THE EFFECT OF FORMULATIVE MATERIALS ON THE VIABILITY AND EFFICACY OF FORMULATED BENEFICIAL MICROBES

By

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ABSTRACT

THE EFFECT OF FORMULATIVE MATERIALS ON THE VIABILITY AND EFFICACY OF FORMULATED BENEFICIAL MICROBES

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Environmental applications of beneficial microbes for bioremediation or biological control using free-cell form are constantly challenged by poor cell viability which subsequently might affect application efficacy. Therefore, there is a need to formulate the beneficial microbes to enhance their field potential and for easy storage, delivery and application. In this study, the identification and formulations of a hydrocarbon-degrading bacterium (UTAR EPA2) and a biocontrol bacterium (UPM 39B3) were carried out. To improve microbial viability and efficacy, the effect of various types of formulative materials in formulation development was investigated. The isolates were formulated separately with various combinations of formulative materials, comprising of clay-based carrier materials such as bentonite (B) and kaolin (K), enrichment materials such as non-fat skim milk (N) and sucrose (S) and a UV- protectant agent, *p*-amino benzoic acid (P). Formulated cells were exposed to sunlight for 6 hours to determine the effect of UV radiation on UTAR EPA2 and UPM 39B3 cell viability and their subsequent efficacy. In this study, UTAR EPA 2 and UPM 39B3 were identified as *Pseudomonas aeruginosa* and *Serratia marcescens*, respectively. Results on cell viability showed that all formulations investigated benefited both UTAR EPA2 and

UPM 39B3 cells when exposed to sunlight (UV radiation). The better cell viability for UTAR EPA2 bentonite-based formulations observed might be attributed to the presence of bentonite clay, the carrier material. However, both the formulated UTAR EPA2 and UPM 39B3 isolates did not produce significantly higher application efficacy and the additive material, *p*-amino benzoic acid, showed inhibitory effect on both formulated UTAR EPA2 and UPM 39B3 cells. This study also showed that higher cell viability did not necessary improve application efficacy as the formulative materials might also influence the efficacy activities. For example, higher cell viability of UPM 39B3 formulation did not significantly increase the efficacy of inhibiting the growth of FocR4, a fungal pathogen. In conclusion, bentonite- based formulations were the suitable formulations for both UTAR EPA2 and UPM 39B3 isolates. However, the formulation containing bentonite clay only (B) was more suitable as relatively higher cell viability and hydrocarbon degradation and pathogen growth inhibition efficacies were achieved.

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APPROVAL SHEET

This project report entitled "**THE EFFECT OF FORMULATIVE MATERIALS ON THE VIABILITY AND EFFICACY OF FORMULATED BENEFICIAL MICROBES**" was prepared by FANG MEI TENG and submitted as partial fulfilment of the requirements for the degree of Master of Science (MSc) at Universiti Tunku Abdul Rahman.

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SUBMISSION OF FINAL YEAR PROJECT /DISSERTATION/THESIS

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I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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LIST OF ABBREVIATIONS

amu	Atomic Mass Units
ANOVA	Analysis of Variance
AOC	Appellation of Origin Controlled
API	Analytical Profile Index
B	Bentonite
Bt	*Bacillus thuringiensis*
BTEX	Benzene, Toluene, Ethylbenzene and Xylene
CEC	Cation Exchange Capacity
CFU	Colony Forming Units
DNA	Deoxyribonucleic Acid
FAME	Fatty Acid Methyl Ester
FC	Free-cell
FDA	Food and Drug Administration
FID	Flame Ionization Detector
FocR4	*Fusarium oxysporum* f.sp. *cubense* race 4
GC	Gas Chromatography
GC-FID	Gas Chromatography-Flame Ionization Detector
GC-MS	Gas Chromatography-Mass Spectroscopy
HPLC	High Performance Liquid Chromatography
IR	Infrared
K	Kaolin
LB	Luria-Bertani
LBA	Luria-Bertani Agar

LC	Liquid Chromatography
N/NFSM	Non-fat Skim Milk
NCBI	National Center for Biotechnology Information
OTA	Office of Technology
P/PABA	*p*- aminobenzoic Acid
PAH	Polycyclic Aromatic Hydrocarbons
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PVP	Polyvinyl Pyrrolidone
rDNA	Ribosomal Deoxyribonucleic acid
rRNA	Ribosomal Ribonucleic Acid
S	Sucrose
SAS	Statistical Analysis System
TIC	Total Ion Current
TSA	Trytic Soy Agar
U.S.	United States of America
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

Formulation is an important strategy which involves processing of active ingredient with various formulative materials to create an effective product (Hutchins & Boucher, 2006). Active ingredient in the formulation can be bacteria, fungi, virus, nematodes, antibiotics and other elements. The significance of formulation in improving the active ingredient activity has been shown in many studies. Formulation has been widely used in many areas, such as agricultural (Meyer, 2003), environmental bioremediation (Gordon, 1994; Higa & Parr, 1994; Raupach & Kloepper, 1998), pharmaceutical (Akers & DeFelippis, 2000) and industrial sectors (Tanaka, Tosa, & Kobayashi, 1993). Formulation plays a key role in addressing four main objectives which ultimately aim for better product achievements. The objectives are to stabilize microbial agents during distribution and storage (Hutchins & Boucher, 2006), aid in handling and application of the product (Brar, Verma, Tyagi, & Valero, 2006; Hutchins & Boucher, 2006), enhance activity of active ingredients in field, and to protect the active agent from adverse environmental conditions (Brar et al., 2006).

There are many types of formulative materials used in the formulation exercise. Although the component and composition varies in different applications and studies, the aim is to generally establish the active ingredient with a suitable carrier materials and additives to produce a desired end-

product. Selection of formulative materials with their potential functions depends on the types of the formulation desired, target of application, environment, mode of action, method of application, the product-environment interactions and cost effectiveness (Brar et al., 2006). In other words, there is no universal formulative material that can improve the performance for all active ingredients against all targets, or under all environmental conditions. Therefore, the type of formulation and formulative material selected and the relative amounts used must be tailored to the specific conditions of each application (Tu & Randall, 2005).

Microbes used for various field applications benefit greatly from the formulation developed. In formulated form, microbial cell viability is sustained during storage, transportation and when applied to the field, formulation provides protection to the active ingredient from harmful environmental factors such as from UV irradiation from the sunlight which could cause damage to microbial DNA (Davies-Colley, Donnison, & Speed, 1997; Coohill & Sagripanti, 2008). Consequently, formulated microbial cells result in a more consistent level of cell viability and better efficacy in applications. This is highly favoured as application in free-cell forms resulted in inconsistent results as microbes are not likely to be active and effective in all kinds of environments and ecosystems (Raupach & Kloepper, 1998).

In this study, carrier materials, enrichment materials and protectant additives were used to formulate beneficial isolates. The beneficial isolates used in this study have biological control and hydrocarbon bioremediation potential, and

are targeted for field application. Thus, formulation of these isolates might allow easy handling, protect microbial cells against the adverse environmental conditions, and sustain cell viability during storage and post-application.

This study attempts to investigate the effect of various formulative materials on the viability and efficacy of two beneficial bacteria isolates with the aim of selecting the most compatible formulation. Several formulative materials, bentonite and kaolin clays; non-fat skim milk and sucrose; and *p*-amino benzoic acid, were tested for their role as carrier material, enrichment material and protectant additive, respectively. The viability and efficacy of the formulated bacterial isolates on their application as hydrocarbon biodegradation and fungus inhibitor were compared to the free-cell form.

Thus, the objectives of this study are

i) to establish formulation processes using compatible carrier, additives and enrichment materials,

ii) to establish the influence of various formulative materials on microbial cell viability,

iii) to establish the influence of various formulative materials on microbial cell efficacy, and

iv) to identify the suitable formulation that provides good cell viability and efficacy on their applications.

CHAPTER 2

LITERATURE REVIEW

2.1 Microbial Formulation

Microbial formulation involves the incorporation of microbe(s) with necessary materials to achieve desired effects. Microbial formulation is widely used in many applications where the active ingredient in the formulation is the desired microbes. Microbial formulation is getting more common and popular for environmental applications especially agricultural applications and hydrocarbon bioremediations as compared with chemical treatments (Paau, 2004). Through the addition of formulative materials, the formulated products could have a longer shelf-life and better field performance while having improved handling and application. These are the main aims and the principles of developing suitable microbial formulations (Filho, Alves, August, Pereira, & Alves, 2001).

An effective formulation has also been shown to ensure better microbial viability during storage under ambient conditions with minimum loss of desirable characteristics (Couch & Ignoffo, 1981; Filho et al., 2001). Formulations should also have the lowest risk to the applicators, non-target organisms, crops or target areas and the environment (Tu & Randall, 2005). In fact, it is a challenge to develop a formulation with high viable cell recovery, longer shelf life, better cost effectiveness and a more user- and environmental-friendly product.

The common active ingredient for microbial formulation is the live microorganisms. The viability of these microorganisms will have to be maintained at acceptable level during the formulation process and storage period of these products. Upon application, the microorganisms must be activated from their "dormant" state readily to be applicable in the field (Johnson, Pearson, & Jackson, 2001). Live microorganisms without formulation have shorter shelf-life and loss their field activity, especially when they are exposed to detrimental environmental conditions such as intense sunlight irradiation, fluctuating temperature, and uncertain pH conditions (Gnanamanickam, Vasudevan, Reddy, Kloepper, & Defago, 2002; Warrior, Konduru, & Vasudevan, 2002).

Natural sunlight (UV radiation) especially UV-B (280-310 nm) and UV-A (320-400 nm) is the main factor for inactivation of many microbes such as *Bacillus thuringiensis* (Bt) which is a common biocontrol agent (Pusztai, Fast, Gringorten, Kaplan, Lessard, & Carey, 1991). In addition, most microbes need an acceptable range of temperature and pH for their growth and metabolisms. Thus, for microbial formulation used for environmental applications, the incorporation of formulative materials that are able to buffer the pH of the environment, provide physical barrier and protection to reduce the impact of extreme temperature and UV radiation on the microbes is essential for better microbial activity.

In the market, there are many formulated beneficial microbes products available where the microbes and composition of the formulation are

depending on the application, final product forms and specific market needs (CK Life Sciences group, 2008). For examples, the NutriSmart® and NutriWiz® are the products that consist of unique microbial formulations for agricultural applications to enhance plant resistance to disease, plant growth and nutrient availability (CK Life Sciences group, 2008) while EPIZYM-HC (Epicore BioNetworks) is a bioremediation product used for degradation of contaminating hydrocarbons by converting them into carbon dioxide (Epicore BioNetworks Inc., 2010). The ultimate goal of establishing a microbial formulation is to formulate microbial cells for better product shelf-life and field performance.

2.1.1 Common Beneficial Microbes Used in Various Applications

The identification of suitable bacteria available is important before developing a formulation. Of many beneficial microbes available, *Pseudomona*s and *Serratia* species are some of the versatile beneficial microbe strains exploited for environmental applications. For example, *Pseudomonas aeruginosa* and *Serratia marsescens* are environmentally important ubiquitous species known to degrade hydrocarbons (Deziel, Paquette, Villemur, Lepine, & Bisaillon, 1996; Sattler, 1997). These petrophiles are useful in cleaning up contaminated sites because they are able to degrade hydrocarbon compounds by using enzymes produced in their metabolism (Alexander, 1999). Soil beneficial bacteria, *Pseudomonas*, *Serratia*, *Rhizobium* and *Bacillus* species are commonly formulated for enhancing the plant growth and suppressing plant diseases (Rawat, Izhari, & Khan, 2011). For example, endophytic *Serratia* species such as *S. plymuthica* and *S. marcescens*, are beneficial to their host

(plant) by promoting plant growth or acting as the biocontrol agent for mainly fungal diseases (Ryan, Germaine, Franks, Ryan, & Dowling, 2008). Besides, oligosaccharide- elicitors which are produced by the degradation of the fungal cell wall with chitinolitic enzymes excreted by *Serratia marcescens* have been reported to play an important role in signal transduction essential in triggering various plant defence mechanisms (Lotan & Fluha, 1990). Commonly, endophytic microbes with these capabilities are identified and used as biocontrol agents. For these microbial inoculations to be effective, it must be compatible with the environment conditions. This means that the microorganisms must be formulated with formulative materials that aid in delivery the microorganisms to the targeted environment sites and protect the inoculants from the abiotic and biotic stresses (Gentry, Rensing, & Pepper, 2004; Paau, 2004).

For pharmaceutical and food industries, beneficial microbes (probiotics) or their useful metabolites which are useful for human health are also gaining much popularity and credibility. Encapsulation of beneficial microbes such as probiotics has been considered as potentially inexpensive and a safe approach to be used as functional food, supplements, or pharmaceutical products (Manojlovic, Nedovic, Kailasapathy, & Zuidam, 2010). In addition, granulation using food grade materials is commonly employed by pharmaceutical industry for tablet making. Tablets containing beneficial microbe or its beneficial metabolites are convenient and suitable for oral consumption (Tousey, 2002). Common beneficial microbial strains, *Lactobacillus* species and *Bifiobacteria* species, are usually incorporated in the

functional foods such as yogurt or processed into pharmaceutical products to improve human intestinal health by modulating intestine microbial composition (Stanton et al., 2001).

