment might result from the dye industries, textile industries and also petroleum industries waste stream to the environment (Ji et al., 2017). DBT is known to have slight motility when is released into soil and some indigenous soil microorganisms at contaminated sites could degrade DBT when stimulated. Biodegradation screening test indicated that DBT are not readily biodegradable, however, lab studies showed that DBF was degraded in a few days (Oramas-Royo et al., 2017). Hence, the study of DBF-degrading bacteria is important in the bioremediation of DBF as these bacteria unable to degrade DBF on itself in the environment.

Florene (FL) solids is identified as a small, white crystalline plates and is moderately soluble in water. FL did not exist in the environment naturally and were formed during the incomplete combustion of coal, oil, gas wood, garbage and tobacco (Eeshwarasinghe et al., 2018). FL production and its use in manufacturing industries results in its release to the environment through various waste streams. Exposure to FL may cause respiratory problems, gastrointestinal problem, photosensitivity and irritation to the skin and may lead to cancer. Isolation of bacteria that existed in FL contaminated area

could help in bioremediation of FL as it uses FL as carbon source for metabolic activities (Singh, Kawamura, Yanase, & Barrie, 2017). This could help to reduce the existing concentration of FL in the environment as well as can be used to treat FL containing waste produced by the industries.

4.1.2 Biodegradation of heterocyclic hydrocarbon compounds using free and immobilized bacteria

High concentration of xenobiotics usually limits the degradation of pollutants and toxic compounds by natural microbial activities as it inhibits the growth of the microorganism (Ahmad et al., 2012). Hence, cell immobilization is found to be an attractive strategy to produce a robust cell as a biocatalyst. Immobilized cells are also less likely to be affected by predators, toxins or parasites (Nawaz, Franklin, & Cerniglia, 1993). Chen et al., (2013) conducted a study on comparison between free cell bacteria and immobilized cell for the production of butanol and found that immobilized cells produced higher biomass and provide higher cell stability for long term butanol production. This shows that cell immobilization can overcome the limitation of slow growth rate and low butanol production from the free cells. In addition, immobilized cells are viable to be used in continuous process as they exhibit high recovery at lower cost, high reusability, as well as protecting the cells from environmental stress (Martins et al., 2013). These advantages encourage researches to investigate the applications of immobilized cells in the biodegradation of various toxic compounds (Wang et al., 2007; Martins et al., 2013). Techniques of immobilization varied from adsorption on surfaces, covalent bonding to carriers, entrapment in polymer gel and self-aggregation. Calcium alginate, glass beads, polyacrylamide gel, silanized magnetite, agarose, polyurethane foam and carrageenan are the most commonly used matrices for cell immobilization. The choice of immobilization

technique and mechanical properties of the matrices are vital factors affecting the stability of biocatalysts.

4.2 Materials and methods

4.2.1 Preparation of 0.1% (w/v) substrate

Each substrate was made at 10% (w/v) stock solution. For the stock solution, 10.0 mL of dimethylformamide (DMF) was used to dissolved 1.0 g of the substrate. For this experiment, 1.0 g of Carbazole (CAR), Dibenzofuran (DBF), Dibenzothiophene (DBT) and Fluorene (FL) was added in separated bijou bottles. Each substrate was added with 10 mL of DMF, hence 10% (w/v) stock was prepared. To reduce the concentration of substrate to 0.1% (w/v), the following calculation was carried out to find out the volume from the 10% (w/v) stock.

$$M_1V_1 = M_2V_2 \quad (11)$$

Where;

M_1 = Initial concentration of the stock

M_2= Final concentration needed

V_1 = Volume from the stock solution

V_2 = Final volume of the solution

4.2.2 Utilization test of *T. profundimaris* M02 on CAR, DBT, DBF and FL

Utilization of CAR, DBT, DBF and FL by *T. profundimaris* M02 was tested using a simple growth plate analysis. A double layer agar containing ONR7a media with 0.1% (w/v) was prepared and *T. profundimaris* M02 was grown on the plate using streaking method. Positive utilization of the substrate would show growth on the plate. This will indicate that this bacterium is capable to degrade the compounds.

4.2.3 Preparation of free suspended and immobilized *T. profundimaris* strain M02

Cultivation and harvesting of the cell pellet were done the same as the procedure in Section 3.2.1, where the cells were cultivated in ONR7a media with marine broth as the nutrient supply at 9:1 ratio. After 24 hours of cultivation, the cells were harvested using centrifuge machine where it was spun at 7000 rpm for 10 minutes to obtain the cell pellets. In this experiment, the cell mass used was at 1.25 g for 100 mL media where it was investigated to be the optimum cell mass loading for cell immobilization. For free suspended cells, the same cell mass loading was also used in order to investigate the difference of the cells performance when it was free and immobilized.

Preparation of free cell suspension was relatively simple. After the cell was spin down, the cells were washed three times using PBS buffer to remove the remaining nutrients from the cell pellets. Once the cell pellet was obtained, the tube was weighed, and the weight of the tube was deducted with the weight of the tube without the cell. This is done to obtain the mass of the cell pellet. The cell pellet was kept in the tube at -4ºC upon using.

The preparation of immobilized cells was done following the best condition of immobilization as in Section 3.2**.** Calcium alginate was used as the immobilization matrix at 4.0% (w/v) concentration and 1.25 g of cell mass loading was used. The immobilized cells were kept in sterile distilled water before the experiment.

4.2.4 Degradation of heterocyclic hydrocarbon compounds

The degradation of CAR, DBT, DBF and FL was done in 250 mL conical flask with 100 mL ONR7a media supplemented with 0.1% (w/v) substrate. Two flasks were prepared, one for free suspended cells and the other was for immobilized cell. The media was incubated on an orbital shaker for 36 hours at 150 rpm. Sampling was done every 6

hours and all experiment was done in triplicates. Degradation of the substrates was expressed as the rate of degradation and calculated using the formula (12).

$$Rate\ of\ Degradation = \frac{(C_0 - C_n)}{(t_n - t_0)} \quad (12)$$

Where, C_0 = Initial substrate concentration T_0 = Initial time

C_n = Substrate concentration at time, n T_n = Time at given hour

4.2.5 Gas Chromatography Flame Ionization (GC FID) analysis

The extraction of these compounds was also done following the procedure in Section 3.2.8. 1.0 mL of sample was added with equal volume of ethyl acetate and the solution was vortexed until a two-layer solution was observed. The hydrocarbons were dissolved in the upper layer of solution and the layer was taken and transferred into vial tubes before analysing using GC FID. Quantitative analysis of substrate degradation is determined using gas chromatography with flame ionization detector (SHIMADZU GC 14B, Japan) shown in Figure 3.5. Sample of known concentrations with no bacterial culture are used as standards for this experiment and a calibration curve was plotted. The graph of calibration curve for all substrates are attached in Appendix A. Detection of the compound was done using HP-5 fused silica capillary column (50mm x 0.32 mm x 0.25 μm) at 250ºC at the injector, 300ºC at the detector, with column heated to 200 - 250ºC at 5ºC per minute and split less column with helium as the carrier gas.

4.3 Results and discussion

4.3.1 Degradation of heterocyclic compounds by *T. profundimaris* M02

Degradation ability of *T. profundimaris* M02 on degrading other heterocyclic hydrocarbon compounds was investigated since *T. profundimaris* M02 was isolated from CAR degraded environment and was maintained by supplying only CAR as its sole carbon

source. Its ability of degrading other heterocyclic hydrocarbon compounds was unknown. Growth test on plate indicated that *T. profundimaris* M02 was able to utilize DBT, DBF and FL for their metabolic activity. Table 4.1 shows the result of growth and utilization test of *T. profundimaris* M02 on CAR, DBF, DBT and FL. Rapid growth was measured based on the appearance of bacteria on the plate after 2 days whereas utilization of the compounds was measured based on the appearance of clear zone or metabolites on the plate. From the observation, *T. profundimaris* M02 showed fast growth on all plates, however the production of metabolites/clear zone could only be seen on CAR and DBF plates. Degradation test was carried out to investigate the ability of this bacterium to degrade these compounds. Figure 4.1 shows the degradation of *T. profundimaris* M02 on CAR, FL, DBT and DBF. From Figure 4.1, it could be observed that this bacterium could degrade and utilize these compounds for their metabolic activities. The degradation profiles also show that at 24th hour incubation, this bacterium can degrade DBF better than CAR, indicating its ability to degrade compounds was not restricted to only CAR. This results also corresponds with the utilization test where it showed better utilization on CAR and DBF plate. To further improve this bacterium capabilities, immobilization technique was adapted.

Table 4.1: Growth of *T. profundimaris* M02 when supplemented with different substrates and its utilization of the respective substrates. (++) indicates that the cells can grow or utilize the cells rapidly while (+) indicates that only moderate growth or utilization are observed. (-) indicates no growth or utilization.

	CAR	**DBF**	**DBT**	**FL**	**Control**
Growth	++	++	++	++	-
Utilization	++	++	+	+	-

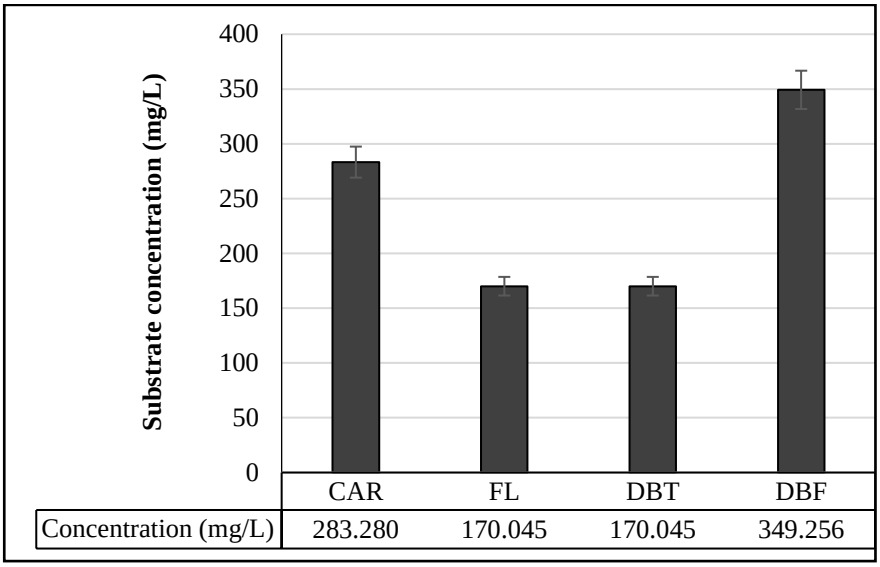


Figure 4.1: Average substrate degradation by *T. profundimaris* M02 strain M02 in ONR7a with 0.1% (w/v) substrate concentrations at 24-hour incubation

4.3.2 Degradation of CAR by free and immobilized *T. profundimaris* strain M02

In order to compare the performance of immobilized cells with the conventional method of biodegradation, an experiment of CAR degradation using free suspended cells and immobilized cells was done. The result of this experiment showed similar trend of concentration of CAR degraded, with immobilized cells showing higher concentration at every hour compared to free suspended cells. At the end of the cycle, immobilized cells showed 705.95 mg/L of CAR degradation, compared to 431.35 mg/L in free cells suspension. The results were tabulated in Table 4.1. From Figure 4.2, it could also be observed that free cells experienced a decrease in rate of degradation of CAR over time until the 18th hour but remain almost stationary after the 24th hour. Immobilized cells showed a spike in the degradation rate at the first 6th hour of incubation but experiencing lowered rate after that until it increased again after the 30th hour. This might due to the cell's growth or the diffusion of substrate in the cell. The cells death might become limiting factor of CAR degradation. However, after 30th hour, sufficient growth of cells at

the beads surface might causes the increase in CAR degradation hence increasing the degradation rate. On the other hand, the spike at the first 6th hour of the incubation may due to the adsorption of CAR into the alginate beads as stated by Moslemy et al (2002), where in their experiments, gellan gum beads showed the adsorption of gasoline hydrocarbons during the incubation (Moslemy et al., 2002).

Table 4.2: Average concentration of CAR degraded by free and immobilized *T. profundimaris* strain M02 in ONR7a with 0.1% (w/v) initial CAR concentration

	Concentration of CAR (mg/L)	
Hour	**Free Cell**	**Immobilized cell**
0	0	0
6	60.72	165.34
12	97.02	210.15
18	131.08	283.35
24	283.28	337.48
30	356.54	453.11
36	431.35	705.95

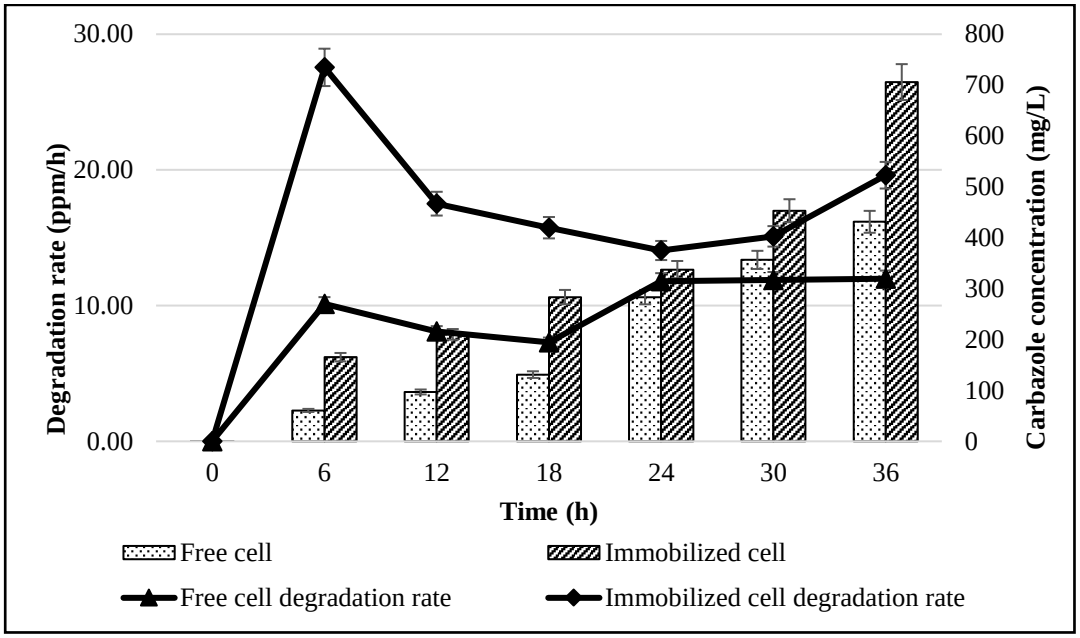


Figure 4.2: Bar graph shows the average concentration of CAR degraded by free and immobilized *T. profundimaris* strain M02 in ONR7a with 0.1% (w/v) initial CAR concentration and line graph shows the degradation rate of CAR by free and immobilized *T. Profundimaris* strain M02

4.3.3 Degradation of DBT by free and immobilized *T. profundimaris* strain M02

The experiment was repeated using different type of substrate, where in this experiment, DBT was used. The results of this experiment were illustrated in Figure 4.3. From the graph, slight difference of DBT degradation compared to with CAR degradation. the first 12th hour of the cycle showed almost identical value of DBT concentration degradation. However, after the 12th hour, more distinct difference of the degradation was observed and at the end of the cycle immobilized cells show the higher concentration of DBT at 472.51 mg/L and free cells at 341.78 mg/L. The degradation of DBT was shown in Table 4.3. On average, the increase in degradation performance of immobilized bacteria was at 6.91%. the degradation rate of DBT showed that at the first 6th hour of incubation, free cells outrun immobilized cells. This showed that *T. profundimaris* M02 degrade DBT faster than the substrate diffusion into the beads. However, the degradation rate of free

cells rapidly decreased after the 6th hour, indicating that most cells were already vulnerable to the toxicity of the media causing the cell concentration to be the limiting factor of the substrate degradation. On the contrary, immobilized cells showed an increasing rate of degradation after the 12th hour showing that the alginate beads provide protection towards the cells hence increasing the cells viability for a better and sustainable degradation of DBT.

Table 4.3: Average concentration of DBT degraded by free and immobilized *T. profundimaris* strain M02 in ONR7a with 0.1% (w/v) initial DBT concentration

	Concentration of DBT (mg/L)	
Hour	**Free Cell**	**Immobilized cell**
0	0.00	0.00
6	77.16	68.82
12	112.86	119.01
18	128.74	212.92
24	170.05	344.19
30	226.69	432.45
36	341.78	472.51

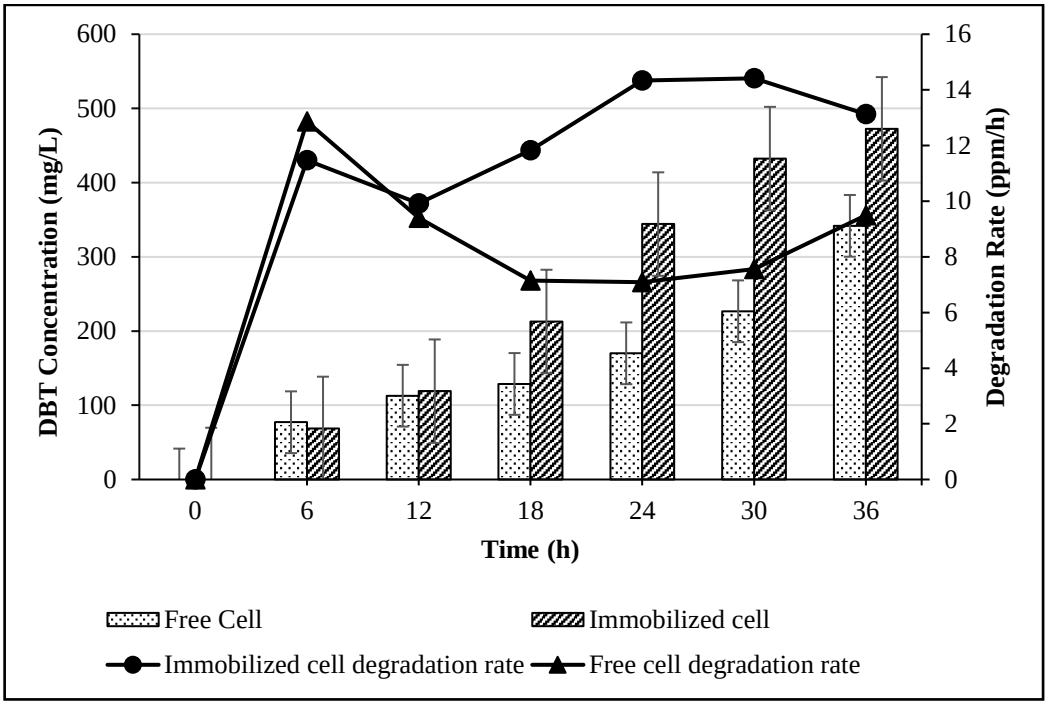


Figure 4.3: Bar graph showed the average concentration of DBT degraded by free and immobilized *T. profundimaris* strain M02 in ONR7a with 0.1% (w/v) initial DBT concentration and line graph shows the degradation rate of DBT by free and immobilized *T. Profundimaris* strain M02

4.3.4 Degradation of DBF by free and immobilized *T. profundimaris* strain M02

On the degradation of DBF by immobilized and free suspended bacteria, the degradation was similar at the first 6th hour of the cycle. At 12th cycle and onwards, the difference of both free and immobilized was noticeably different where immobilized cells were showing higher degradation at every hour. At the end of the cycle, immobilized cells showed 659.27 mg/L of total DBF degradation while free suspended cells showed 463.91 mg/L. Table 4.4 showed the concentration of total DBF degraded by free and immobilized cells. The degradation rate of for both immobilized and free cells were illustrated in Figure 4.4 showed a rapid increase at the first 6th hour. However, free cells showed a decrease after that while immobilized cells continued to peak before it started to decrease after the 12th hour. Immobilized cells rate of degradation kept decreasing until the 30th hour and

then it started to be stationary while the free cells have a slight fluctuation at every hour. The growth of free cells might not be stable due to its toxic environment, causing cells to experience death at some point, but at the same time, new cells started to grow that caused the slight increase in degradation rate. The growth of cells in immobilized cells however are relatively stable as it showed uniform pattern of decrease, showing less cells embedded were available to degrade DBT but cells growing at the outer layer of the beads were enough to keep degrading DBT until the cell's availability become the limiting factor.

Table 4.4: Table of average concentration of DBF degraded by free and immobilized *T. profundimaris* strain M02 in ONR7a with 0.1% (w/v) initial DBF concentration

	Concentration of DBF (mg/L)	
Hour	**Free Cell**	**Immobilized cell**
0	0.00	0.00
6	87.86	96.12
12	150.11	301.58
18	235.31	412.64
24	349.26	492.08
30	391.58	557.81
36	463.91	659.27

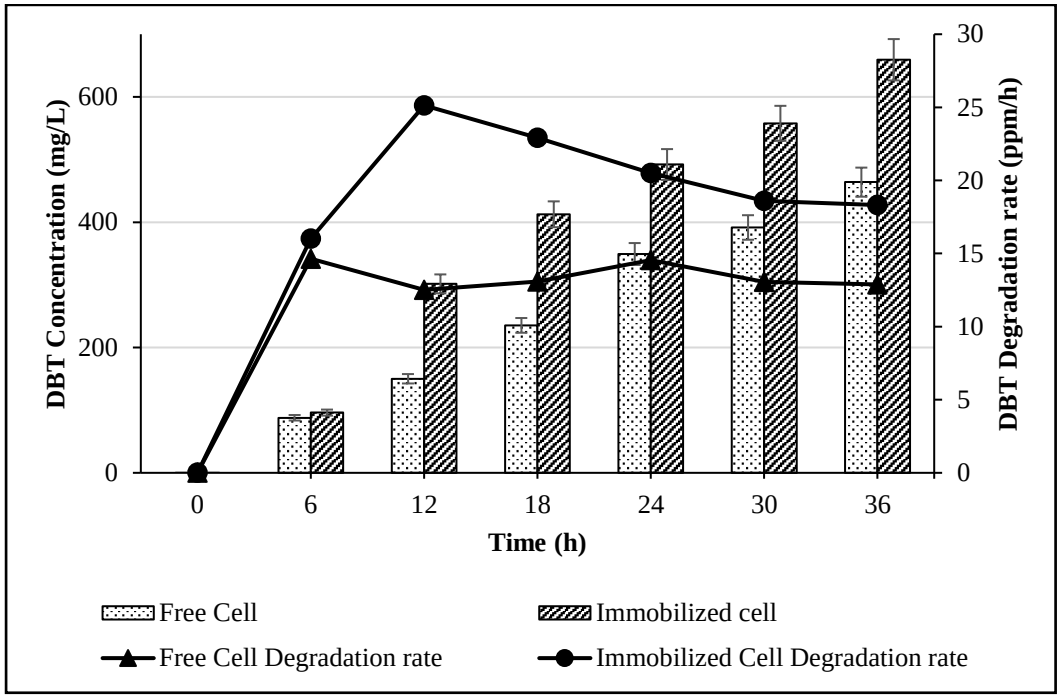


Figure 4.4: Bar graph shows the average concentration of DBF degraded by free and immobilized *T. profundimaris M02* strain M02 in ONR7a with 0.1% (w/v) initial DBF concentration and line graph shows the degradation rate of DBF by free and immobilized *T. profundimaris* strain M02.