2.1.2 Microbial Formulation for Agricultural Applications

For agricultural purposes, microbes are formulated to provide better soil quality, promote plant defence system and improve plant growth. The soil supporting agriculture activities may lack of sufficient microbial activity to promote healthy plant growth. The cause of insufficient microbial activity in the soil and poor soil quality is primarily due to heavy agricultural reliance on pesticides to control disease (Pimentel, Hepperly, Hanson, Douds, & Seidel, 2005). The synthetic chemicals of pesticides might have destroyed the essential bacteria and fungi found in healthy soil. When microbial population is depleted, the plant growth system becomes stressed and gives rise to many problems (Pimentel et al., 2005).

The beneficial microbes can affect plant growth through improving nitrogen fixation, acting as biocontrol of soil-borne diseases, enhancing mineral uptake, and weathering of soil minerals (Bashan, 1986). Thus, many formulated beneficial microorganisms are invented and created to promote healthy and vigorous plant growth (Neyra, Arunakumari, & Olubayi, 1997). In addition, certain bacteria also exhibit activity against plant-parasitic nematodes and have potential to be used as microbial pest management agents (Meyer, 2003).

Effective biological control of pests or diseases relies on the successful establishment and maintenance of a threshold population of suppressive organisms on the planting materials and the soil. The efficacy is impaired or insufficient if the population of suppressive organisms is below the threshold (Warrior et al, 2002). Varying degrees of efficacy have been achieved in the laboratory or greenhouse with different preparations and varying levels of performance using microbes as biocontrol agents. The application of many biocontrol agents at the glasshouse and field stage is often hampered by the susceptibility of the viable cells to the unfavourable environmental conditions. Many formulations did not show good efficacy in the field because many environmental factors were not taken into account during the development of formulation.

Researchers had reported that the biopesticide formulation containing *Bacillus thuringiensis* was not effective when applied to the field as it was washed-off by rainfall and the activity was degraded by sunlight (Tamez-Guerra, McGuire, Medrano-Roldan, Galan-Wong, Shasha, & Vega, 1996). As a result, many biocontrol agents only showed impressive control efficacy in suppressing the growth of the pathogen at the laboratory stage, or at best, the glasshouse stage (Elliot & Lynch, 1995). Inappropriate formulation caused many potentially useful bacteria reported in the scientific literature not well accepted in the commercial market. Among the potential biological pathogen control agents, only a few have resulted in providing commercially acceptable control of pathogens or diseases (Warrior et al, 2002).

This variability in performance is attributed to factors such as stability or poor viability, sensitivity to UV light, desiccation, and fluctuating environmental conditions (Warrior et al, 2002). Therefore, to ensure that the biocontrol agents remain viable when they are introduced into the soil, they must be protected from these unfavourable conditions which may diminish their viability and subsequently affect their efficacy towards the pathogens. These effects could be minimised or overcome by the addition of formulative materials and by preparing the final product in a form that is specific to the pathogen/disease-crop (Warrior et al, 2002).

2.1.3 Microbial Formulation for Hydrocarbon Bioremediation

In hydrocarbon bioremediation, microbes that have the ability to degrade hydrocarbon are formulated with carrier materials to increase their degradation activity when applied to target site. Environmental variables can greatly influence the rate and extent of biodegradation. However, lack of sufficient knowledge about the effect of various environmental factors on the rate and extent of biodegradation by different microbial strains is another source of uncertainty (U.S. Congress, Office of Technology [OTA], 1991). Thus, formulation is generally employed to protect the cells from environmental stresses and maintain the cell viability to an acceptable level which is adequate to initiate and regulate the degradation process. Without formulation, microbial population may be too low to sufficiently degrade the hydrocarbon effectively (Gentry et al., 2004). Researchers have found that formulations with nitrogen- and phosphorus-rich compounds (Zaidi & Imam, 1999), clays (Gentry et al., 2004; Schmitz, Goebel, Wagner, Vomberg, & Klinner, 2000)

and polyvinyl alcohol (Cunningham, Ivshina, Lozinsky, Kuyukina, & Philp, 2004) increased the hydrocarbon biodegradation. With high inoculums level, the time required for complete degradation might not necessary be shorter because the rates of biodegradation depend on many factors and variables such as electron acceptor availability, chemical nature of hydrocarbons, oxygen, nutrients, pressure, salinity, temperature and pH (U.S. Congress, OTA, 1991).

The formulation of hydrocarbon-degrading microbes is most commonly performed using fine porous solid materials such as straw, clays and sawdust, as they act as absorbents to help in agglomerating oil films and form oil/water emulsions (Gentry et al., 2004; Rowsell, 1993). Formulation with high absorbing carrier materials is desirable because it aids in the physical gathering of the spilled oil. Some of these adsorbents such as clay also serve as nutrients for the microorganisms (Brar et al., 2006). Rong, Huang and Chen (2007) also reported that the clay minerals and iron oxide found in the clay stimulated the exponential growth of *B. thuringiensis*. Therefore, with formulation, the ability of hydrocarbon-degrading microbes to degrade oil in the agglomerated form is enhanced when compared to non-formulated microbes (Rowsell, 1993).

Clearly, agglomeration of hydrocarbon and the hydrocarbon degrading microbes is crucial. The ability can be enhanced using formulation developed from various types of clays such as attapulgite, bentonite, kaolin, montmorillonite and carbonaceous asphaltenic. These clays are amphiphilic in nature due to the presence of polar oxygenated products on their surface

(Ramesh, 2004). The enhanced amphiphilic nature of the clay particles increases the stability of the oil and water emulsion (Tu & Randall, 2005). It then increases the bioavailability of hydrocarbon to the hydrocarbon degrading microbes (Ramesh, 2004). Formulation is therefore important to stabilise the hydrocarbon and water interface especially for aqueous or polar environments such as marine to allow degradation by formulated microbes (Ramesh, 2004).

The impacts and effects as a result of the oil/hydrocarbon spill are tremendous to the ecosystem, environment and economy. Certain indigenous bacteria present naturally in the environment have been found to be able to degrade the oil (Chianelli et al., 1991; Prichard, 1991). However, these bacteria might not be sufficient enough to effectively degrade the oil due to abiotic stresses from the environment such as fluctuating or extreme temperature, water content, pH and nutrient availability along with presence of potentially toxics (Gentry et al., 2004). The small number of the indigenous microbial population is not sufficient to elicit a rapid response to degrade oil spill. Thus, it is necessary to formulate microbes with the oil degradation ability to improve their viability and efficacy. This is vital to overcome the environmental stresses that might reduce microbial viability and efficacy for degradation of oil.

2.2 Microbial Formulation Types and Formulation Techniques

Different formulation techniques are available to produce the desired type of formulation. There are mainly four types of formulations according to their physical structures, namely dry solids (dusts, granules, powders and briquettes), liquids (emulsions, and oil- or water-based suspensions), capsules

(Applicator Core, 2006; Rhodes, 1993) and gases (Applicator Core, 2006). For microbial cells, suitable formulations include dry solid formulations or liquid formulations (Brar et al., 2006).

Dry solid formulations are preferred over liquid formulations because they provide extended shelf-life, can be applied with simple equipments and are easier to transport and store (Lumsden, Lewis, & Fravel, 1995). Moreover, most dry formulations especially granular or powder formulation can also be made into liquid- or water based suspensions which will increase the flexibility of dry formulation for various applications (Lumsden et al., 1995). Among dry formulations, granules are preferred for various applications. Granules are easy to transport, store, apply, less drift and have low risks of operator safety if compared with dusts and powders which may pose detrimental effects on the operator's inhalation system (Brar et al., 2006).

Formulation technique or technology combines colloid and surface chemistry through chemical process engineering (Mollet & Grubenmann, 2001). Some techniques require a simple mixing operation while some require an entire series of complicated engineering procedures such as comminution, dispersion, emulsification, agglomeration or drying (Mollet & Grubenmann, 2001). Generally, granulation and encapsulation are the two most commonly employed formulation techniques (Summers & Aulton, 2002; Brar et al., 2006). Granulation and encapsulation would enhance the microbial activity as shown in the study where the survival of probiotic *Lactobacillus plantarum*

was enhanced by granulation and encapsulation techniques (Woraharn, Chaiyasut, Sirithunyalug, & Sirithunyalug, 2010).

2.2.1 Granulation

Granulation is the process of collecting particles together by creating bonds between them (Tousey, 2002). Bonds are formed by compression or by using a binding agent (Food and Drug Administration, [FDA], 1999; Tousey, 2002). It involves the incorporation of necessary and suitable formulative materials to achieve the final desired solid product. There are three types of granules. Exterior granules with a sticker and granules without a sticker are the types of granules where the microbes are attached to the outer surface of a carrier by a sticker or without a sticker. For incorporated granules, all constituents are mixed into pasta to form matrix and later sieved to achieve desired size (Mayer, Wassmer, & Doerr, 2000).

Granulation also reduces the hazard associated with the generation of toxic dust or powder that may arise when handling dust and powder formulations (Summers & Aulton, 2002). Dusts and powders are fine particles that can pose serious inhalation hazard for the applicator and drift hazard for the sprayer (Brar et al., 2006). Granules, on the other hand, have a discrete mass of around 5-10 mm^3 (Green, 2000). Granules, being denser than the parent powder mix, occupy less volume per unit weight. They are, therefore, more convenient for storage or shipment (Summers & Aulton, 2002).

Traditional wet granulation, which is still commonly used, is the process of mixing the powder mixture and solution, suspension or slurry which contain a binder followed by drying (Tousey, 2002; Kirchholtes & Sas, 2005). Wet granulation which involves harsh spray drying steps will cause the loss of active ingredient. Thus, the water activity and temperature used for drying need to be optimised to prevent dramatic loss of the active ingredient (Brar et al., 2006). On the other hand, dry granulation converts primary powder particles into granules without using liquid solution and drying process because some active ingredients or formulative materials to be granulated may be sensitive to moisture and heat (Summers & Aulton, 2002). However, more processes are needed in dry granulation because forming granules without moisture requires compacting and densifying the powders (Tousey, 2004).

Granulation has been used in developing granulation products with beneficial microbes, molasses from sugar cane, grape or sugar beets and other plant growth promoting compounds such as gibberellins, cytokinins and indole acetic acid (Lin, Zhou, & Chu, 2008). The potential of microbial formulation using granulation was also reported in the study of using *Bacillus thuringiensis israelensis* against floodwater mosquitoes in the field through various types of granular formulations (Mulla, Federici, Darwazeh, & Ede, 1982; Rydzanicz, DeChant, & Becker, 2010). The study showed that the granulated bacterial formulations improved the efficacy of *Bacillus thuringiensis israelensis* in the field.

2.2.2 Encapsulation

Encapsulation involves the incorporation of various ingredients within a capsule or formation of the wall/layer around the active ingredients. The active ingredient is usually encapsulated in a capsule made of sugars, gums, proteins, natural and modified polysaccharides, lipids and synthetic polymers (Lee, 1996). These encapsulation techniques include spray drying, spray chilling, extrusion, fluidized bed coating, liposome entrapment, coacervation, inclusion complexation and rotational or centrifugal suspension separation (Desai & Park, 2005). The encapsulation technique has been expansively exploited to produce smaller size and high efficient microcapsule microbial formulations (Winder, Wheeler, Conder, Otvos, Nevill, & Duan, 2003).

Encapsulation is employed as a mean to protect the valuable content from being destroyed by the environment while allowing small entities to pass in and out of the membranes. Encapsulations increase the residual stability due to the slow release of formulation and protect the active ingredient from extreme environmental conditions (Brar et al., 2006). The microbial suspension encapsulated in a capsule can control the release of the active materials and minimize deterioration for long-term storage (Tamez-Guerra, McGuire, Behle, Shasha, & Wong, 2000; Desai & Park, 2005). Besides, the capsules aid in delivery of the active ingredients to the target sites (Shasha & McGuire, 1992).

A study on the effect of encapsulation showed that lactic acid bacteria encapsulated in calcium alginate beads had a good diffusion properties and the capsules offered space for the cell growth (Ivanova, Teunou, & Poncelet,

2006). Another study showed that the encapsulated bacterial consortium within the gellan gum microbeads showed improved biodegradation activity in the removal of gasoline as compared to free-cells (Moslemy, Guiot, & Neufeld, 2002). In contrast, Sabaratnam and Traquair (2001) reported that *Streptomyces* in alginate-kaolin capsules used to suppress *Rhizoctonia* damping-off in tomato transplants had least cell viability if compared with granular and powder formulations after 24 weeks of storage.

2.3 Formulative Materials

Formulative materials are compounds which can be added to a formulation to enhance the effectiveness of the active ingredient (Tu & Randall, 2005). There are many types of formulative materials used in microbial formulation, such as enrichment materials (or nutritional amendment), ultraviolet (UV) protectants, inert carrier materials, dispersants, surfactants and binders (Brar et al., 2006). Some of these materials may have multiple roles in a formulation. Clays, for example, are not only served as a carrier material but also provide protection from UV, temperature, desiccation (Brar et al., 2006) and as an enrichment medium (Vettori, Gallori, & Stotzky, 2000; Tu & Randall, 2005).

The selection of appropriate formulative material and formulation type is important as they have different impacts on the active ingredient. The selection of suitable formulative materials and desired formulation type is dependent on the nature of the active cells and factors related to the site of application such as application to aquatic or terrestrial landscapes, temperature, climate and water chemistry (Sabaratnam & Traquair, 2001; Tu

& Randall, 2005). The compatibility and beneficial interaction between the active ingredient and formulative materials play an important role in maintaining the microbial cells in metabolically and physiologically competent state in order to obtain the desired benefit when applied (Xavier, Holloway, & Leggett, 2004).