4.3.5 Degradation of FL by free and immobilized *T. profundimaris* strain M02

Degradation of FL by free and immobilized cells was noticeably different from the 6th hour of the cycle and immobilized cells continued to degrade at higher concentration of FL compared to the free cells. At the end of the cycle, immobilized cells showed 497.79 mg/L of the FL degraded while free cells showed 341.78 mg/L of total FL degradation. Concentration of FL degraded by free and immobilized cells were shown in Table 4.5**.** This displayed an average of 14.38% difference of total FL degradation between free and immobilized cells. Degradation rate of FL were expressed in Figure 4.5 and at first 6th hour of incubation was at the highest rate. The degradation rate then rapidly decreased before showing an almost stationary trend of degradation rate until the 36th cycle. The trend was almost similar for both immobilized and free cells, only the difference is that the rate of

immobilized cells was at two times higher than the free cells. This showed that the cells degradation activity doubled when it was immobilized in calcium alginate beads.

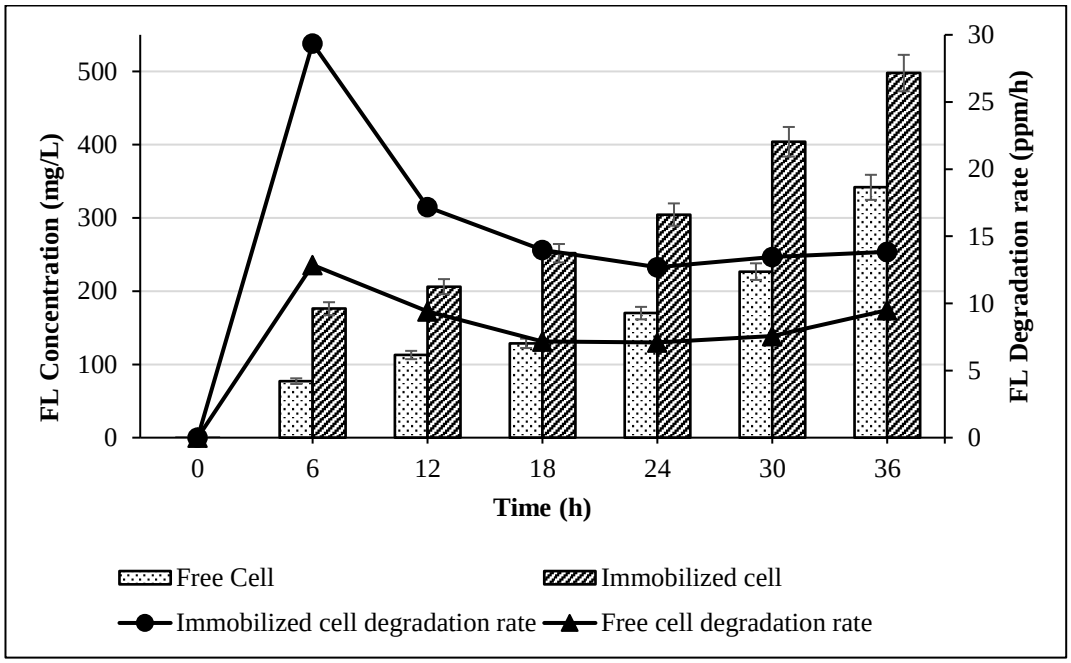


Figure 4.5: Graph of the average concentration of FL degraded by free (●) and immobilized (▲) *T. profundimaris* strain M02 in ONR7a with 0.1% (w/v) initial FL concentration

Table 4.5: Average concentration of FL degraded by free and immobilized *T. profundimaris* strain M02 in ONR7a with 0.1% (w/v) initial FL concentration

	Concentration of FL (mg/L)	
Hour	**Free Cell**	**Immobilized cell**
0	0.00	0.00
6	77.16	176.12
12	112.86	206.16
18	128.74	251.81
24	170.05	304.59
30	226.69	403.99
36	341.78	497.79

4.3.6 Degradation comparisons of *T. profundimaris* strain M02 on CAR, FL, DBT and DBF

Overall, the degradation of CAR, FL, DBT and DBT showed the same results where immobilized bacteria degrade higher concentration of the substrate as well as no extended lag period of cell growth compared to free bacteria. Concentration of total substrate degradation by free and immobilized cells were shown in Figure 4.6. From Figure 4.6, immobilized cells showed higher concentration of substrate degradation in all compounds. The high performance of encapsulated cells could be explained by the effect of protection given by the gel matrix. Moslemy et al (2002) also listed out the protective actions of the gel matrix towards the cells. Firstly, the gel matrix adsorbs the hydrocarbons hence lowering the concentration of dissolved hydrocarbon in the cell microenvironment within the porous microbeads (Moslemy et al., 2002). The formation of microcolonies within the gel matrix at the outer layer of the beads offered a diffusion barrier to the cells located in the inner layers, thus limiting the exposure to the increasing levels of toxic hydrocarbons. In addition, the formation of microcolonies within the gel matrix might also limit the loss of intracellular material from the damaged cells (Moslemy et al., 2002). It could be concluded that immobilized cells gave better performance on degrading the substrate as the matrix acted as the cells host and protected the cells from the toxicity of the substrates.

Therefore, aside from protecting the cells embedded, immobilization also improved the degradation capability of *T. profundimaris* M02 to degrade other heterocyclic hydrocarbon compounds. This could eliminate the needs for further isolating and characterizing a specific compound degrading bacterium thus saving more time. Further improvement could be made by only using one strain of bacteria and maintaining one strain of bacteria would be more cost effective.

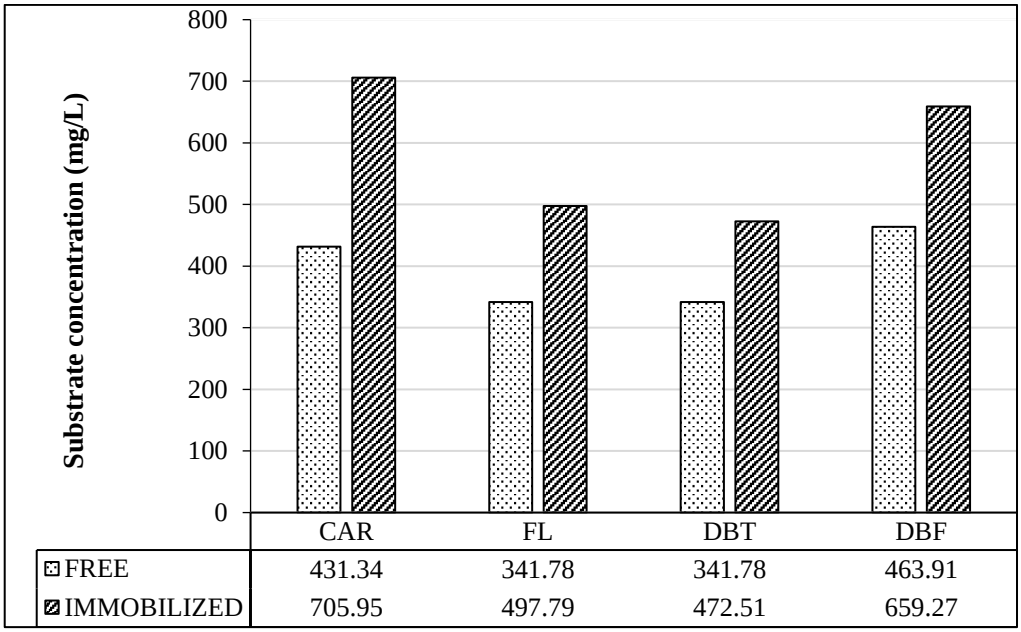


Figure 4.6: Graph of the average substrate degradation by free and immobilized *T. profundimaris* strain M02 in ONR7a with 0.1% (w/v) substrate concentrations.

4.4 Conclusion

From the utilization test, it is concluded that *T. profundimaris* strain M02 could grow on CAR, DBF, DBT and FL. However, full utilization was only observed on CAR and DBF as production of metabolites was observed on the plates. The degradation test also exhibited higher compounds degradation on CAR and DBF.

Degradation of the compounds by free and immobilized cells showed identical results for all compounds where immobilized cells exhibited higher compounds concentration degraded. Immobilized cells also showed 20.41%, 16.27%, 6.91% and 14.38% of performance increase in degrading CAR, DBF, DBF and FL, respectively. This indicated that although this strain is weak in degrading other compounds, immobilizing this strain improved degradation of these compounds. Degradation rate of free and

immobilized cells also exhibited significance difference as immobilized cells showed an extended lag phase thus causing the increases the concentration of compounds degraded.

CHAPTER 5

ACTUAL EXPERIMENT OF DEGRADATION OF CARBAZOLE (CAR) USING IMMOBILIZED *T. profundimaris* STRAIN M02 USING SEAWATER IN BIOREACTOR

5.1 Introduction

Bioreactors are basically tank in which living organisms can carry out biological reaction. It can be applied in bioremediation strategies as it is able to provide optimum controlled environment for the biodegradation of hydrocarbon polluted media and eliminates most of the rate limiting factors such as oxygen supply, temperature and optimal pH. Besides, the reactor should be easy to maintain and operate, as well as being able to function under aerobic and an aerobic condition. Immobilized system can provide massive populations of bacteria inside the bioreactor and can handle high flowrates (Kariminiaae-Hamedaani, Kanda, & Kato, 2003). The system can effectively remove the chemical oxygen demand (COD) under certain conditions, as well as retaining its performance level over the operation period (Kariminiaae-Hamedaani et al., 2003).

Cells encapsulation for bioreactor use has several advantages over conventional suspension culture methods. It allows larger cell densities attainment inside a bioreactor, protects fragile mammalian cells from shear forces and simplifies product purification (Riley et al., 1995). As immobilization are known to increase the cell productivity, developments in engineering application are possible as encapsulated cells are cultivated in bioreactors to manufacture valuable biological products such as antibodies, vaccines, interferons and growth factors (Riley et al., 1995). Besides that, Jena, Roy, & Meikap (2005) emphasizes that immobilized cell bioreactors are better than free culture bioreactors based on the experiments done by comparing these two bioreactors and said

that semi fluidized bed bioreactor is a novel and efficient bioreactor, which can be adapted for wastewater treatment (Jena et al., 2018).

The use of immobilized cells also reported in the production of propionic acid by Dishisha, Alvarez, & Hatti-Kaul in 2012. In their study, production of propionic acid was higher when immobilization was applied to the cells. Their results also show a significant decrease in lag phase of the cells when immobilized hence increasing the productivity of propionic acid (Dishisha et al., 2012).

This study was done to understand the cells reaction in a real-world condition as well as its performance in a controlled environment. The use of cell immobilization was also implied to eliminate most of the problems emerged when up-scaling studies in bioreactor as mentioned in Section 2.7. Hence, seawater was used as the media and the study was done in a batch stirred tank bioreactor.

5.2 Material and Methods

5.2.1 Microorganism and cultivation

T. profundimaris strain M02 were cultivated in 100 mL ONR7a media with marine broth as its nutrient at 9:1 ratio. After 24 hours of cultivation, the broth was centrifuged at 7000 rpm for 10 minutes and cell pellets were obtained. Since this was a scale up study, the ratio of the cells obtained was at 1:10 from the lab scale study. Therefore, the total cell pellets used in this study was 12.5 g.

5.2.2 Preparation of calcium alginate beads for cell immobilization

Calcium alginate was used as the immobilization matrix as it shows promising results from the previous lab scale study in Section 3.2.2. The preparation of calcium alginate beads was done following the method by Usha et al. (2010). Calcium alginate

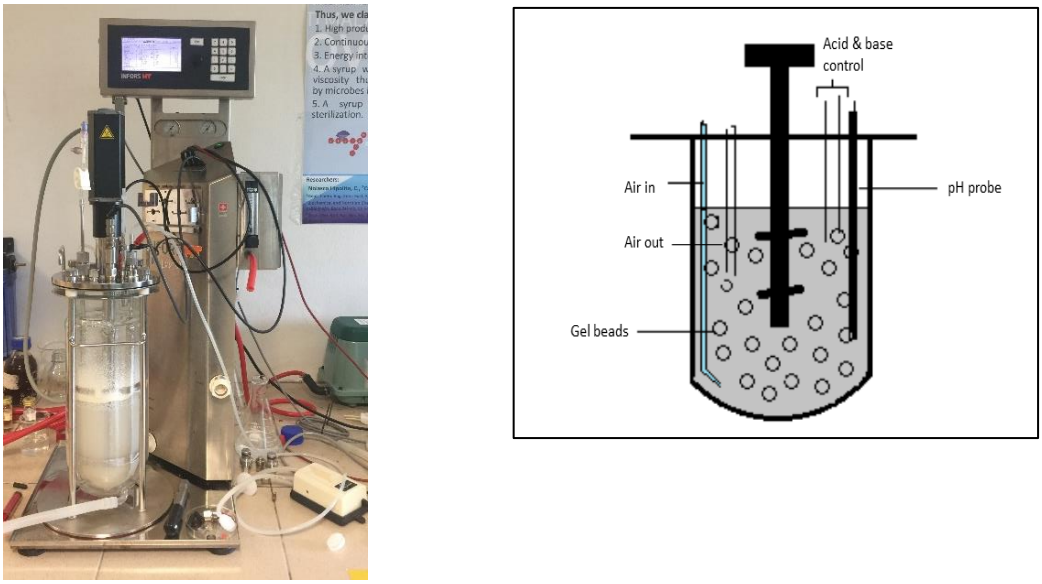
(4% w/v) are dissolved with 100 mL distilled and autoclaved at 121^0C for 15 minutes. After the mixture cooled down, bacterial suspension was added into the mixture and stirred gently. The alginate/suspension mixture were extruded into cold, sterile 0.2M $CaCl_2$ using a syringe. Beads formed were stored in fresh $CaCl_2$ for 2 hours to harden it. Lastly, the beads were washed with sterile distilled water before using it for experimentation.

5.2.3 Bioreactor Set-up for biodegradation experiment

Benchtop bioreactor (Infors HT, Labfors 4, Switzerland) with 3-5 L capacity vessel was used as the inoculum for the experiment. Figure 5.1(a) showed the set-up of the bioreactor and Figure 5.1(b) was the close-up view of the bioreactor vessel with seawater media and 0.1% (w/v) CAR.

Seawater was used in this experiment as it was the origin of the isolated cells and because the target area of using these developed cells in the future were mainly in contaminated sea area. The seawater was collected at Damai Beach Resort (Figure 5.2). The water was filtered using 0.22 μm filter (MILIPORE Express^{TM} PLUS) to remove excess substrate and bacteria and autoclaved at 121°C for 15 minutes before use. For every batch of degradation, 1000 mL of seawater was used and 0.1% (w/v) of CAR was used as the cell's sole carbon source.

The bioreactor was set at 150 rpm stirring rate, pH maintained at 7.0 to 7.3, air supply at 10 mL/min and incubated at room temperature. 5M of NaCl or 5M HCl of acid and base were used to control the pH of the media. All probes and tubing were pre-sterilized before use.



(a) (b)

Figure 5.1: (a) Full bioreactor set up with the control centre attached and (b) Schematic diagram of bioreactor set-up

Figure 5.2: Damai Beach Resort was the sampling site of seawater. The circle area indicates the seawater sampling range.

5.2.4 Degradation of CAR by *T. profundimaris* M02 using batch bioreactor

Freshly harvested cell pellets were aseptically added into 1.0 L sterilized seawater medium with 0.1% (w/v) CAR as the sole carbon source in a 3.5 L jacketed glass bioreactor. The temperature was set at room temperature and air was supplied using pump with flow rate of 10 mL/min. pH was measured using pH electrode connected to the control base for peristaltic pump control to add 5M of NaCl or 5M HCl to maintain the pH. Samples were collected using super save sampler for extraction of substrates. The same conditions were repeated for the degradation using immobilized cells and control experiment. The control experiment were calcium alginate beads with no bacteria embedded within.

The inoculation was run for 36 hours, the same length of time as the lab scale experiment and the degradation of CAR in bioreactor was expected to be faster than of the lab scale experiment. Sampling of the samples were done at 6-hour interval and all experiments were done in triplicates.

5.2.5 Growth kinetic of cell in bioreactor

The specific growth rate of cell, μ was calculated from equation 13.

$$\mu = t \cdot \ln\left(\frac{X}{X_0}\right) \tag{13}$$

Where X and X_0 were biomass concentration at time t and t = 0 respectively.

The logistic equation is as in equation 14 and is written as equation 15 in order to determine the carrying capacity coefficient of the cell growth.

$$\frac{dX}{dt} = kX\left(1 - \frac{X}{X_{max}}\right) \tag{14}$$

$$k = \frac{1}{x} \cdot \frac{\Delta X}{\Delta t} \div \left(1 - \frac{\bar{X}}{X_{max}}\right) \quad (15)$$

Where, $\bar{X}$is the average biomass concentration during Δt and X_{max} is the maximum biomass concentration in the experiment.

5.2.6 Gas Chromatography Flame Ionization (GC FID) analysis

The extraction of CAR was done similar with the method in Sections 3.2.8 and 4.2.5, where 1.0 mL of aliquot sample was sampled from the reactor using syringe and added with equal volume of ethyl acetate. The solution was vortexed, and the upper layer of the solution was obtained for analysis in GC-FID.

In this study, GC FID (Shimadzu GC-2010 Plus, Japan) with autosampler (Shimadzu AOC-20i, Japan) was used. Figure 5.3 shows the setup of the machine. Sample of known concentrations with no bacterial culture was used as standards for this experiment and a calibration curve set. Detection of the compound was done by using HP-5 fused silica capillary column (50mm x 0.32 mm x 0.25 μm) at 250ºC at the injector, 300ºC at the detector, with column heated to 200 - 250ºC at 5ºC per minute and split-less column with compressed air/nitrogen as the carrier gas. The peak graph obtained from the detection was attached in Appendix C.

Figure 5.3: GC-FID used for the detection of CAR.

5.3 Results and discussion

5.3.1 Performance of biodegradation of CAR in bioreactor in comparison with lab scale experiment

The degradation of CAR was compared between the experiment done in Erlenmeyer flask in 100 ml media and stirred tank bioreactor in 1000ml media. From the experiment, both free and immobilized cells showed better overall performance when inoculated in bioreactor. However, immobilized cells showed a steadier CAR degradation as it showed uniform increase in the CAR concentration degraded over time in both flask and bioreactor experiment as seen in Figure 5.4 and 5.5. Degradation rate of immobilized cells for both flask and bioreactor experiment also exhibited steady trends although the degradation rate of immobilized cells in lab scale experiment decreased after 6 hours of incubation but slowly increased after 24th hour. From Figure 5.5, free cells in bioreactor exhibited fluctuated trend of degradation rate over time while free cell incubated in flask showed an early stationary phase after only 24-hour incubation. This showed that even

though an optimal environment for the cell growth was provided, it still does not protect the cells form the harsh and toxicity of the compounds hence affecting the degradation performance of the cells. Besides that, cells exhibited better performance when incubated in bioreactor were due to the preserved pH level in the environment. Presence of concentrated level of compounds might change the pH level of the media and disrupted the performance of the cells in compounds degradation. Therefore, as the pH level in the bioreactor were maintained at 7.0 to 7.3, it ensured the best performance of the cells. Nikakhtari, Song, Kumar, Nemati, & Hill (2010) stated that the reason for difference of degradation rate in lab and bioreactor experiment was due to the mixing rate. Lab experiment study exhibits lower mixing rate hence the mass transfer was less efficient (Nikakhtari et al., 2010).

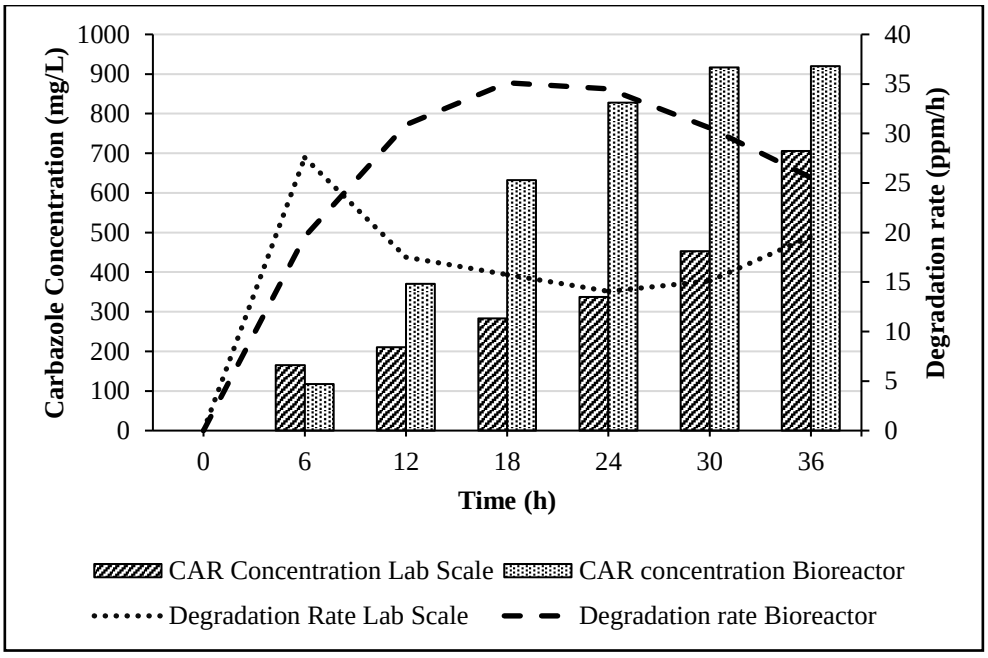


Figure 5.4: The line graph showed the biodegradation rate of immobilized cells in lab scale experiments (•••) and up-scale experiments in bioreactor (---), while bar graph showed the concentration of CAR degraded by immobilized cells in lab scale experiment and up-scale experiment in bioreactor.

Table 5.1: Table of average concentration of CAR degraded by immobilized *T. profundimaris* strain M02 in ONR7a with 0.1% (w/v) initial CAR concentration in lab scale experiment and up-scale experiment

	Concentration of CAR (mg/L)	
Time (hour)	**Lab Scale Experiment**	**Experiment in Bioreactor**
0	0.000	0.000
6	165.34	117.59
12	210.15	370.54
18	283.35	632.09
24	337.48	827.87
30	453.11	916.50
36	705.94	919.72

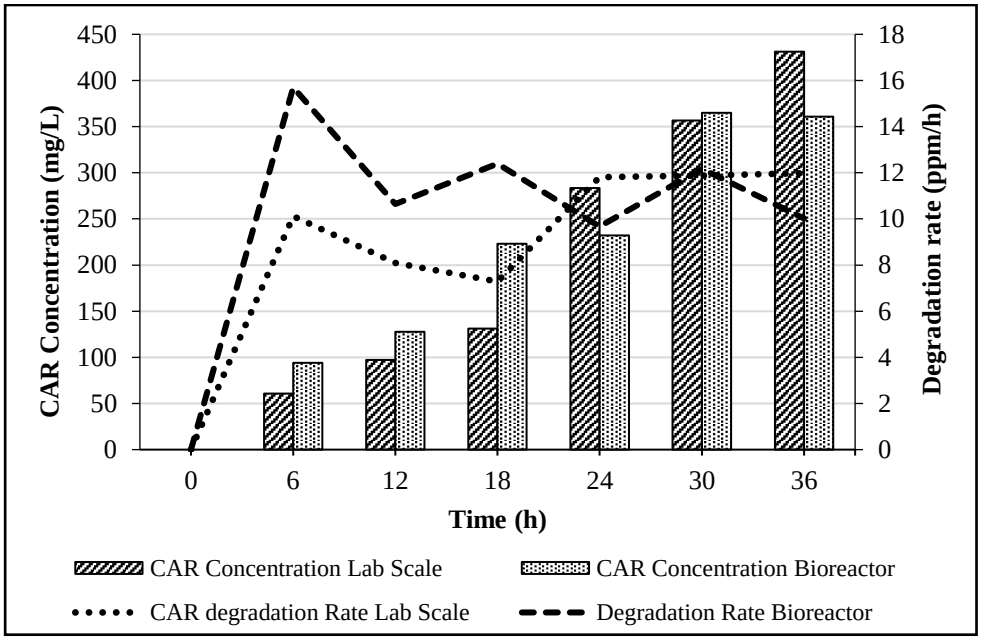


Figure 5.5: The line graph shows the biodegradation rate of free cells in lab scale experiments (•••) and up-scale experiments in bioreactor (---), while bar graph shows the concentration of CAR degradation of free cells in lab scale experiment and up-scale experiment in bioreactor.