Careful selection of suitable materials in the formulation is essential as certain formulative materials used in formulation may have adverse impacts on human health and environment. For example, xylene was initially used as the preservative agent for *Bacillus thuringiensis* (Bt) formulations (Fortin, Lapointe, & Charpentier, 1986). However, xylene is a health hazard material which exhibits a wide range of toxicity and neurological effects toward human and animals. Subsequently, sorbitol is employed in the recent development of formulation for safer Bt formulation (Brar et al., 2006; Linda, 2008). Biodegradable, non-toxic, non-irritating and inert formulative materials are highly preferred as to produce a more eco-friendly and safer formulation, especially when the formulations are meant for environmental applications.

2.3.1 Carrier Materials

Carriers are used in dry solid formulation of dusts, wettable powders, granules and water-dispersible granules. A carrier is the major portion of a formulation. Carrier materials or absorbent carriers are inert materials with high absorptive capacities and are normally added into formulations to give strength and shape to the active ingredient (Knowles, 1998). A good carrier should have the capacity to facilitate delivery of the necessary concentration of viable cells in

the most optimal physiological state (Bashan, 1986). Absorbent carriers can be mineral or vegetable.

There are four main classes of carrier which are silicate clays, carbonates, synthetics and botanicals (Knowles, 1998). Attapulgite, montmorillonite, kaolin, mica, talc and vermiculite are some of the examples of silicate carriers used for granular formulations. Among silicate clays, montmorillonite (bentonite) and kaolin are widely used in the manufacture industry and suitable for various applications (Adamis, Williams, & Fodor, 2005). On the other hand, carbonate carriers such as calcite and dolomite containing minerals such as calcium and magnesium which are important nutrients for plants. They have been widely used in agricultural applications and are also used as the carriers for herbicides, insecticides, and fungicides (Yechiel & Myers, 2010). The synthetic carriers such as calcium silicate, precipitated silica and fumed silica are frequently used in numerous applications in many different industries. They are commonly used as a carrier for liquid and solid formulations and act as a flow agent to reduce the caking of powders (Evonik Degussa GmbH, n.d.). In contrast with other carriers, agro-byproducts such as corn cob grits, ground grains, rice bulls, soybean, walnut shell and coconut shell are common botanical carriers (Knowles, 1998).

The roles of a carrier material in a formulation are not only providing strength but also to improve the stability during storage, handling and delivery of the active ingredient (Applicator Core, 2006; Brar et al., 2006). Selection of a suitable carrier material and its concentration present in a formulation are

important for an effective active ingredient formulation. One of the main roles of the carrier is to spread the active ingredient over a large surface area of particles so that the formulated active ingredient can be applied uniformly in the field (Knowles, 1998). Thus, a carrier material with high adsorptive capacity is desired. Many active ingredients are chemically unstable during long-term storage at elevated temperatures. This is due to the natural compounds that present in the carrier which has the active acidic, basic or catalytic sites (Knowles, 1998; Brar et al., 2006). Thus, the compatibility of the active ingredient with the carrier material is another important factor to be considered in selecting a suitable carrier material. A good combination between the active ingredient and carrier material can enhance the shelf-life and stability of the product.

Clay is a widely distributed and abundant mineral resource that has major industrial importance for enormous variety of uses (Ampian, 1985). Clays have high adsorption power, good colloidal properties and high amenability to modification that make them the most suitable material for designing solid formulations (Lagaly, 2001). Clay minerals have various sites that have negative charges from isomorphous substitution (Salah, 2010). Ion-exchange capabilities of clay minerals could possibly influence microbial metabolism through the preferential trapping and release of cations (McCollum & Galyean, 1983).

Cation exchange capacity (CEC) is the amount of exchangeable cations per unit weight of dry soil (Donahue, 1983) and exchange kinetics or rates are largely dependent on the type of clay minerals (Sparks, 2003). Cation exchange is an important factor in soil fertility, correcting soil acidity and basicity, altering physical properties of soil and purification of water (Gowariker, Krishnamurthy, Gowariker, Dhanorkar, Paranjape, & Borlaug, 2009). The CEC of the soil also influences the availability of nutrients in the soil for microbial use, as charged nutrient particles also will be held or removed through the soil based on its charge (Sylvia, Fuhrmann, Hartel, & Zuberer, 2005). Similarly, due to the presence of charged organic molecules in microorganisms, the CEC of the carriers influence the ability of microbes to uphold their presence in the formulation or soil. The nutrient cations from formulative material are adsorbed at the negative charged sites of the carrier. These adsorbed cations resist removal by water leaching, but can be exchanged by other cations through mass action (Salah, 2010). Leaching of nutrient cations from formulation has negative impacts on microbes which subsequently reduce the formulation's field efficiency (Nennemann et al., 2001).

In addition, clay with high CEC and absorptive capacities is suitable and useful as a carrier especially for formulations used for soil applications such as fertilizer. Nutrient cations or microbes in the formulation used for plant growth promotion will not leach but adsorbed on the cation exchange sites when other ionic water pollutants were introduced in the soil (Gowariker et al.,

2009). Thus, nutrient cations or microbes in the formulation applied to the plant are able to permeate far into the soil before being leached out.

Among the clay minerals used, the montmorillonites and kaolinite are recognised for the high cation exchange capacity and rapid exchange rate, respectively (Grim, 1968). Small amounts of bentonite and kaolin are also used as a catalyst in the refining of petroleum (Adamis et al., 2005). In addition, among many types of clays, bentonite and kaolin have been widely used in many formulation studies especially for environmental applications. Bentonite and kaolin are eco-friendly and have low toxicity toward aquatic organism (Adamis et al., 2005).

2.3.1.1 Bentonite

Bentonite is a highly colloidal and plastic clay that is hydrated from aluminum silicate. Montmorillonite, a clay mineral of the smectite group is the main component of bentonite (Adamis et al., 2005). Bentonite is special as it has the ability to form thixotrophic gels with water if large quantities of water is absorbed and bound on the surface and between the clay layers (Adamis et al., 2005). This provides a protection layer to the entrapped molecules between the clay layers. Due to the high adsorptive property and large surface area, bentonite is widely used in industry and domestic uses such as serving as a filler, stabiliser, or extender in adhesives, paints, cosmetics, and medicines. Bentonite is also used as a carrier in pesticides and fertilisers and as an absorbent for grease, oil and animal wastes (Hosterman & Patterson, 1992;

Hanchar, Stroes-Gascoyne, & Browning, 2004). In addition, it can be used to clarify wine and vegetable oil and to purify wastewater (Patterson & Murray, 1983; Kuzvart, 1984; Hosterman & Patterson, 1992).

Bentonite also has high cation exchange capacity (Adamis et al., 2005). This cation exchange capacity allows the minerals in the clay to bind with inorganic cations, organic cations, bio-organic particles and proteins (Potter & Stollerman, 1961; Weber, Perry, & Upchurch, 1965; Lipson & Stotzky, 1983). Besides, bentonite also plays a role in protecting the microbial cells by shielding them from the spilled toxic materials or by buffering the toxic materials into less toxic compounds (Masato, Kenichi, & Kazue, 2000). The addition of clay with high buffering capability such as bentonite clay into microbial formulation could improve microbial cell viability by providing them a suitable environment for growth. Stotzky (1986) and Burns (1989) both reported that the addition of the clay particles could improve microbial activity due to their buffering ability to maintain the pH within the suitable values.

2.3.1.2 Kaolin

Kaolin or china clay is formed by rock weathering. The main component of kaolin is kaolinite, which is made up of tiny sheets of triclinic crystals with pseudohexagonal morphology (Adamis et al, 2005). The cation exchange capacity of kaolinite is considerably less than that of montmorillonite (bentonite). The kinetic of cations exchange for both kaolinite and montmorillonite is rapid if compared to other clay mineral such as vermiculite (Malcom & Kennedy, 1969). The rate of the exchange reaction is slightly

higher for kaolinite, almost instantaneous, if compared to montmorillonite (Grim, 1968).

For kaolin clays, the tetrahedral sheets of adjacent clay sheets are held tightly by hydrogen bonds, thus, only planar external surface (edge) sites are available for ionic exchange. With montmorillonites (bentonite), the inner peripheral space is not held together by hydrogen bonds, but instead it is able to swell with adequate hydration and thus allow for rapid passage of ions into the interlayer (Sparks & Carski, 1985). Thus, kaolinite has higher reaction rate because it has only external exchange sites if compared with montmorillonites which contains both external and internal exchanges sites (Sparks, 2003). This makes kaolinite less preferable to montmorillonites in formulation because the absorbed molecules such as proteins (Wallace, Headley, & Weber, 1975), bacteria, and viruses (Steel & Anderson, 1972; Schiffenbauer & Stotzky, 1982; Lipson & Stotzky, 1983) can be easily removed from the kaolin particles because the adsorption is not strong and limited to the surface of the particles (external exchange sites) (Weber et al., 1965; Adamis et al., 2005).

Nevertheless, kaolin is able to enhance the performance of some microbial products and has been used successfully as an inert carrier for some biopesticides (Rasad & Rangeshwaran 2000). Kaolin clay has been used in many formulation studies. For example, better stability and shelf life were obtained when kaolin and wheat flour are used to formulate the mycoherbicide agent, *Colletotrichum truncatum* (Connick, Jackson, Williams, & Boyette, 1997). Besides, a study showed that the addition of kaolin particles (300

mg/L) into *Pseudomonas fluorescens* suspension stimulated the bacterial activity in all pH values tested (pH 5, 7 and 9). This suggested that kaolin could reduce the effect of pH changes towards the active ingredient, *Pseudomonas fluorescens* (Pereira, Vieira, & Melo, 2000).

2.3.2 Enrichment Materials

Enrichment materials or nutritional amendments are nutrient-rich ingredients or compounds which permit retention of microbial cell viability. There are various nutritional amendments used in formulations, all with the general aim of providing necessary nutrients. The active ingredient such as bacteria is usually provided with carbon source to ensure higher viable cell recovery after a long period of storage (Lee & Chang, 1993). The carbon source used plays an important role in supporting growth and in the production of bioactive compounds. Small molecules like sugars, organic acids and alcohols and complex molecules such as proteins, polysaccharides and lipids can be used as the carbon source (Barnett, 1981). Skim milk contains only disaccharide lactose which consists of one molecule of glucose bound to one molecule of galactose (Eddleman, 1998). Sucrose is a disaccharide composed of glucose and fructose joined by an α (1,2) glycosidic linkage. Since most bacteria do not digest fats or do so very slowly, skim milk and sugar are highly suitable (Eddleman, 1998).

In many formulation exercises, non-fat skim milk and sucrose are employed not only because of they can be used as nutrients and carbon sources for bacterial cells, but also for other additional benefits (Lee & Chang, 1993;

Abadias, Benabarre, Teixido, Usall, & Vinas, 2001). Non-fat skim milk and sucrose also serve as rehydrating agents and osmoprotectants. They prevent severe desiccation of cells upon sunlight exposure and protect cells from osmotic stress (Costa, Usall, Teixido, Garcia, & Vinas, 2000; Gouffi & Blanco, 2000). Enhanced survival of *Rhizobium* spp. (Bushby & Marshall, 1977; Mugnier & Jung, 1985) and yeast (Abadias et al., 2001) have been observed when appropriate protective media containing combinations of skim milk and other protectants such as 5% or 10% lactose or glucose, and 10% fructose or sucrose were used (Abadias et al., 2001).

2.3.3 UV Protectants

Natural sunlight has ultraviolet (UV) radiation, and the radiation causes lethal or deleterious effect towards active ingredients especially bacteria (Green, 2000). Ultraviolet radiation inhibits bacterial cell proliferation by damaging bacterial cell DNA. The DNA damage is ascribed to both direct interaction of the radiation energy with DNA and indirect effect result from interaction of DNA with radicals formed by radiation (Ward, 1988). The massive DNA damage could lead to bacterial cell death.

In addition, certain useful metabolites produced by beneficial microbes are also susceptible to UV radiation causing rapid photolysis. Subsequently, this could cause the reduction of the microbial formulation effectiveness when applied in the field (Bull et al., 1984; Iwata, MacConnell, Flor, Putter, & Dinoff, 1985). For example, avermectins have short-half life when they are exposed to the sunlight which causes a rapid loss of avermectins residues for

the control of target species. Avermectins are a series of 16-membered macrocyclic lactone derivatives with potential anthelmintic and insectidical properties (Omura & Shiomi, 2007). Thus, exposing avermectins to the sunlight could limit their utility in crop protection (Peterson, Alvares, & Mookerjee, 1996). The presence of a UV radiation screen to protect the active ingredients (bacteria or metabolite) from UV radiation of the natural sunlight is important. It is to ensure that the active ingredients remain active and viable for expression in the field.

Several formulative materials that protect metabolites or microbes from the deleterious effects of sunlight are available, and they include Congo Red, folic acid, lignin, molasses, alkyl phenols, *p*-amino benzoic acid, and clay minerals such as montmorillonite (bentonite) and kaolinite (kaolin) (Yang, Pan, Chen, Kao, & Tsai, 1995; Behle, McGuire, Gillespie, & Shasha, 1997; Vettori et al., 2000). These UV radiation sunscreens are either absorbent or reflectant. They either absorb the UV radiation or form a protective layer to protect the active ingredients (Brar et al., 2006). A study showed that p-amino benzoic acid, a common UV protectant, scavenges reactive oxygen species and protects DNA against UV and free radical damage (Miao, Yang, Ling, & Sano, 1995). It is a precursor of folic acid biosynthesis for certain bacteria (Donnelly, 2001) and is used as a sunscreen because of its UV absorbing property especially against UV-B (Murphy & Hawk, 1986; Hu, Chen, Chen, & Sano, 1995).

2.4 Techniques for Cell Biomass Assessment

Many studies require the quantitative determination of microbial growth and viability. There are many available techniques and the two most widely used methods for determining the viability of bacteria are the plate count and spectrophotometric (turbidimetric) methods.

2.4.1 Viable Plate Count

Viable plate count method is used most frequently for assessing the viability of bacterial cells. A sample needs to be serial diluted in a solution that will not inhibit or enhance the microbial growth during analysis. Original sample without dilution have too many cells that would be impossible to count due to agar medium densely populated with colonies. After the cells have been diluted, they are spread on an agar medium or mixed with melted agar medium and incubated until colonies form (Madigan, Martinko, & Parker, 1997). Plate counting requires an incubation time before the results can be scored and colony forming unit (CFU) can be obtained.

The plate count technique works well for microbial cells that pinch off the cross wall in a short time after cell division (Coleman, 2010). Bacterial colonies may arise from chains or clusters of bacteria, resulting in under estimation of the true bacterial number (Pepper & Gerba, 2009). The temperature of incubation and medium conditions must also be optimized to achieve the largest colonies possible so that they are easily counted (Pepper & Gerba, 2009). Despite some limitations, plate count is the most common method used for viability study and is an indirect measurement of cell density,

revealing information related only to viable bacteria (Claus, 1989; Bunthof & Abee, 2002).

The equipments necessary for performing viable plate counts are readily available in any microbiology lab. They are cheap if compared with other methods (Wigg, Phillips, Wheatland, & Berry, 2003). Finally, by using a selective medium it is possible to determine the number of bacteria of a certain class, even in mixed populations (Claus, 1989). Thus, viable plate count is suitable to determine the cell viability using common media such as Luria-Bertani agar and nutrient agar at room temperature.

2.4.2 Spectrophotometric Methods

The traditional spectrophotometric method is based on turbidity and indirectly measures all bacteria (cell biomass), dead or alive (Reynolds & Farinha, 2005). Traditional spectrophotometric method is simple and non-destructive, but the sensitivity is limited to about 10^7 cells per ml for most bacteria and does not provide information related only to live cells (Todar, 2011). These disadvantages made this method not suitable for the cell viability study. However, advance and specific spectrophotometric instruments toward certain microbial strain are used for quantification of viable bacteria in research especially for slowly growing microorganisms such as *Mycobacterium paratuberculosis* (Shin, Han, Manning, & Collins, 2007). In addition, bacterial viability kit available in the market such as LIVE/DEAD® *Bac*Light™ allows researchers to reliably distinguish and quantify live and dead bacteria by flow cytometry within minutes. It is also useful to quantify viable but uncultivable

microbes (Life Technologies Corporation, 2011). However, this approach is not commonly applied for estimating cell concentration due to the high cost involved (Wigg et al., 2003).

According to Beer's law, the absorbance at bacterial absorption range will directly reflect the bacterial concentration. However, deviations from Beer's law will occur when others particles that absorb the light at the bacterial absorption range present in the sample solution. The spectrophotometric methods are less suitable for formulated cells with different materials as the formulative material particles may increase the turbidity of the suspension and thus, affecting the absorbance value.

2.5 Techniques for Efficacy Assessments

2.5.1 Hydrocarbon Degradation Activity

Analytical methods for hydrocarbon pollution studies are of two basic types: spectroscopic and chromatographic methods. Spectroscopic methods include infrared (IR) absorption, ultraviolet (UV) absorption and UV-fluorescence spectrometry techniques. Each technique has its advantages and disadvantages.

For spectroscopic methods, such as UV-absorption and UV-fluorescence, they are more suitable to estimate hazardous hydrocarbons especially the aromatic petroleum hydrocarbons. UV spectrometries are selective and very sensitive to poly aromatic hydrocarbons (PAHs), but results may be influenced by the presence of many other compounds in higher concentrations (Pavlova &

Ivanova, 2003). IR spectrometry is a global quantification method of petroleum hydrocarbons. This method is well suited for rapid screening of petroleum pollution in widespread and extended environmental studies (Ge, Brown, & Alberts 1994; Kovacheva & Belcheva, 1994). It is simple and effective in producing immediate results. However, these spectroscopic methods require intense clean-up procedures and great care to avoid contamination (Morel et al., 1991). Spectroscopic methods also do not give direct information on the structure of compounds in the samples.

In recent years, capillary gas chromatography with flame ionization detector (GC-FID) has been widely used in hydrocarbon analyses. High Performance or high pressure liquid chromatography (HPLC) using fluorescence detection has been employed to screen petroleum-related aromatic compounds in sediments and sludge (Manoli & Samara, 1996). However, to further verify the results, gas chromatography-mass spectroscopy (GCMS) technique is often used to identify and provide structural information of the individual petroleum compounds present in the environmental samples (Volkman, Holdsworth, Neill, & Bavor, 1992).

Chromatography is physical chemical technique that separates the components in a mixture. This separation relies on the differences of molecules in partitioning behaviour or affinities relative to the interface between a flowing mobile phase (eluent) and a stationary phase (sorbent) (Moskovitz, n.d.). Different compounds have differential affinity toward the mobile and stationary phases. Thus, for a given condition and system, each component has

its unique retention time (Moskovitz, n.d.). GC and LC are some of the common separation techniques used for hydrocarbons detection where gas and liquid are used as the mobile phase, respectively. For example, HPLC was employed to analyse the residual polycyclic aromatic hydrocarbons in the biodegradation study conducted by Yuan, Wei and Chang (1999). GC was also commonly used by researchers in hydrocarbons detection and hydrocarbons biodegradation efficiency monitoring (Raghaven & Vivekanandan, 1999; Michaud, Giudice, Saitta, Domenico, & Bruni, 2004).

2.5.2 Inhibition of Fungal Pathogen

The type of quantification method used to assess the fungal growth inhibition after treatment depends largely on the experimental design. For example, if the sample is needed for further analysis, quantification method which is destructive is not preferred. Dry weight or biomass of fungal mycelium and recovery of viable fungal cells after treatment are always used as the parameter of growth inhibition (Aryantha & Lunggani, 2007).

Determination of mycelia dry weight is the traditional direct method of quantifying filamentous fungal growth (Granade, Hehmann, & Artis, 1984). The mycelium dry weight is obtained through measuring the dried and filtered mycelium suspension. In the study conducted by Ilondu (2011), the anti-fungal activities of the aqueous extracts of *Carica papaya*, *Chomolaena odorata* and *Acalypha ciliata* leaves on the growth of the pathogenic fungi was determined by measuring the mycelia dry weight. The filtered fungal mycelia were dried at 70 ˚C for 24 hours in a dessicator (Ilondu, 2011). Mycelial dry weight

measurement, although accurate, is tedious and time consuming (Granade et al., 1984). It is also less suitable for study involves granular microbial formulation. As solid particles and bacteria of the microbial formulation are hardly separated from the fungus, thus, they might affect the mycelial dry weight measurement.

Cultivation method is one of the simple and common methods used to determine the recovery of viable fungal cells after treatment (Reeslev, Miller, & Nielsen, 2003; Aryantha & Lunggani, 2007). For example, the difference in the number of colony forming units (CFU) before and after radiation experiments were used to determine the inactivating effect of microwave radiation on tested fungal or actinomycetal spores (Gorny et al., 2007). This method does not require extraction and filtration techniques and no expensive instrument is needed. However, incubation with suitable conditions is required before the CFU can be recorded. Cultivation method is suitable to determine the viable cells after treatment.

Fungal biomass can also be estimated by determining the ergosterol content in the fungal membrane (Gessner & Chauvet, 1993; Schnurer, 1993). Ergosterol is a suitable marker for assessing the abundance and estimating biomass of fungi in atmospheric aerosols (Lau, Lee, Chan, & Fang, 2006). However, determination of the ergosterol is rarely used in practice because it requires advanced equipments such as high performance or high pressure liquid chromatography (HPLC) and highly trained personnel (Reeslev et al., 2003).

In addition, ergosterol content depends on the physiological state and general growth conditions of the fungus (Gessner & Chauvet, 1993).

CHAPTER 3

MATERIALS AND METHODS

3.1 Sources of Bacterial Isolates and *Fusarium oxysporum* f.sp. *cubense* race 4 and Preparation

The bacterial isolates, UTAR EPA2 and UPM 39B3, were obtained in pure cultures. The isolate UTAR EPA2 has showed potential to degrade hydrocarbon-based substrate (Tan, 2007), while isolate UPM 39B3 has showed potential role as a biological control agent (Ting, Sariah, Jugah, Son & Gurmit, 2004). These cultures were established on Luria-Bertani agar (LBA) (Pronadisa) plates and incubated at room temperature (27 ± 2 ˚C) for 24 to 36 hours prior to use in subsequent studies. The fungal pathogen *Fusarium oxysporum* f.sp. *cubense* race 4 (FocR4) used in efficacy assessment of UPM 39B3, was obtained as filter paper culture from Prof. Dr. Sariah Meon from University Putra Malaysia. The culture was established on potato dextrose agar (PDA)(Merck) plates and incubated at room temperature (27 ± 2 ˚C) for 7 days prior to use in subsequent studies.

3.2 Establishment of Standard Growth Curve for Isolates

The established bacterial isolates (UTAR EPA2 and UPM 39B3) were used to construct a standard growth curve individually. The standard growth curves (Appendix A) were constructed to standardize the concentration of bacterial inoculum used for every formulation exercise. The optimum wavelength for maximum absorbance by each isolate, by using the wavelength between 400 nm and 600 nm, was determined prior to standard growth curve construction.

To construct the standard growth curve, a volume of 10 ml sterile deionised water was pipetted into the culture plate to dislodge 48-h-old bacteria cells from the agar using a sterilised glass spreader. Different dilutions of bacterial suspension were then prepared by performing a serial dilution with sterile deionised water. The absorbance value for the serial dilution 10^{-2} to 10^{-10} of the bacterial suspension was then read using a spectrophotometer (GENESY20) at 600 nm and 540 nm for isolate UTAR EPA2 and UPM 39B3, respectively. For each bacterial dilution prepared, plate count was also performed by plating the bacterial cells onto three LBA plates (triplicates). The average number of colonies formed was recorded as colony forming units (CFU) per millilitre (CFU/ml) and expressed as $\log_{10}$ CFU/ml. A standard growth curve of fungal pathogen FocR4 (Appendix B) was also constructed using similar method with slight modifications, PDA plates and 7 days-old FocR4 were used. The optimum wavelength for maximum absorbance of FocR4 is 600 nm.

3.3 Identification of Bacterial Isolates

3.3.1 Colony Morphological Observation

The physical characteristics of a colony are termed the colony morphology (Seeley, Vandemark, & Lee, 1990). The basic elements used to characterise the isolate's colonies cultured on the LBA plates in this study are the basic shape of the colony (form), the surface appearance of the colony, pigmentation of growth and the shape of colony's edge. The form of the colony can be punctiform, circular, filamentous, irregular, rhizoid or spindle. The surface of the colony can be smooth, glistening, rough, dull, wrinkled, granular,

contoured or papillate. The colony edge can be entire, undulate, filamentous, curled, erose or lobate. The pigmentation of the colony vary from species and the medium used for cultivation, some are white, buff, green, light yellow, straw yellow, deep yellow, red and others (Seeley et al., 1990).

Isolates UPM 39B3 and UTAR EPA2 were grown overnight on LB plate at room temperature (27 ± 2 ˚C). The characteristics of colonies formed were recorded.

3.3.2 BIOLOG

The BIOLOG identification method characterizes and identifies microbes based on carbon source metabolic fingerprint. The bacterium was streaked on tryptic soy agar (TSA) and incubated at room temperature for 48 hours. The isolate suspension was inoculated into the 96-well microplate (GN2 Microplate) and incubated for 16-24 hours at 30 ˚C. The bacteria suspension (150 ul) was pipetted into each well and added with sodium thioglycolate (for encapsulation purpose) before pipetting into the microplate. The biochemical reaction of the isolate towards various carbon sources in the wells of the microplate produced calorimetric changes detectable via the Microplate Reader. Isolate was then identified based on their similar phenotypic results in the database. These procedures were performed for both isolates UTAR EPA2 and UPM 39B3. Both isolates were sent to Focus Biotech Sdn Bhd for BIOLOG analysis.

3.3.3 Molecular Verification of Isolates' Identities

Based on the colony morphological characteristics and BIOLOG identification, UPM 39B3 was preliminarily identified as *S. marcescens*. While for UTAR EPA2, the identified species obtained from BIOLOG identification was not correspondent to the morphological characteristic of UTAR EPA2. Thus, to further confirm the identity of UPM 39B3, a set of primer pair specific for 16S RNA gene of *S. marcescens* (Smar16SM and Smar16SNR) (Wilson, Tatford, Yin, Rajki, Walsh, & Larock, 1999) was used for polymerase chain reaction (PCR). For UTAR EPA2, the genomic DNA was extracted and sent for 16S rDNA sequencing.