Table 5.2: Table of average concentration of CAR degraded by free *T. profundimaris* strain M02 in ONR7a with 0.1% (w/v) initial CAR concentration in lab scale experiment and up-scale experiment

	Concentration of CAR (mg/L)	
Time (hour)	**Lab Scale Experiment**	**Experiment in Bioreactor**
0	0.000	0.000
6	60.72	94.21
12	97.02	127.75
18	131.08	222.98
24	283.28	232.20
30	356.54	365.05
36	431.35	360.93

5.3.2 Degradation comparisons between free and immobilized cells in bioreactor

The degradation by free and immobilized cells in bioreactor showed the same results as the lab scale experiments in Section 4.3.6, where immobilized cells exhibit higher concentration of CAR degradation. As explained by Moslemy et al. (2002), immobilized cells were protected by the gel matrix from existing toxic, and when in bioreactor it provided another protection from the bioreactor impeller. The fast stirring impeller as well as the constant flow of air from the sparger caused conditions inside the bioreactor vessel to be harsh. Free cells might not take the conditions and it unable to keep up with the increasing mass transfer rate that is occurring.

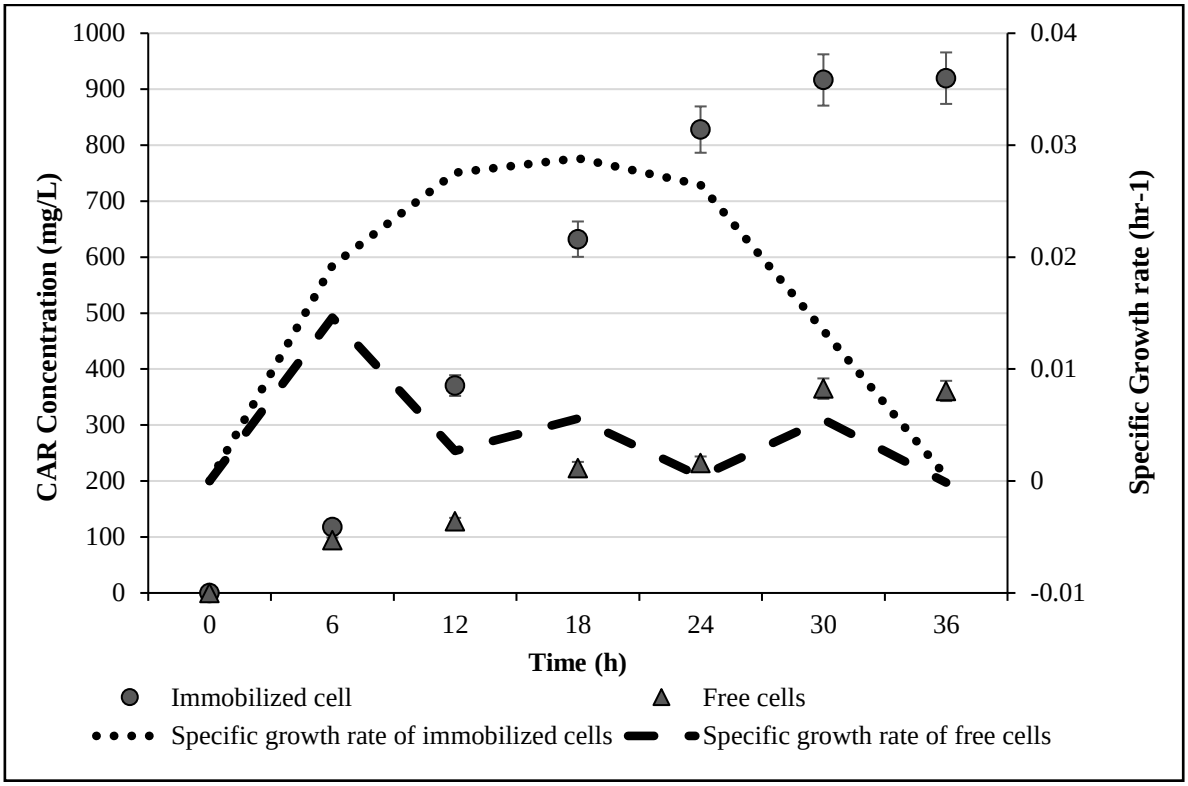


Figure 5.6: Scatter graph showed the concentration of CAR degraded at 36-hour incubation by free (▲) and immobilized (●) cells in bioreactor at optimum conditions. Line graph showed the specific growth rate of immobilized cells (•••) and free cells (---) in the bioreactor.

5.3.3 Growth kinetic of cells

The growth kinetic of cells were important to understand the cells behaviour during bioremediation process. Loyola-Vargas & Vázquez-Flota (2006) explained that the specific growth rate referred to the steepness of a curve and defined by the increased rate of biomass of a cell population per unit of biomass concentration. It was possible to calculate in batch cultures as at a defined period of time, the increase rate of biomass per unit of biomass concentration is constant and measurable (Loyola-Vargas & Vázquez-Flota, 2006). This time period occurred between lag phase and stationary growth phase. During this period, the increase in cell population fitted a straight-line equation between ln*x* and *t*.

Form Figure 5.5, it could be observed that immobilized cells experienced extended lag phase at the first 6 hours of incubation compared to free cells. Over time, immobilized cells also exhibited higher specific growth rate compared to free cells and this indicates immobilization technique prolonged the growth of the cells.

Table 5.3: Table of specific growth rate of 12.5g of free and immobilized cells when inoculated in bioreactor with 1.0 L seawater and 0.1% (w/v) CAR.

	Specific growth rate of cell, μ (hr^{-1})	
Time (hour)	**Free cells**	**Immobilized cells**
0	0.0000	0.0000
6	0.0146	0.0192
12	0.0027	0.0275
18	0.0056	0.0288
24	0.0004	0.0265
30	0.0055	0.0134
36	-0.0001	0.0004

5.4 Conclusion

Biodegradation of CAR exhibited better performance when it was inoculated in bioreactor. Aside from having higher concentration of CAR degraded, inoculation in bioreactor also showed higher degradation rate. This might due to the controlled environment in the reactor as well as higher rate of mass transfer. Constant supply of air into the reactor gave out steady supply of oxygen, hence reducing the cells competition for oxygen. Therefore, having the inoculation in a bioreactor is better for the cell performance as well as its sustainability.

Comparisons of the degradation between free and immobilized cells in bioreactor showed higher degradation of CAR in immobilized cells. This corresponded with the

previous lab scale experiment where immobilized cells also showed higher performance. Besides that, immobilization also prolonged the growth of the cells as its specific growth rate were higher than free cells. This affected the degradation of CAR as high growth of cells caused more CAR to be degraded by the cells. Hence, immobilization of the cells would be a great improvement for bioremediation of compounds in bioreactor.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Best condition for cell immobilization of isolated marine bacteria *T. profundimaris* M02 was achieved using calcium alginate instead of gellan gum. The matrix concentration showed highest concentration of CAR degradation when used at 4% (w/v) concentration and the cell mass loading of 1.25g showed the highest degradation of CAR. These immobilization conditions were used to proceed on the next part of the study. On the other hand, reusability of these immobilized bacteria was tested using repetitive batch study. From the results, it showed that after the first cycle of the test, immobilized cells could only retain only half of its degradation ability. Although it might not be suitable for degradation of CAR at high concentration, these cells could be used to degrade traces of CAR at lower concentrations.

The isolated marine bacteria *T. Profundimaris* M02 were isolated from CAR contaminated environment and its degradation ability of degrading other compounds were unknown. From utilization test, this bacterium could grow on DBT, DBF and FL, however complete utilization can be only observed on DBF plate. Cell immobilization was used to further increased the degradation capability of this bacteria and from the results, it showed that immobilization increased the compound degradation. Therefore, it could be concluded that cell immobilization was not only improving the cells performance, it also could replace the need for a specific compound bacteria isolation as a single cell strain had shown promising results on degrading other compounds when immobilization technique was adapted.

Performing this experiment in bioreactor showed significant increase in the performance of the immobilized cells from the lab scale experiment. The total CAR degradation in up-scale experiment was close to 100% due to the controlled and optimum environment in the bioreactor. The comparison study between free and immobilized cells showed that the degradation of CAR was higher and steadier using immobilized cells. This showed that even at high volume and high concentration, the performance of immobilized bacteria still remained. Therefore, immobilized cells were a great method for large scale bioremediation as it showed prominent results in this experiment.

6.2 Limitations

During this project, metabolites or by-products produced by the cell during the degradation was unable to be identified because of the unavailability of Gas Chromatography Mass Spectrometry (GC MS) machine. Besides that, observation of dissolved oxygen in the reactor was not able to be conducted due to the faulty pO_2 probe of the bioreactor. Therefore, no information was able to be provided about the metabolism and health of cells.

6.3 Recommendations

Improvements can be made from this study to further understand the cells behaviour and conditions during the cell's immobilization. Viability of the cells encapsulated in the matrix should be observed and the carrying capacity coefficient also should be identified. This will further improve the understanding and the limit of the cell's degradation ability. Besides that, metabolites production of the bacteria from the compound degradation can be analysed using Gas Chromatography Mass Spectrometry (GC MS). From this analysis, the degradation pathway of the compounds can be sketched

out and therefore more information can be obtained from the pathway. Identification of the metabolites produced may also help in understanding the effects of these metabolites towards the cells and the gel matrix.