3.3.3.1 Genomic DNA extraction

The DNA of the isolates UTAR EPA2 and UPM 39B3 was first extracted using the iNtRON G-spin^{TM} bacterial genomic DNA extraction kit (NHK Biotechnology Sdn Bhd, Malaysia). The cells from each of the bacterial suspension, with absorbance value of 0.8 to 1.0, were harvested by centrifugation at 13,000 rpm for 1 min. The pellet was then mixed well with 300 µl of G-buffer solution and incubated at 65 ˚C for 15 min and constantly inverting the sample. Then, binding buffer (250 µl) was added and mixed well with the cell lysates before loading on a G-spin^{TM} column which was attached with the tube and centrifuged at 13, 000 rpm for 1 min. Then, washing buffer A (500 µl) was pipetted to the column and centrifuged at 13,000 rpm for 1 min. The pellet was resuspended using washing buffer B (500 µl) and centrifuged again at 13, 000 rpm for 1 min. The column was then de-attached from the tube and placed on a 1.5 ml microcentrifuge tube. Then, the elution

buffer (250 µl) was added directly into the column, followed with 1 min incubation at room temperature (27 ± 2 ˚C). The microcentrifuge tube attached with the column was then centrifuged for 1 min at 13,000 rpm. The extracted DNA in elution buffer was kept at -20 ˚C until used.

3.3.3.2 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) using extracted genomic DNA of UPM 39B3 was performed using Biometra T Personal PCR machine. The PCR reagents (iNtRON i-Taq™ Plus DNA Polymerase) were used. PCR was performed with each reaction consisted of 0.8X magnesium chloride ($MgCl_2$)-free PCR buffer, 2.5 mM $MgCl_2$, 0.8 mM dNTPs, 25 pmol of forward and reverse primers, 2.5 U i-Taq and 0.2 µl of DNA template. The primer pairs, Smar16SM (5'-TGG TGA ACT TAA TAC GCT CAT CAA-3') and Smar16SNR (5'-CCC CTC TAC GAG ACT CTA GCT-3'), were used (Wilson et al., 1999). PCR was performed with a hot-start at 96 ˚C for 7 minutes followed by 35 repeating cycles of denaturation (96 ˚C for 1 minute), annealing (65 ˚C for 1 min) and elongation (72 ˚C for 1 min). The PCR process was hold at 72 ˚C for 10 min before completed (Wilson et al., 1999).

3.3.3.3 Electrophoresis and DNA sequencing

Agarose gel electrophoresis was performed to analyse the PCR products. The PCR product was separated using 1.7% (w/v) agarose gel at 80V. For the amplified DNA of UPM 39B3, iNtRON MEGAquick-spin™ PCR and Agarose Gel DNA Extraction Systerm (NHK Biotechnology Sdn Bhd, Malaysia) were used to extract and purify the PCR product from the gel. The

extracted and purified PCR product was sent for DNA sequencing. For UTAR EPA2, the extracted genomic DNA was sent for 16S rDNA sequencing (Macrogen Inc., Korea). The obtained DNA sequences of both isolates were analysed using the BLASTN software (National Center for Biotechnology Information [NCBI]) to search for homology nucleotides sequence from GenBank database.

3.4 Formulation of Isolates

The isolates UTAR EPA2 and UPM 39B3 were first established on LBA plates as 48-h-old cultures. To each isolate, bacterial cells was then dislodged from the agar using LB broth and the resulting bacterial suspension was adjusted using LB broth to approximately 15 $\log_{10}$ CFU/ ml.

For each isolate, the bacterial suspension without treatment served as free-cell formulation (FC). For preparing formulation, the bacterial suspension was incorporated into the various combinations of formulation accordingly for this study. Clay-based carrier materials (bentonite and kaolin), enrichment materials (sucrose and non-fat skim milk) and additives (*p*-amino benzoic acid) were used as formulative materials in the solid formulation. For each of the clay type, seven combinations of formulations were prepared by mixing together the formulative materials and bacterial suspension, in quantities according to their respective combinations. One control for each clay type was also prepared by substituting the bacterial suspension with sterile distilled water. The amount of formulative materials and bacterial suspension used in

the formulation exercise was determined through several trials to ensure that the resulting formulation obtained was in granular form.

For bentonite-based formulations, each combination comprised of 180 g of bentonite clay inoculated with 180 ml of bacterial suspension (15 $\log_{10}$ CFU/ ml) and respective enrichment materials and additives according to Table 3.1. For formulation containing bentonite clay (B) only, no other formulative material was incorporated except the bacterial inoculums as the active ingredient. For the formulations, BN and BS, bentonite clay was mixed with the enrichment materials, 15 g of non-fat skim milk (N) and 15 g of sucrose (S), respectively. The additive material, *p*-amino benzoic acid (P), was added at a rate of 1.5 g for every 180 g of bentonite clay, into separately prepared bentonite formulations, bentonite with *p*-amino benzoic acid only (BP), bentonite with non-fat skim milk and *p*-amino benzoic acid (BNP) and bentonite with sucrose and *p*-amino benzoic acid (BSP). A formulation mixture containing all enrichment and additive materials, bentonite with non-fat skim milk, sucrose and *p*-amino benzoic acid (BNSP), was also prepared using similar compositions. The control of the study, bentonite clay (BC) was prepared by mixing 180 g bentonite clay with 180 ml of sterile distilled water (Table 3.1).

Table 3.1 Compositions of each formulation

Formulation type	[a]B, (g)	[a]N, (g)	[a]S, (g)	[a]P, (g)	Bacterial suspension, (ml)	Sterile Deionised water, (ml)
FC	-	-	-	-	180	-
BC	180	-	-	-	-	180
B	180	-	-	-	180	-
BN	180	15	-	-	180	-
BP	180	-	-	1.5	180	-
BS	180	-	15	-	180	-
BNP	180	15	-	1.5	180	-
BSP	180	-	15	1.5	180	-
BNSP	180	15	15	1.5	180	-
Formulation type	**[a]K, (g)**	**[a]N, (g)**	**[a]S, (g)**	**[a]P, (g)**	**Bacterial suspension, (ml)**	**Sterile Deionised water, (ml)**
KC	240	-	-	-	-	240
K	240	-	-	-	240	-
KN	240	10	-	-	240	-
KP	240	-	-	1.0	240	-
KS	240	-	10	-	240	-
KNP	240	10	-	1.0	240	-
KSP	240	-	10	1.0	240	-
KNSP	240	10	10	1.0	240	-

[a] B: bentonite; BC: cell-free bentonite; FC: free-cell formulation; K: kaolin; KC: cell-free kaolin; N: non-fat skim milk; P: *p*-amino benzoic acid, S: sucrose and -: none.

For kaolin-based formulations, each combination comprised of 240 g of kaolin clay and was inoculated with 240 ml of bacterial suspension (15 $\log_{10}$CFU/ ml) except formulation comprising of solely kaolin clay (K) which was not incorporated with any other formulative materials. For kaolin formulations with a combination of enrichment materials, KN and KS, 10 g of non-fat skim milk (N) and 10 g of sucrose (S) was incorporated, respectively. For formulations with incorporation of *p*-amino benzoic acid, *p*-amino benzoic acid was added at a rate of 1.0 g for every 240 g of kaolin clay, into separately prepared formulations: kaolin with *p*-amino benzoic acid (KP), kaolin with non-fat skim milk and *p*-amino benzoic acid (KNP) and kaolin with sucrose and *p*-amino benzoic acid (KSP). Similarly, the formulation KNSP was a combination of all enrichment and additive materials which was prepared with similar compositions. The formulation (KC) was used as the control. KC was prepared by mixing 240 g of kaolin clay with 240 ml of sterile distilled water (Table 3.1).

After mixing the ingredients, the mixture was manually molded and dried in an oven (Memmert, Germany) at 30 ±2 ˚C for 3 days. After drying, the granules were ground and sieved through a 1.0 mm sieve (Cole-Parmer, 16 mesh). The resulting finer granules were then stored in 1000 ml glass Schott bottles at room temperature (27 ± 2 ˚C) for subsequent experiments. In this study, free-cells formulation (FC), bentonite clay (BC) and kaolin clay (KC) formulations were used as the controls. Free-cell formulation served as the control to compare the effect of formulative materials on the viability and

efficacy of formulated isolates. BC and KC were without bacterial cells and served as negative controls.

3.5 Viability Assessments

The assessment was designed to determine the viability of each formulation upon storage. The assessment was performed at monthly interval over a 6-month period by dilution plate count on LBA. The viability test was carried out on the following day after the formulations were prepared and recorded as the initial cell viability. The bacterial cell viability of both UTAR EPA 2 and UPM 39B3 was recorded for all the formulation investigated monthly for six months.

A total of 14 formulations (B, BN, BS, BNP, BSP, BNSP, K, KN, KS, KNP, KSP and KNSP) and three controls (FC, BC and KC) were sampled for the viability test. Each formulation (1.0 g of each bentonite-based formulation and 2.0 g of each kaolin-based formulation) was sampled and suspended in 15 ml of sterile deionised water. A double amount of kaolin-based formulation was sampled due to double amount of kaolin clay was used to achieve the kaolin-based formulations compared with amount of bentonite clay used for the bentonite-based formulations. A serial dilution was then performed until 10^6 dilution times. From each diluted sample, 0.05 ml aliquot was pipetted and spread onto LB agar plates (duplicates). The plates were then incubated at room temperature (27 ± 2 ˚C) for 2 days. Colony enumeration was then conducted after the incubation period. To determine the cell viability of the three controls, 0.5 ml of free-cells formulation (FC), 1.0 g of cell-free

bentonite clay (BC) and 2.0 g of cell-free kaolin clay (KC) were sampled and cell viability was assessed using similar method.

3.6 Efficacy Assessments

3.6.1 Efficacy of Formulated UTAR EPA2 in Degrading Various Hydrocarbon Substrates

A simple biodegradation test was conducted to determine the degradation activity of formulated isolate UTAR EPA2 on three different hydrocarbon substrates; commercial petrol, mineral oil (Acros Organics) and toluene (Merck). This efficacy assessment was conducted at a monthly interval for 6 months. For hydrocarbon degradation assessment, a total of 14 clay-based formulations (B, BN, BS, BNP, BSP, BNSP, K, KN, KS, KNP, KSP and KNSP) and free-cell formulation (FC) were sampled. Each formulation (1.0 g of each bentonite-based formulation, 2.0 g of each kaolin-based formulation and 0.5 ml of free-cell formulation), was mixed with 20 ml of sterile deionised water and 1.0 ml of hydrocarbon substrate. The mixture was then incubated at room temperature (27 ± 2 ˚C) for 10 days.

The hydrocarbon substrate residue detected after the incubation was quantified. The value was then used to reflect the amount of hydrocarbon substrate degraded, the higher value of hydrocarbons substrate residue detected, the lesser amount of hydrocarbon substrate were degraded. Toluene is a simple aromatic hydrocarbon. The residue of toluene detected can be directly used for the calculation. However, petrol and mineral oil contain mixture of different hydrocarbons. Comparison is difficult to be made if without common peaks from gas chromatography mass spectrometer (GCMS)

analysis. Some easily biodegraded compounds such as short aliphatics were not detected in GCMS analysis after treatment. Thus, the peaks of some common hydrocarbons residues that were consistently detected in the petrol and mineral oil were used as the key compounds to determine the amount of petrol and mineral oil degraded.

The residue of hydrocarbon substrate was first extracted using acetone (Merck) followed by hexane (Merck) as the solvents. Acetone (75% v/v) was first added into a 50 ml tube which contained the mixture of treated hydrocarbon substrate with formulation and vortexed for 30 s before 75% (v/v) of hexane was added and vortexed for another 1 min. This was followed by 5 min of centrifugation (Sigma 3K30, Sartorius, Germany) at 9000 rpm and 4 ˚C. The top clear layer formed was collected and analysed using GCMS, while the bottom layer containing clays, bacterial cells and aqueous solution was discarded.

The GCMS analysis of extracted hydrocarbon substrates (toluene, petrol and mineral oil) was conducted using the GCMS-QP2010 Plus Gas Chromatograph/Mass Spectrometer from Shimadzu. For each sample, one microlitre was injected into the gas chromatograph with the AOC (appellation of origin controlled) 20I Auto Injector. The BPX5 (SGE) chromatographic column with an internal diameter of 0.25 mm and 30 m in length with a constant column flow of 1.38 ml/min (toluene and petrol analysis) and 1.58 ml/min (mineral oil analysis) was used. Helium was used as the carrier gas.

The GC parameters used for the GCMS analysis of the extracted toluene and petrol was similar where the GC was programmed at an initial temperature of 100 ˚C and was then increased to 220 ˚C at the rate of 6 ˚C/ min. The temperature of the injection port was 240 ˚C, with different split ratio for toluene and petrol, which were 1:80 and 1:20, respectively. The MS operating parameters, ion source temperature and interface temperature were 200 ˚C and 315 ˚C, respectively. Total ion current (TIC) spectra were recorded in the mass range of 40– 400 atomic mass units (amu) in scanning mode.

On the other hand, analysis of extracted mineral oil by GCMS was carried out using the same equipment, but with modifications in the GC program and MS parameters for better peaks separation. The GC was programmed at an initial temperature of 100 ˚C and was then increased to 200 ˚C at the rate of 10 ˚C/ min. The temperature was then further increased to 250 ˚C at 5 ˚C/ min and held for 3 min before increasing to 300 ˚C at the rate of 3 ˚C/ min and held for 5 min. Other than that, the temperature of the injection port was 310 ˚C with the split ratio of 1:40. The MS operating parameters, ion source temperature and interface temperature were 200 ˚C and 310 ˚C, respectively. TIC spectra were recorded in the mass range of 40– 550 atomic mass units (amu) in scanning mode.

The amount of a key compound residues detected in the sample was determined by comparing the particular peak area with the respective standard curve constructed using similar extraction method and GCMS analysis conditions (Appendix C). The equation, [(Vo–Vr)/Vo] x 100%, was employed

to calculate the percentage of hydrocarbon substrate degradation. Vo is the original volume (ml) of hydrocarbon substrate added to the sample and Vr is the volume (ml) of hydrocarbon substrate in the sample after treatment based on the key compound residue detected.

3.6.2 Efficacy of Formulated UPM 39B3 in Inhibiting the Growth of *Fusarium oxysporum* f.sp. *cubense* race 4 (FocR4)

The number of FocR4 colonies recovered upon exposure to the formulated bacterial cells was assessed to reflect the efficacy of the formulated UPM 39B3 isolate cells in inhibiting the growth of the pathogenic FocR4. The efficacy of free-cell formulation (FC) of UPM 39B3 was used as the control. This efficacy assessment was carried out monthly for a duration of 6 months. In each efficacy assessment, the pathogen, FocR4, was first cultured for 7 days on PDA plates. The FocR4 culture was then dislodged from the agar using 10 ml of sterile deionised water. The fungal suspension was then adjusted to 6.5 $\log_{10}$ CFU/ ml (Abs 0.980 at 600 nm) with sterile deionised water.

In this *in vitro* assessment, a total of 14 clay-based formulations (B, BN, BS, BNP, BSP, BNSP, K, KN, KS, KNP, KSP and KNSP) and free-cell formulation were sampled. Each formulation (1.0 g of the bentonite-based formulation, 2.0 g of the kaolin-based formulation or 0.5 ml of free-cell formulation) was mixed with 20.0 ml of sterile deionised water in a 50 ml test tube. After mixing well, 1.0 ml of FocR4 suspension prepared as described above was then inoculated. A negative control was prepared by inoculating 1.0 ml of FocR4 into 20.0 ml of sterile deionised water.

The tubes inoculated with the fungus were then incubated at room temperature (27 ± 2 ˚C) for 7 days. For assessment, each tube was vortexed before a serial dilution with sterile deionised water, 0.05 ml of solution from each 10^{-1}, 10^{-2} and 10^{-3} dilution was used for plating. The number of FocR4 colonies recovered from the treatments was enumerated using similar procedure as described in the viability assessment study at section 3.5. The PDA medium supplemented with 1 g/L streptomycin (AMRESCO Inc., USA) was used to select FocR4's growth. The percentage of inhibition of FocR4 was calculated as [(X – Y)/X] x 100, where X is the CFU/ml of FocR4 in the control tube and Y is the CFU/ml of FocR4 treated with formulated cells. The percentage of formulated UPM 39B3 in inhibiting the growth of FocR4 was calculated from replicates.

3.7 The Effect of Sunlight (UV radiation) on Viability and Efficacy Assessments

Sunlight is one of the factors which can cause the reduction of bacterial cell viability. This occurs when these microbial formulations are applied to the environment, which subsequently will also affect their efficacy. Thus, in this study, the effect of UV radiation from the sun on the bacterial cell viability of the formulations and their efficacy was investigated. Two sets, one set for cell viability assessment and another set for efficacy assessments, were prepared for each formulation in this study.

Bacterial cell viability of each isolates with sunlight exposure was accessed monthly for a duration of 6 months. Each formulation was exposed under the sunlight for 6 hours before the cell viability was assessed as described at

section 3.6. On the other hand, efficacy of each isolates with the UV exposure was conducted monthly for a duration of 3 months. The procedure was similar with the efficacy assessment as described at section 3.6. Similarly, the formulations were exposed under the sunlight for 6 hours prior to the efficacy assessments.

3.8 Statistical Analysis

The data obtained from viability and efficacy tests was analysed with Analysis of Variance (ANOVA) using SAS Program (Statistical Analysis System) V 6.12. With the same software, the treatment means were compared using Tukey's Studentized Range Test and significant differences were determined at the P=0.05 level.

CHAPTER 4

RESULTS

4.1 Identification of Beneficial Isolates

Identification of isolates was carried out to provide additional information on the beneficial isolates used in this study. The morphology of the both isolates cultured on the LB agar was consistent. Thus, the morphology of the bacterial colonies was used to assist in identifying the bacteria. The colonies of the isolate UTAR EPA2 were circular, rough and water soluble green pigmented with an entire edge (Figure 4.1 A) while isolate UPM 39B3's colonies were circular, smooth and water insoluble red pigmented with an entire edge (Figure 4.1 B).

Both isolates were for BIOLOG analysis based on their carbon source utilisation. The BIOLOG results indicated that UTAR EPA2 has a similar carbon-utilisation patterns with *Flavimonas oryzihabitans* which achieved 89% probability, the highest if compared with other bacterial species (less than 5%), and similarity value of 0.58 (Table 4.1 & Appendix D). Thus, based on the BIOLOG result, UTAR EPA2 was most probably *Flavimonas oryzihabitans*. However, the green pigmentation observed from the UTAR EPA2 colonies was not the characteristic of *Flavimonas oryzihabitans*. Therefore, the molecular method was employed to further verify the identity of UTAR EPA2.

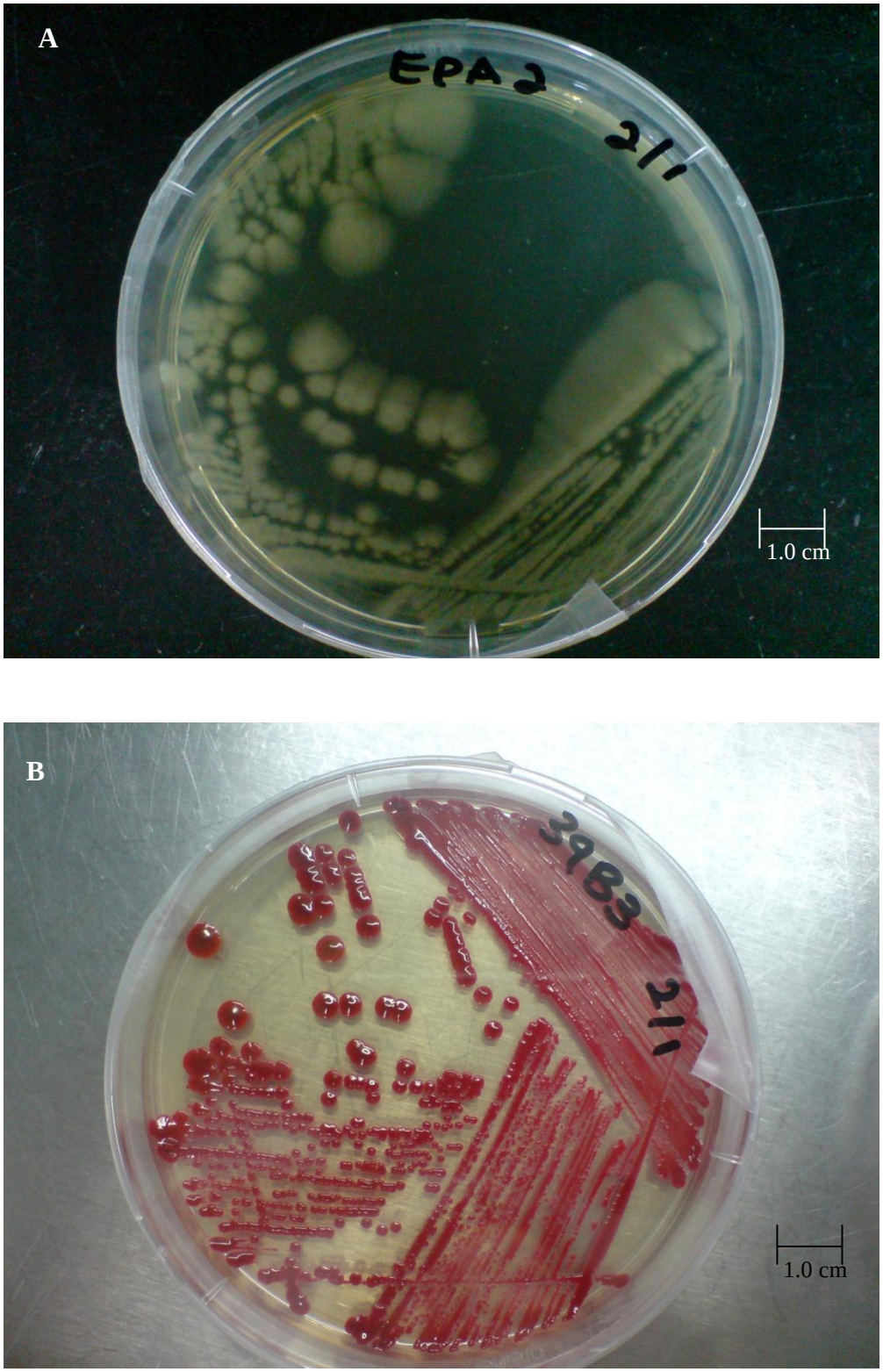

Figure 4.1: The isolates UTAR EPA2 (A) and UPM 39B3 (B) on the LB agar and incubated at room temperature.

Table 4.1: Identification of isolates UTAR EPA2 and UPM 39B3 by using BIOLOG system and molecular identification.

Isolate	BIOLOG system			Molecular identification	
	Identity	Probability (%)	Similarity	Identity	Identical (%)
UTAR EPA2	*Flavimonas oryzihabitans*	89	0.58	*Pseudomonas aeruginosa*	99
UPM 39B3	*Serratia marcescens*	100	0.64	*Serratia marcescens*	98

The genomic DNA of UTAR EPA2 was sent to partially sequence the 16S rRNA gene of the bacteria (Macrogen, Korea). The obtained partial 16S rRNA gene sequence of UTAR EPA2 was blasted using the BLASTN (NCBI). The blast result showed that UTAR EPA2 was 99% identical to *Pseudomonas aeruginosa* based on the GenBank database (Table 4.1 and Appendix E). Consequently, UTAR EPA2 was identified as *Pseudomonas aeruginosa* according to molecular typing and the morphological characteristics.

In contrast, isolate UPM 39B3 was identified as *Serratia marcescens*, with 100% probability and similarity value of 0.64, based on the carbon-utilisation properties from the BIOLOG result (Table 4.1, Appendix D). To further confirm its identity, PCR was carried out using specific primer pair for the amplification of the 16S rRNA gene of *Serratia marcescens*, Smar16SM and Smar16SNR (Wilson et al., 1999). The agarose gel electrophoresis showed that an expected 215 bp DNA fragment was amplified from isolate UPM 39B3 (Figure 4.2). In contrast, two DNA fragments with different sizes were

amplified from isolate UTAR EPA2 while a larger DNA fragment was amplified from the *Pseudomonas aeruginosa* sample (Figure 4.2). To further verify, the PCR product of isolate UPM 39B3 was then sent for DNA sequencing. The DNA sequencing result of the partially amplified 16S rRNA gene of UPM 39B3 showed 98% identical to *Serratia marcescens* after BLASTN analysis based on the GenBank database (Table 4.1 & Appendix E). Thus, UPM 39B3 was identified as *Serratia marcescens* according to the colony morphologies, BIOLOG system and molecular typing.

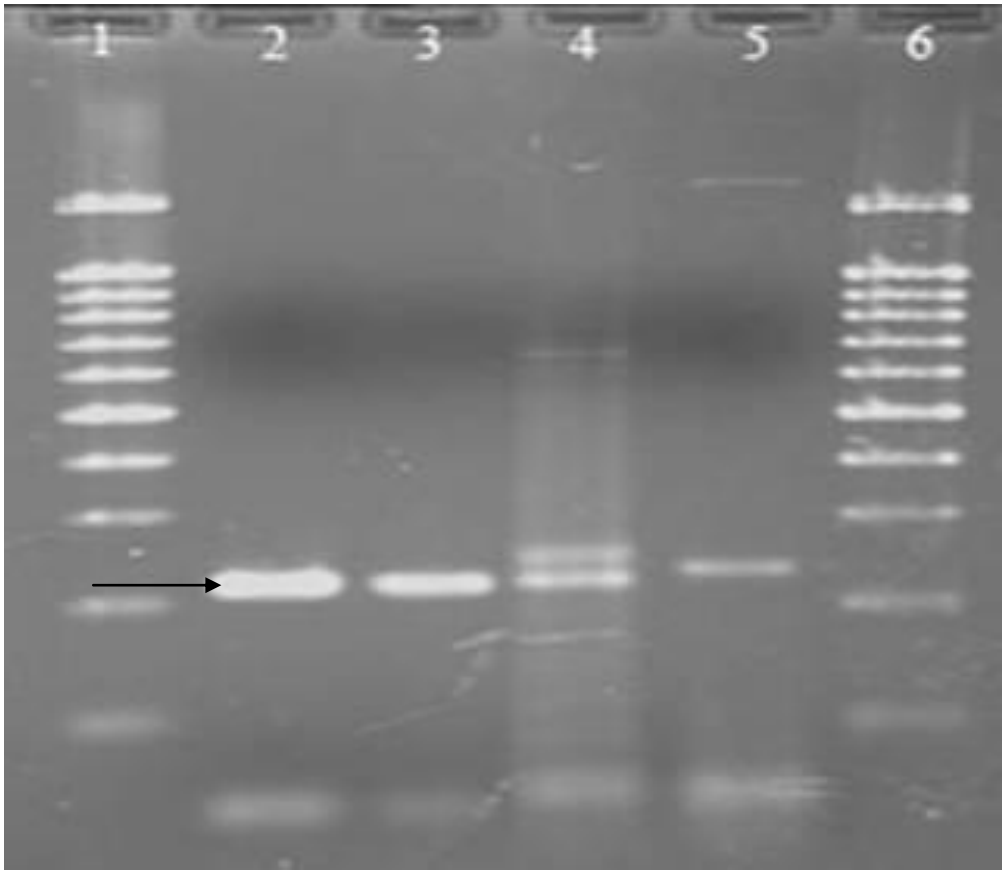

Figure 4.2: The amplified PCR products of partial 16S rRNA gene from the genomic DNA of isolate UPM 39B3. Lane 2 and 3: The amplified 215 bp (⟶) from UPM 39B3; Lane 4: UTAR EPA2; Lane 5: *Pseudomonas aeruginosa*. Electrophoresis was conducted on 1.7% agarose gel.