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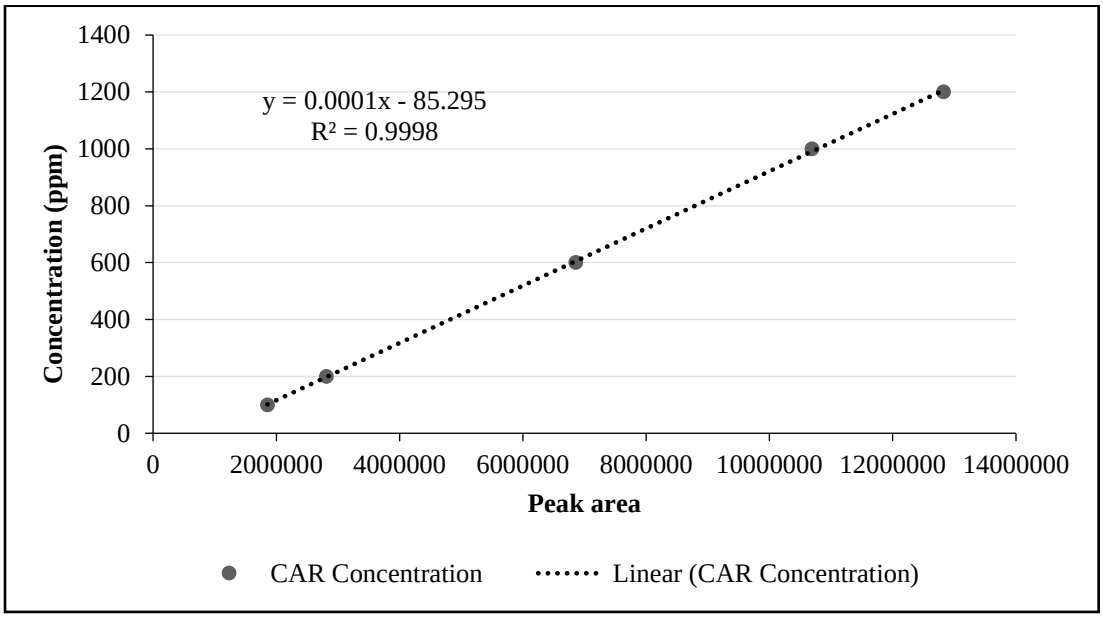
APPENDICES

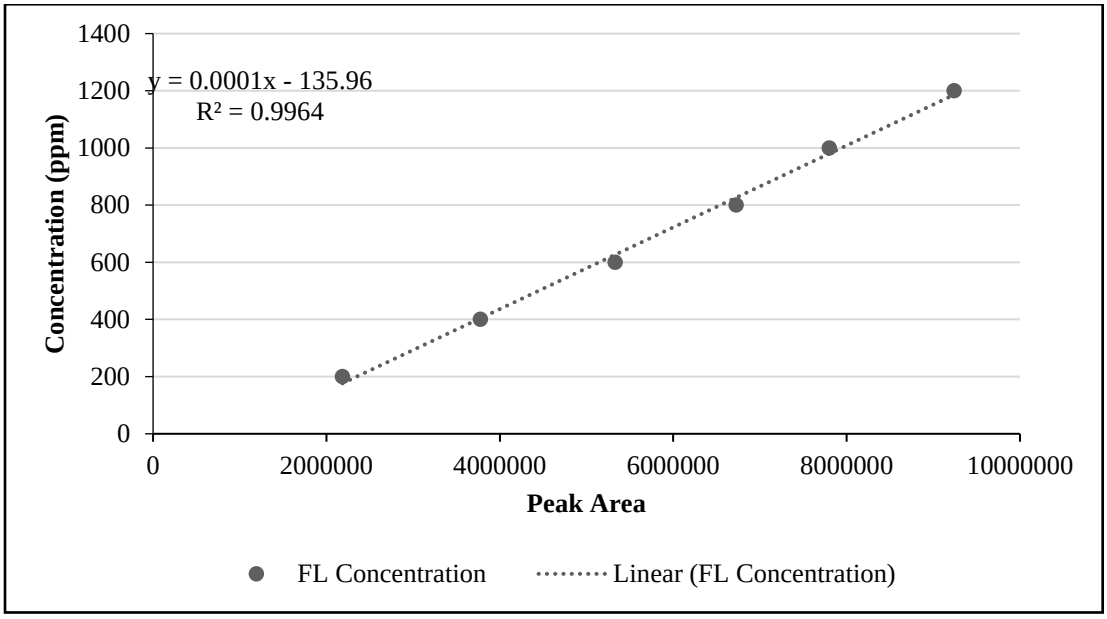
Appendix A

a) Calibration for CAR, DBT, DBT and FL

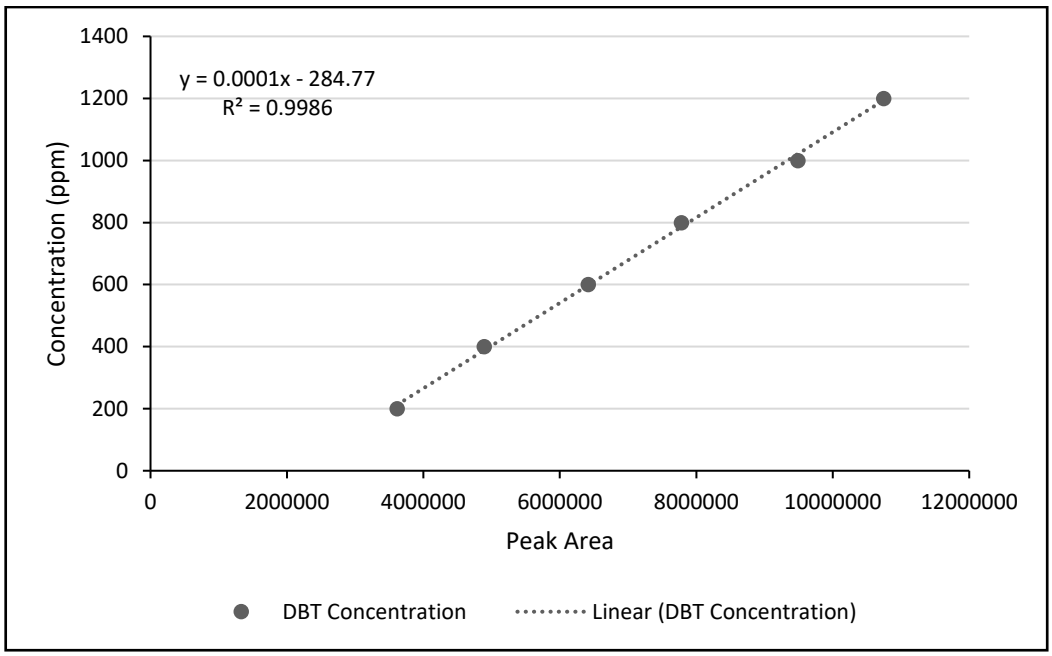
i. Calibration standard for Carbazole



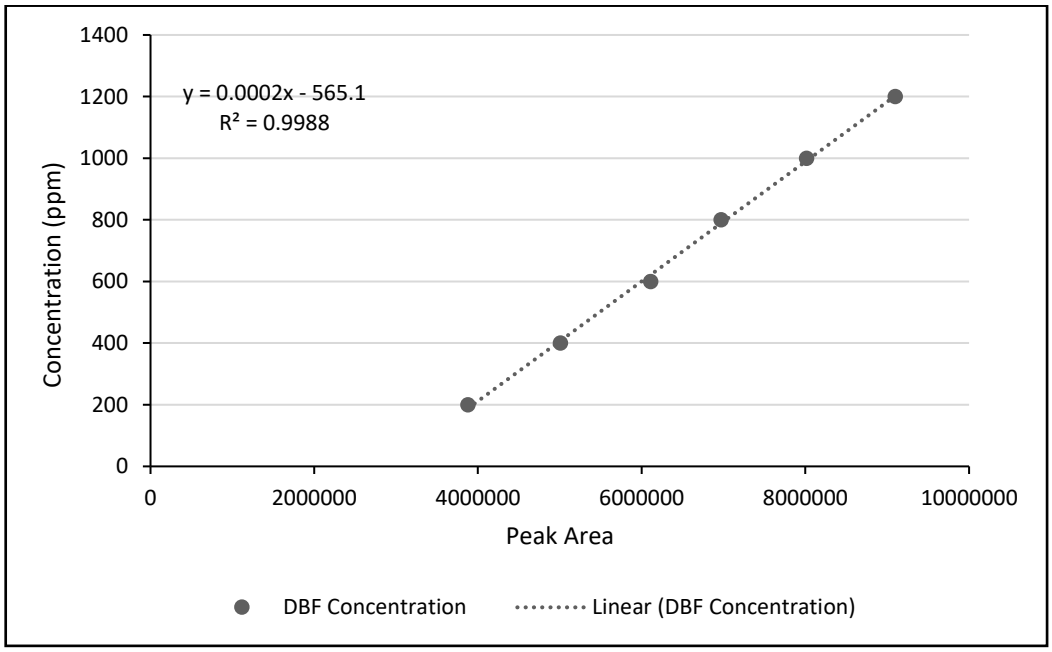
ii. Calibration standard for Fluorene



iii. Calibration standard for Dibenzothiophene



iv. Calibration standard for Dibenzofuran (DBF)



Appendix B

a) Different Matrix Experiment

Matrix Type	Hour	Replicate 1	Replicate 2	Replicate 3	Average	Standard Deviation
Gellan Gum	0	0.000	0.000	0.000	0.000	0.000
	6	12.705	121.023	80.882	71.537	44.712
	12	112.346	364.036	134.051	203.477	113.877
	18	195.562	489.803	205.225	296.863	136.486
	24	199.278	523.742	283.013	335.344	137.534
	30	384.699	738.858	337.529	487.029	179.108
	36	650.731	861.288	512.177	674.732	143.531
Calcium Alginate	0	0.000	0.000	0.000	0.000	0.000
	6	130.3447	216.437	262.716	203.166	54.849
	12	236.1371	320.659	412.495	323.097	72.018
	18	359.6956	352.523	480.706	397.642	58.809
	24	412.5419	395.136	494.087	433.922	43.132
	30	493.6451	508.884	572.075	524.868	33.955
	36	777.4633	727.127	826.344	776.978	40.507

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b) Different Matrix Concentration Experiment

Matrix Concentration	Hour	Replicate 1	Replicate 2	Replicate 3	Average	Standard Deviation
3.00%	0	0.000	0.000	0.000	0.000	0.000
	6	133.1582	129.6875	103.4611	122.10	13.257
	12	237.3055	154.4174	164.2373	185.32	36.977
	18	335.732	192.5048	260.3718	262.87	58.499
	24	357.812	242.4011	267.9863	289.40	49.490

	30	369.661	247.7419	312.9445	310.12	49.813
	36	541.9258	298.8428	411.5412	417.44	99.326
4.00%	0	0.000	0.000	0.000	0.000	0.000
	6	130.344	216.437	262.715	203.166	54.849
	12	236.137	320.65	412.494	323.097	72.018
	18	359.695	352.522	480.706	397.642	58.809
	24	412.541	395.136	494.086	433.922	43.132
	30	493.645	508.883	572.075	524.868	33.955
	36	777.463	727.126	826.343	776.978	40.507
5.00%	0	0.000	0.000	0.000	0.000	0.000
	6	110.186	116.124	100.331	108.880	6.513
	12	412.145	188.717	223.819	274.894	98.104
	18	439.286	255.462	316.117	336.955	76.479
	24	450.025	297.233	375.190	374.150	62.382
	30	622.052	398.839	386.870	469.254	108.155
	36	798.738	611.015	522.961	644.238	115.011

c) Different Cell Mass Loading

Cell Mass Loading (g)	Hour	Replicate 1	Replicate 2	Replicate 3	Average	Standard Deviation
control	0	0.000	0.000	0.000	0.000	0.000
	6	79.610	80.200	71.170	76.993	4.125
	12	116.495	120.482	110.575	115.851	4.070
	18	125.300	133.720	129.090	129.370	3.443
	24	121.240	130.640	140.080	130.653	7.691
	30	119.580	130.220	145.020	131.607	10.432
	36	133.943	144.252	157.394	145.196	9.597
0.6	0	0.000	0.000	0.000	0.000	0.000
	6	151.300	131.580	135.940	139.607	8.458
	12	266.234	241.927	245.471	247.023	10.721
	18	338.380	316.900	317.380	317.180	10.015
	24	333.760	308.340	309.440	324.227	11.732
	30	346.280	325.040	325.150	337.967	9.987
	36	342.492	319.423	321.945	333.307	10.332
1.25	0	0.000	0.000	0.000	0.000	0.000
	6	130.3447	216.437	262.716	203.166	54.849
	12	236.1371	320.659	412.495	323.097	72.018
	18	359.6956	352.523	480.706	397.642	58.809
	24	412.5419	395.136	494.087	433.922	43.132
	30	493.6451	508.884	572.075	524.868	33.955
	36	777.4633	727.127	826.344	776.978	40.507
2.5	0	0.000	0.000	0.000	0.000	0.000
	6	165.343	307.884	142.179	205.135	73.267
	12	210.153	458.567	210.940	293.220	116.918
	18	283.352	461.772	225.343	323.489	100.608