4.2 Formulation of Microbial Isolates

The physical appearances, size and colour of UTAR EPA2 and UPM 39B3 in free-cell formulation, bentonite-based formulations and kaolin-based formulations stored at room temperature were recorded (Table 4.2). The UTAR EPA2 free-cell formulation appeared as light brown in LB broth while UPM 39B3 free-cell formulation in LB broth appeared red in colour.

Table 4.2: Physical appearances of isolates UTAR EPA2 and UPM 39B3 in free-cell formulation, bentonite-based and kaolin-based formulations.

Parameters	**Free-cell Formulation**		**[a]Bentonite-based Formulations**		**[b]Kaolin-based Formulations**	
	UTAR EPA2	UPM 39B3	UTAR EPA2	UPM 39B3	UTAR EPA2	UPM 39B3
Physical appearance	Liquid	Liquid	Granule	Granule	Powder	Powder
Colour	Light Brown	Red	Brown	Brown	Creamy white	Creamy white

Note: NA: not applicable
[a]: bentonite-based formulations include BC, B, BN, BP, BS, BNP, BSP and BNSP
[b]: kaolin-based formulations include KC, K, KN, KP, KS, KNP, KSP and KNSP

The clay-based carrier materials used in the formulation exercise had great impact on the texture and colour of the solid formulations if compared to enrichment materials (sucrose and non-fat skim milk) and additive (*p*-amino benzoic acid) (Figure 4.3). Although the amount of bentonite used for formulation was half of the amount used for kaolin clay, the granules of

kaolin-based formulations were easier to be crunched into powder form if compared to bentonite-based formulation.

The green pigment of the isolate UTAR EPA2 was not clearly seen in both clay-based formulations before grinding (Figure 4.3 A & B). In contrast, for isolate UPM 39B3, the surface of both clay-based formulations was red in colour before grinding (Figure 4.3 C & D). The colour of the formulation obtained usually depended on the colour of the formulative materials used while the pigmentation of bacterial isolate was usually not clearly seen in the formulation after grinding. After grinding, both formulated isolate UPM 39B3 and UTAR EPA2 in all the bentonite-based formulations appeared as brownish granules (Figure 4.4 A & C) and creamy white powders were obtained in all the kaolin-based formulations (Figure 4.4 B & D).

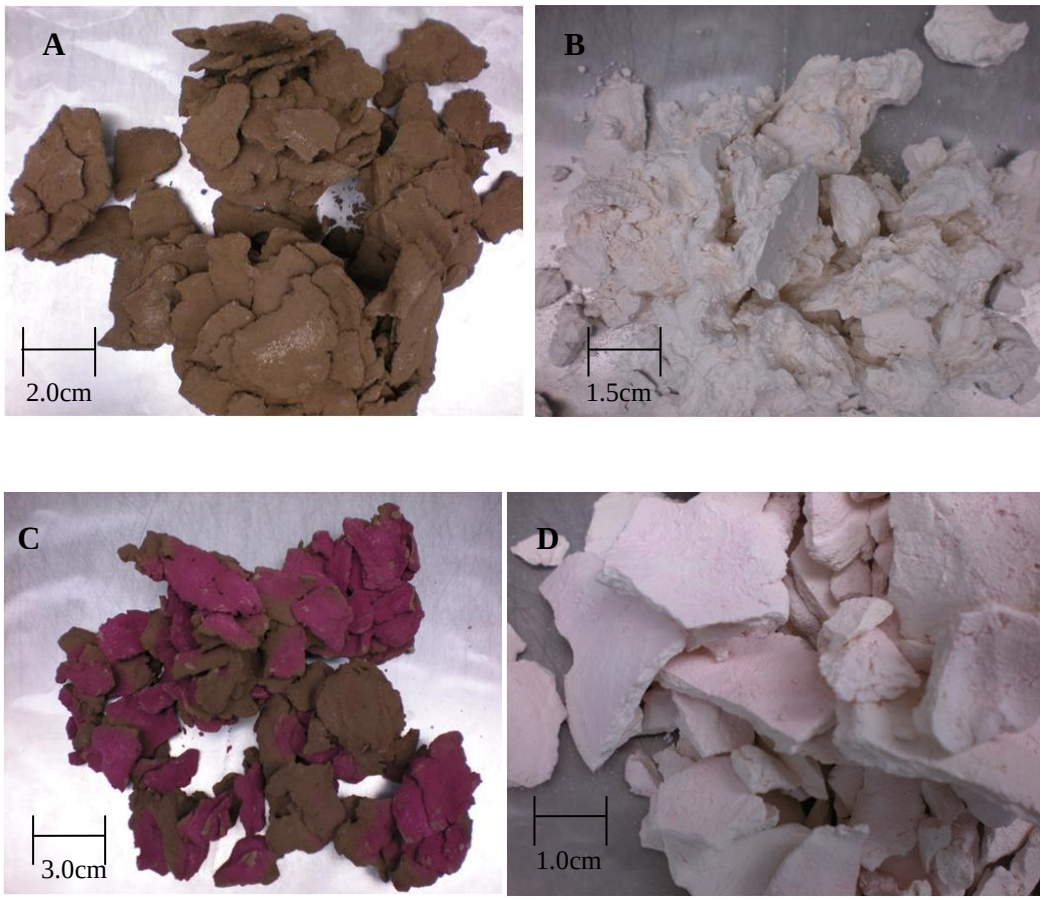

Figure 4.3: The physical appearance, colour and size of benotnite- and kaolin-based formulations before grinding. Isolate UTAR EPA2 in bentonite-based (A) and kaolin-based formulations (B). Isolate UPM 39B3 in bentonite-based (C) and kaolin-based formulations (D)

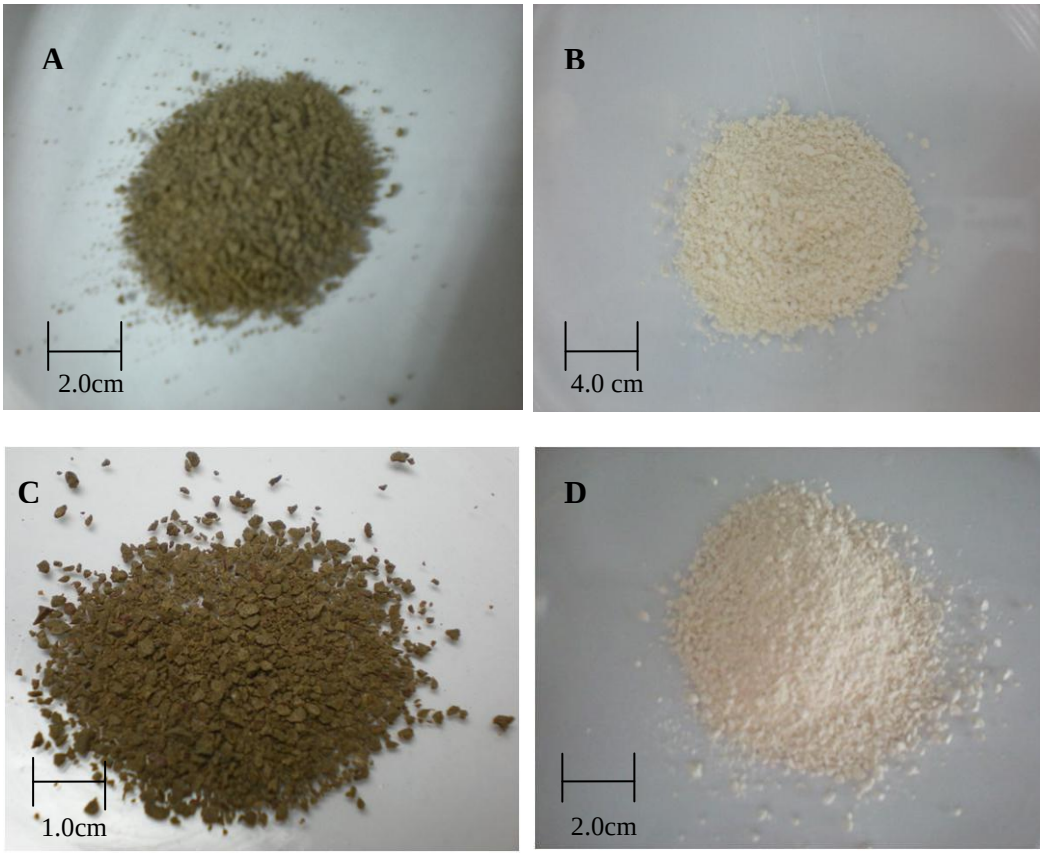

Figure 4.4: The physical appearance, colour and size of bentonite- and kaolin-based formulations after grinding. Isolate UTAR EPA2 in bentonite-based (A) and kaolin-based formulations (B). Isolate UPM 39B3 in bentonite-based (C) and in kaolin-based formulations (D)

4.3 Viability Assessments

The effects of various formulative materials on the cell viability of formulated UTAR EPA2 and UPM 39B3 isolated with or without sunlight exposure were recorded.

4.3.1 Viability of UTAR EPA2 Cells in Various Formulations without Sunlight Exposure

In this study, viability assessment was conducted monthly for six months. The mean viable cell count was the mean of six months viable cell counts. Generally, UTAR EPA2 formulated with bentonite clay only (B) was better in sustaining cell viability if compared to other formulations. The highest cell viable count was obtained for this formulation, B, with the mean viable cell count of 7.35 $\log_{10}$ CFU/ ml (Figure 4.5). Free-cell formulation (FC), with a mean viable cell count of 6.97 $\log_{10}$ CFU/ ml, produced higher cell viability if compared with other bentonite-based formulations. Among the clay-based formulations, bentonite-based formulations (B, BN, BP, BS, BNP, BSP and BNSP) collectively produced higher cell viability compared with kaolin-based formulations (K, KN, KP, KS, KNP, KSP and KNSP), with the collective mean viable cell count of 5.51 $\log_{10}$ CFU/ ml and 3.15 $\log_{10}$ CFU/ ml, respectively.

A comparison among bentonite-based formulations, formulation with bentonite clay only (B) produced the highest cell viability, while addition of enrichment materials, non-fat skim milk (N) and sucrose (S), in the formulation might be able to sustain cell viability as mean viable cell count were 6.70 $\log_{10}$ CFU/ ml and 6.60 $\log_{10}$ CFU/ ml for BN and BS formulations,

respectively (Figure 4.5). On the other hand, the bentonite formulations with addition of *p*-amino benzoic acid (P) showed poor cell viability with mean viable cell count of 4.58 $\log_{10}$ CFU/ml, 3.34 $\log_{10}$ CFU/ ml, 5.24 $\log_{10}$ CFU/ ml and 4.77 $\log_{10}$ CFU/ ml, for BP, BNP, BSP and BNSP, respectively, if compared with the bentonite formulations without incorporation of *p*-amino benzoic acid (B, BN and BS) (Figure 4.5).

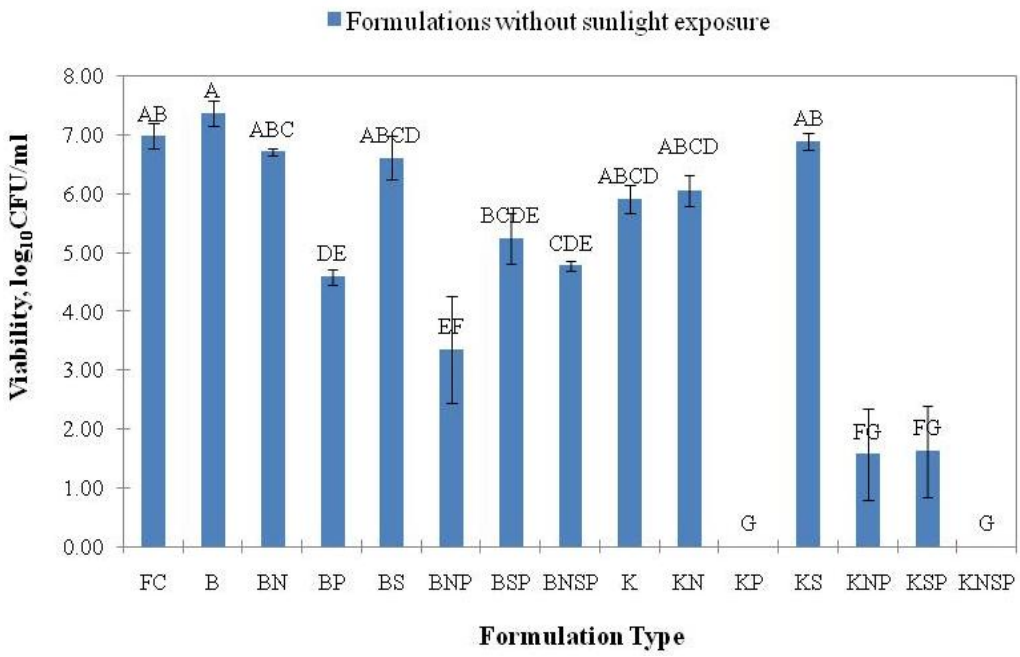

Figure 4.5: The mean viable cell count of UTAR EPA2 cells in various formulations without exposure to 6 h of sunlight. Means with the same letters and same captions are not significantly different ($HSD_{(0.05)}$). The bars on top of each column represent the standard error of the mean. (Note: B: bentonite; FC: free-cell formulation; K: kaolin; N: non-fat skim milk; P: *p*-amino benzoic acid and S: sucrose).