	24	337.483	468.397	246.545	350.808	91.060
	30	453.112	578.778	263.012	431.634	129.802
	36	705.947	666.004	357.330	576.427	155.781
5.0	0	0.000	0.000	0.000	0.000	0.000
	6	39.513	27.972	56.641	41.375	11.778
	12	70.298	35.790	86.008	64.032	20.975
	18	122.056	51.753	114.834	96.214	31.577
	24	148.661	206.783	192.356	182.600	24.711
	30	289.064	289.798	342.291	307.051	24.920
	36	446.836	403.555	492.608	447.666	36.360

d) Reusability Experiment

Day	Hour	Replicate 1	Replicate 2	Replicate 3	Average	Std Dev
0	0	825.56	928.74	932.24	895.51	49.48
	24	574.75	673.25	664.08	637.36	44.43
1	0	817.17	642.47	961.87	807.17	130.59
	24	627.94	480.28	789.69	632.63	126.36
2	0	866.00	882.98	934.30	894.43	29.03
	24	685.74	720.50	792.23	732.82	44.34
3	0	895.37	877.23	857.27	876.62	15.56
	24	753.97	738.25	723.01	738.41	12.64
4	0	778.62	805.75	905.19	829.85	54.41
	24	644.09	661.25	769.66	691.67	55.59
5	0	895.37	866.20	815.75	859.10	32.89
	24	769.36	752.59	704.84	742.26	27.33

e) Degradation of CAR

Matrix Type	Hour	Replicate 1	Replicate 2	Replicate 3	Average	Standard Deviation
Free Cell	0	0.000	0.000	0.000	0.000	0.000
	6	57.067	63.430	61.649	60.716	2.680
	12	68.104	98.391	124.559	97.018	23.068
	18	118.552	122.988	151.701	131.081	14.693
	24	253.630	320.370	275.840	283.280	27.749
	30	263.805	359.594	446.212	356.537	74.498
	36	281.271	457.401	555.363	431.345	113.404
Immobilized Cell	0	0.000	0.000	0.000	0.000	0.000
	6	165.343	307.884	142.179	205.135	73.267
	12	210.153	458.567	210.940	293.220	116.918
	18	283.352	461.772	225.343	323.489	100.608
	24	337.483	468.397	246.545	350.808	91.060
	30	453.112	578.778	263.012	431.634	129.802
	36	705.947	666.004	357.330	576.427	155.781

f) Degradation of DBF

Matrix Type	Hour	Replicate 1	Replicate 2	Replicate 3	Average	Standard Deviation
Free Cell	0	0.000	0.000	0.000	0.000	0.000
	6	10.384	26.230	226.954	87.856	98.570
	12	22.017	120.122	308.176	150.105	118.732
	18	140.699	142.694	422.532	235.308	132.390
	24	176.718	318.947	552.102	349.256	154.741
	30	247.631	342.185	584.927	391.581	142.062
	36	415.323	362.454	613.968	463.915	108.277
Immobilized	0	0.000	0.000	0.000	0.000	0.000

Cell	6	51.541	189.317	47.494	96.117	65.923
	12	306.611	286.776	311.361	301.583	10.648
	18	596.937	314.199	326.779	412.638	130.420
	24	660.261	388.255	427.718	492.078	120.009
	30	732.506	464.710	476.216	557.811	123.618
	36	828.697	552.586	596.537	659.273	121.137

g) Degradation of DBT

Matrix Type	Hour	Replicate 1	Replicate 2	Replicate 3	Average	Standard Deviation
Free Cell	0	0.000	0.000	0.000	0.000	0.000
	6	80.849	113.087	37.534	77.157	30.955
	12	143.347	144.922	50.306	112.858	44.236
	18	158.722	168.027	59.472	128.740	49.127
	24	191.637	195.329	123.169	170.045	33.180
	30	228.740	288.805	162.535	226.693	51.570
	36	356.544	379.223	289.564	341.777	38.063
Immobilized Cell	0	0	0	0	0	0
	6	107.1058	45.5724	53.7913	68.8231	27.2770
	12	187.1345	61.7273	108.1579	119.006	51.7687
	18	272.7076	187.5906	178.463	212.920	42.4398
	24	358.4272	354.0745	320.078	344.193	17.1443
	30	477.3825	416.1924	403.777	432.450	32.1733
	36	538.3795	448.7449	430.3954	472.506	47.1777

h) Degradation of FL

Matrix Type	Hour	Replicate 1	Replicate 2	Replicate 3	Average	Standard Deviation
Free Cell	0	0.000	0.000	0.000	0.000	0.000
	6	80.849	113.087	37.534	77.157	30.955
	12	143.347	144.922	50.306	112.858	44.236
	18	158.722	168.027	59.472	128.740	49.127
	24	191.637	195.329	123.169	170.045	33.180
	30	228.740	288.805	162.535	226.693	51.570
	36	356.544	379.223	289.564	341.777	38.063
Immobilized Cell	0	0.000	0.000	0.000	0.000	0.000
	6	320.402	63.663	144.294	176.119	107.202
	12	324.137	125.441	168.894	206.157	85.290
	18	378.094	195.668	181.673	251.812	89.477
	24	438.602	243.268	231.904	304.591	94.873
	30	506.186	349.759	356.013	403.986	72.311
	36	615.866	430.938	446.573	497.792	83.734

i) Degradation of CAR by Immobilized Cell in Bioreactor by Free and Immobilized Bacteria

Condition	Hour	Replicate 1	Replicate 2	Replicate 3	Average	Standard Deviation
Free Cell	0	0	0	0.000	0.000	0.000
	6	122.726	99.059	60.834	94.206	25.499
	12	161.7926	117.596	103.860	127.749	24.717
	18	212.678	214.968	241.290	222.979	12.982
	24	223.67	230.22	242.723	232.204	7.904
	30	351.335	365.822	377.979	365.045	10.891
	36	353.812	362.951	366.018	360.927	5.185
Immobilized	0	0.000	0.000	0.000	0.000	0.000

Cell	6	118.360	112.700	121.720	117.593	3.722
	12	373.694	371.630	366.291	370.538	3.119
	18	635.489	639.625	621.150	632.088	7.917
	24	833.500	839.470	810.630	827.867	12.429
	30	918.120	922.660	908.710	916.497	5.810
	36	922.780	924.310	912.080	919.723	5.441

j) Mechanical Strength of Ca-AL and GG

i. Calcium Alginate

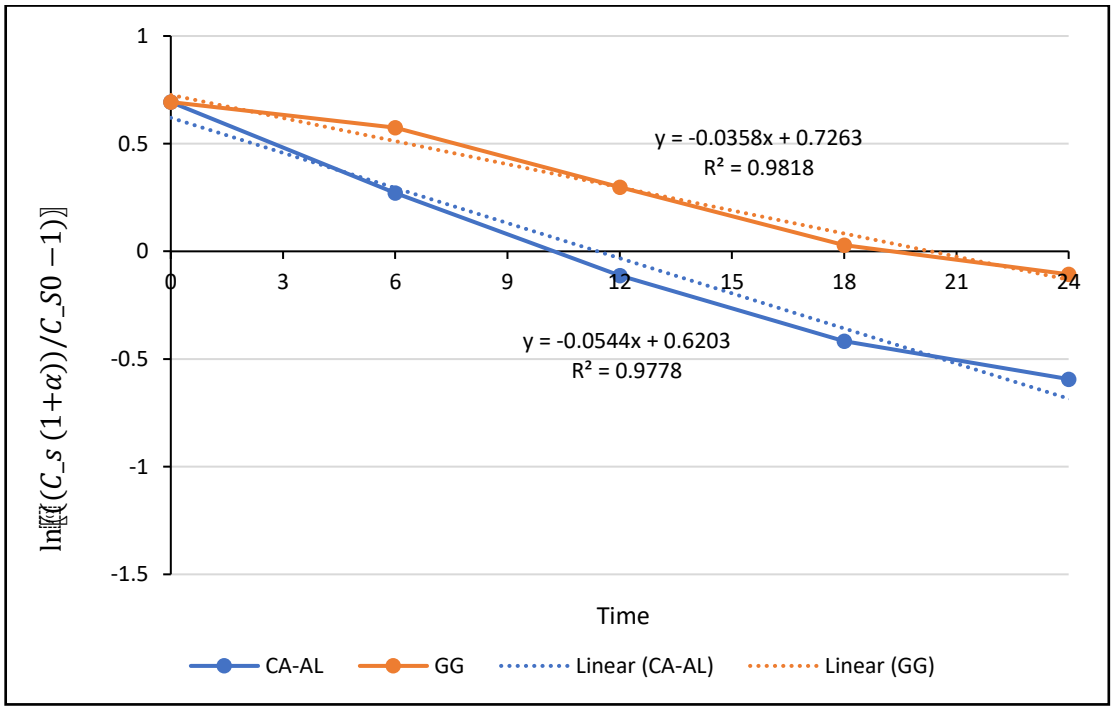
Concentration	Elongation (mm)	Average Elongation (mm)	Standard Deviation	Area (mm^2)	Force Applied (N)	Average Force (N)	Standard Deviation
3%	49.42 39.69 36.7	41.93	5.43	32	3.1 2.4 2.1	2.53	0.42
4%	42.08 40.55 44.51	42.38	1.63	32	4.2 3.5 4.6	4.1	0.45
5%	33.56 47.33 37.88	39.59	5.75	32	4.4 5.6 4.5	4.83	0.54

ii. Gellan Gum

Concentration	Elongation (mm)	Average Elongation (mm)	Standard Deviation	Area (mm2)	Force Applied (N)	Average Force (N)	Standard Deviation
0.50%	13.73 13.16 10.33	12.41	1.48	32	0.34 0.32 0.34	0.33	0.01
0.70%	16.26 19.60 22.67	19.51	2.62	32	0.85 0.8 0.96	0.87	0.07
0.90%	12.53 12.35 10.06	11.65	1.12	32	0.36 0.4 0.34	0.37	0.02

k) Diffusivity test of Ca-al and GG

i. Graph of diffusion data analysis of Gellan Gum and Calcium alginate versus time



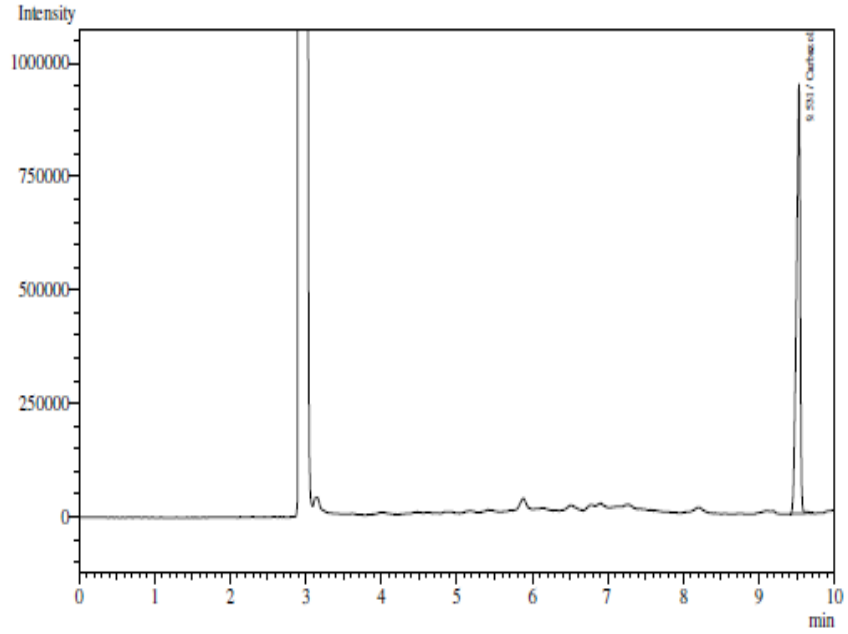
Appendix C

a) GC FID detection peak for substrate

i. Peak sample at Hour 0

Analysis Date & Time : 8/13/2018 2:36:47 PM
User Name : Admin
Vial# : 1
Sample Name : BR0B
Sample ID :
Sample Type : Unknown
Injection Volume : 1.00
ISTD Amount :

Data Name : C:\Documents and Settings\user\Desktop\intan\BR0B.gcd
Method Name : C:\FYP Carbazole Dr Hasma\standard carbazol (500-2500) ppm (hgh).gcm

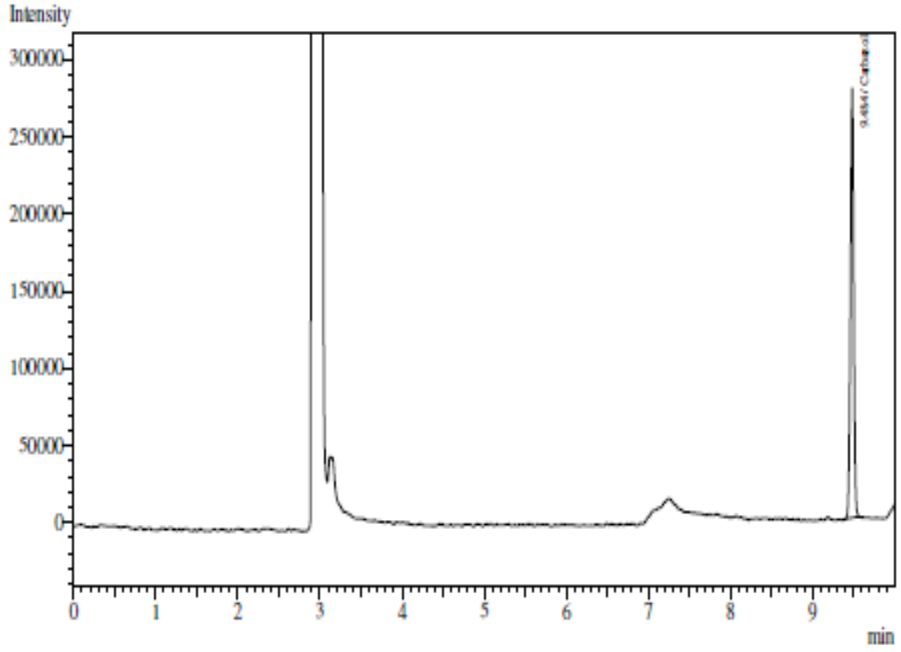


Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	9.531	3309480	933967	1029.553	ppm		1	Carbazol
Total		3309480	933967					

ii. Peak sample at hour 24

Analysis Date & Time : 8/13/2018 5:22:14 PM
User Name : Admin
Vial# : 5
Sample Name : BR24A
Sample ID :
Sample Type : Unknown
Injection Volume : 1.00
ISTD Amount :

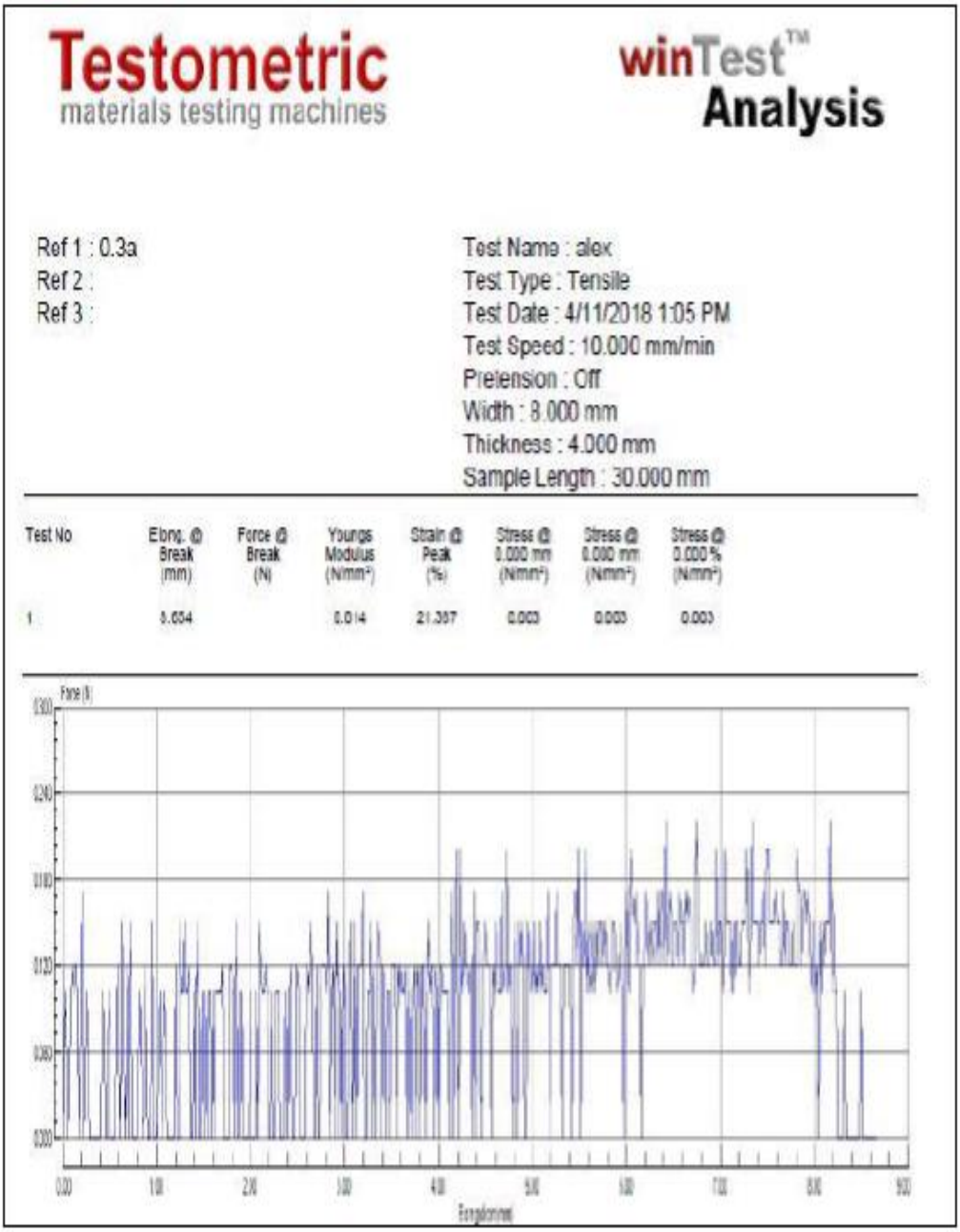
Data Name : C:\Documents and Settings\user\Desktop\intan\BR24A.gcd
Method Name : C:\FYP Carbazole Dr Hasma\standard carbazol (500-2500) ppm (hgh).gcm



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	9.484	772034	276969	305.029	ppm		1	Carbazol
Total		772034	276969					

b) Mechanical Strength test for Gellan gum and Calcium alginate

a. Gellan gum



Testometric
materials testing machines

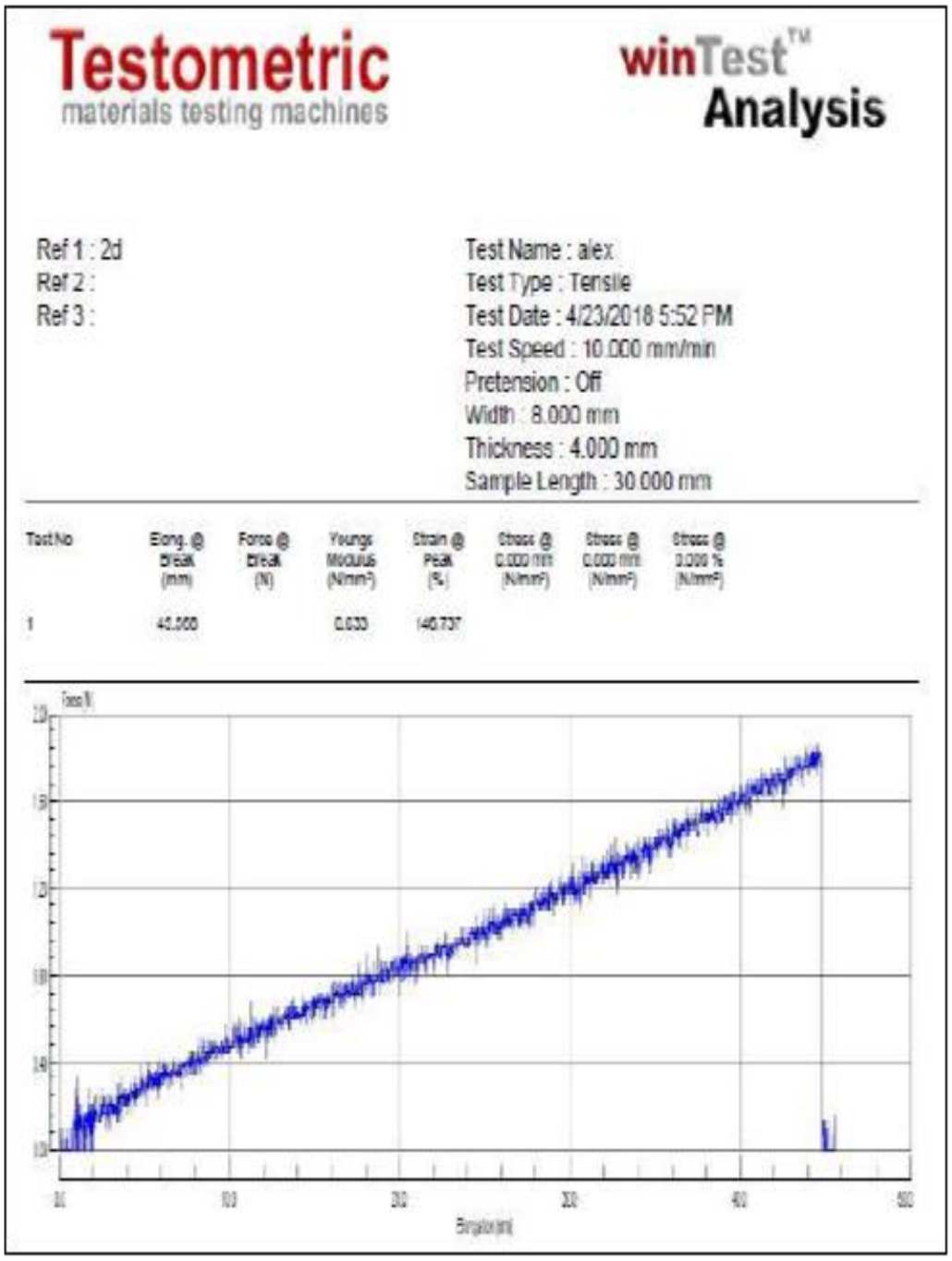
winTest™
Analysis

Ref 1 : 0.3a
Ref 2 :
Ref 3 :

Test Name : alex
Test Type : Tensile
Test Date : 4/11/2018 1:05 PM
Test Speed : 10.000 mm/min
Pretension : Off
Width : 8.000 mm
Thickness : 4.000 mm
Sample Length : 30.000 mm

Test No	Elong. @ Break (mm)	Force @ Break (N)	Youngs Modulus (N/mm²)	Strain @ Peak (%)	Stress @ 0.000 mm (N/mm²)	Stress @ 0.000 mm (N/mm²)	Stress @ 0.000 % (N/mm²)
1	5.654		0.014	21.367	0.003	0.003	0.003

b. Calcium alginate



Appendix D

a) Bioreactor Report

```
Company Name        :  UNIMAS
Company Division    :  Chemical
                    Engineering
Fermentation Title  :  Labfors_0_083
Fermenter           :  Labfors 0


Start time          :  Wednesday, 5
September 2018 9:26:14 AM

Inoculation time    :  Wednesday, 5
September 2018 9:26:14 AM

Time          ,Stirrer,    pH,
      Gas_Mix,

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