Among kaolin-based formulations, the most suitable formulation for cell viability was kaolin clay incorporated with sucrose (KS) as the highest number of viable cells (6.88 $\log_{10}$ CFU/ ml) was recorded (Figure 4.5). Formulations added with *p*-amino benzoic acid such as KP and KNSP were the least suitable

formulation to maintain viability of cells as no viable cell was recovered throughout the experimental period (Figure 4.5). In contrast, high cell viability was obtained for KN and KS formulations with 6.04 $\log_{10}$ CFU/ ml and 6.88 $\log_{10}$ CFU/ ml, respectively (Figure 4.5). Hence, this also suggested that sucrose and non-fat skim milk added in the formulations, KN and KS, played a role in sustaining cell viability.

The effect of addition of enrichment materials and additive in kaolin-based formulations on UTAR EPA2 cell viability was similar to that of the bentonite-based formulations. Addition of *p*-amino benzoic acid in the formulations, BP and KP, rendered inhibitory effects towards UTAR EPA 2 cells, while incorporating sucrose and non-fat skim milk in the formulations showed beneficial effect. The detrimental effect of *p*-amino benzoic acid was less severe in the bentonite formulations incorporated with sucrose and non-fat skim milk, as observed in BNP, BSP and BNSP (Figure 4.5). However, viable cell count remained poor for kaolin-based formulations added with *p*-amino benzoic acid as observed in KNP, KSP, KNSP even in the presence of sucrose and non-fat skim milk (Figure 4.5).

4.3.2 Viability of UTAR EPA2 Cells in Various Formulations with Sunlight Exposure

The type of formulative materials used influenced the viability of UTAR EPA2 cells especially upon sunlight exposure. In general, higher cell viability was obtained for all formulations tested without exposing to sunlight. The presence of clay-based carrier in the formulations might have conferred protection to the cells from sunlight exposure. All the bentonite-based

formulations except BP showed higher mean viable cell count if compared with FC formulation upon exposure to sunlight. For example formulation B achieved a mean cell count of 6.58 $\log_{10}$ CFU/ ml when compared to FC formulation, 2.70 $\log_{10}$ CFU/ ml (Figure 4.6). For kaolin-based formulations, all the formulations obtained higher mean viable cell count than FC formulation except when the formulations were added with *p*-amino benzoic acid upon sunlight exposure. In this study, the collective mean viable cell count for bentonite-based formulations (B, BN, BP, BS, BNP, BSP and BNSP) was generally higher than kaolin-based formulations (K, KN, KP, KS, KNP, KSP and KNSP), at 4.44 $\log_{10}$ CFU/ ml and 2.57 $\log_{10}$ CFU /ml, respectively (Figure 4.6).

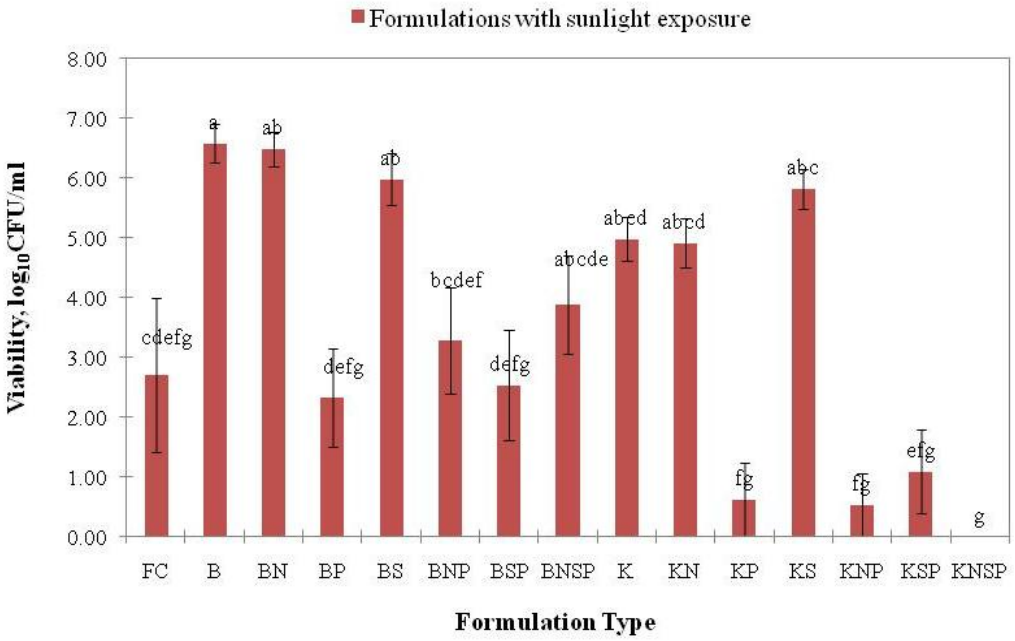

Figure 4.6: The mean viable cell count of UTAR EPA2 cells in various formulations with exposure to 6 h of sunlight. Means with the same letters and same captions are not significantly different ($HSD_{(0.05)}$). The bars on top of each column represent the standard error of the mean. (Note: B: bentonite; FC: free-cell formulation; K: kaolin; N: non-fat skim milk; P: *p*-amino benzoic acid and S: sucrose).

Formulation with bentonite only (B) proved to be the most suitable formulation for maintaining cell viability as highest cell viability was recovered and the cell viability was not significantly affected (Table 4.3) when the formulation, B, was exposed to sunlight for 6 hours. On the other hand, the cell viability for free-cell formulation (FC) decreased significantly (Table 4.3) after 6 hours of sunlight exposure, from viable cell count of 6.97 $\log_{10}$ CFU/ ml (without exposing to sunlight) (Figure 4.5) to 2.70 $\log_{10}$ CFU/ ml (exposing to sunlight) (Figure 4.6). Similarly, significant difference in cell viability was obtained for formulations, BP, K, KN and KS, between the samples exposing and not exposing to sunlight.

Table 4.3: Results of T-test for the comparison of the UTAR EPA2 cell viability between the formulations exposed and without exposed to sunlight.

T-test	t Value	Pr > |t|
FC	3.830	0.009 *
B	2.120	0.079
BN	0.680	0.523
BP	2.480	0.048 *
BS	1.990	0.093
BNP	0.290	0.785
BSP	2.390	0.054
BNSP	1.100	0.313
K	2.850	0.029 *
KN	3.260	0.017 *
KP	-1.000**	0.356
KS	3.660	0.011 *
KNP	0.970	0.371
KSP	0.550	0.600
KNSP	0.000	0.000

Note: FC: Free-cell formulation; B: Bentonite clay; N: Non-fat skim milk; S: Sucrose; P: *p*-amino benzoic acid.

*: Statistically different ($p \leq 0.05$).

**: A negative t-value indicates a reversal in the directionality of the effect, which has no bearing on the significance of the difference between groups.

Among the bentonite-based formulations exposed to sunlight for 6 hours (B, BN, BP, BS, BNP, BSP and BNSP), the mean viable cell count for bentonite-based formulation with addition of sucrose or non-fat skim milk only, BN (6.48 $\log_{10}$ CFU/ ml) and BS (5.98 $\log_{10}$ CFU/ ml), was higher if compared with formulation with addition of *p*-amino benzoic acid (BP), 2.33 $\log_{10}$ CFU/ ml (Figure 4.6). All bentonite-based formulations (except BP) showed better protection to the cells from sunlight exposure as the viable cells recovered from bentonite-based formulations with and without sunlight exposure was not significantly different (Table 4.3).

Kaolin-based formulations were less suitable in maintaining cell viability, especially after exposure to sunlight. Three of the seven kaolin-based formulations, K, KN and KS, recorded significant reduction in the number of cells recovered after exposure to sunlight (Table 4.3). The most suitable kaolin-based formulation was KS with relatively higher number of viable cells after sunlight exposure (5.84 $\log_{10}$ CFU/ ml) (Figure 4.6), although there was a significant reduction in the number of cells after sunlight exposure (Table 4.3). The least suitable formulation was KNSP, where no viable cell was recovered.

The impact of *p*-amino benzoic acid on cell viability after exposure to sunlight did not indicate its beneficial role as a UV protectant. The viable cells recovered from the formulations containing *p*-amino benzoic acid were not significantly higher (Figure 4.5, Figure 4.6 & Table 4.3). On the other hand, addition of enrichment materials (non-fat skim milk and sucrose) aided in sustaining cell viability when the formulations were exposed to 6 hours of

sunlight as observed in BN, BS, KN and KS formulations (Figure 4.5 & Figure 4.6). The UTAR EPA2 formulated with bentonite clay only (B) achieved the highest cell viability during the storage period. Formulation B was also able to sustain cell viability after 6 hours of sunlight exposure and highest viable cell count was obtained stably throughout the experimental period.

4.3.3 Viability of UPM 39B3 Cells in Various Formulations without Sunlight Exposure

In this study, viability assessment was conducted monthly for six months. The mean viable cell count was the mean of six months viable cell counts. Results in this study indicated that free-cell formulation (FC) without sunlight exposure was the most suitable for storage of UPM 39B3 after a 6-month period with a mean viable cell count of 5.99 $\log_{10}$ CFU/ ml (Figure 4.7). The cells in all the clay-based formulations, on the other hand, showed lower viable cell counts than FC after storage for 6 months at room temperature. Among clay-based formulations, bentonite-based formulations (B, BN, BP, BS, BNP, BSP and BNSP) produced higher cell viability if compared with kaolin-based formulations (K, KN, KP, KS, KNP, KSP and KNSP), with collective mean viable cell count of 3.36 $\log_{10}$ CFU/ ml and 0.16 $\log_{10}$ CFU/ ml, respectively.

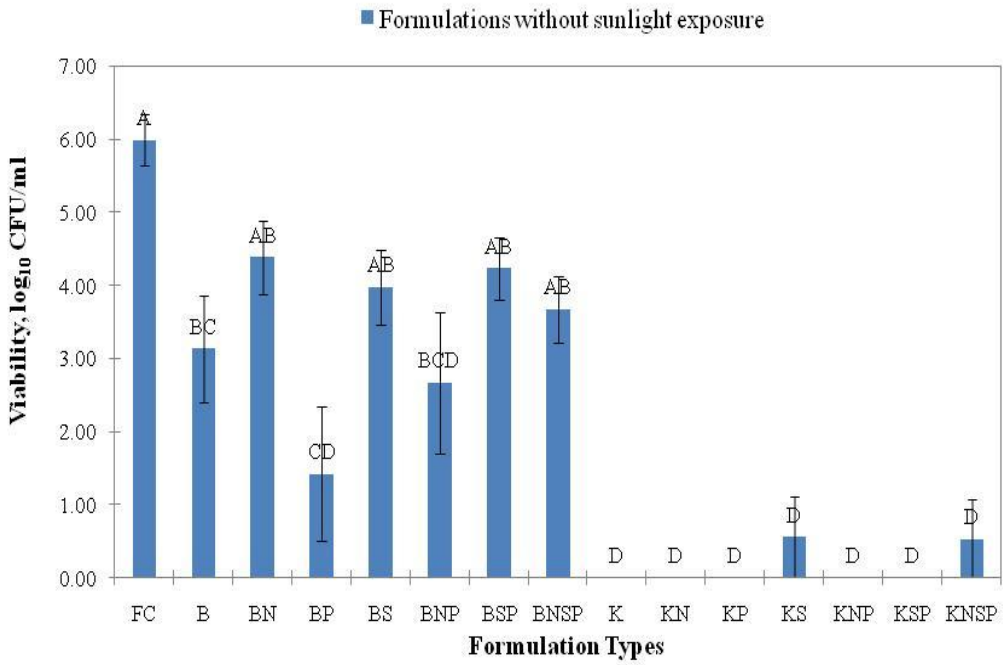

Figure 4.7: The mean viable cell count of UPM 39B3 cells in various formulations without exposure to 6 h of sunlight. Means with the same letters and same captions are not significantly different ($HSD_{(0.05)}$). The bars on top of each column represent the standard error of the mean. (Note: B: bentonite; FC: free-cell formulation; K: kaolin; N: non-fat skim milk; P: *p*-amino benzoic acid and S: sucrose).

Among the bentonite-based formulations, incorporation with enrichment materials (non-fat skim milk or sucrose) showed more beneficial effect on cell viability than the additive (*p*-amino benzoic acid). The phenomenon was observed in BN (4.38 $\log_{10}$ CFU/ ml) and BS (3.98 $\log_{10}$ CFU/ ml) formulations if compared with BP (1.43 $\log_{10}$ CFU/ ml) (Figure 4.7). The presence of non-fat skim milk and sucrose in BN and BS formulations, respectively, produced relatively higher cell viability. They might be able to sustain cell viability even in the formulations containing *p*-amino benzoic acid (BNP, BSP, BNSP). The additive, *p*-amino benzoic acid, was found to reduce the viability of the cells as observed in BP formulation (Figure 4.7). In

general, the kaolin-based formulations were not suitable in sustaining the cell viability as no viable cell was recovered from five kaolin-based formulations (K, KN, KP, KNP and KSP) from a total of seven kaolin-based formulations investigated (Figure 4.7). Addition of sucrose as in the formulations, KS and KNSP, did show beneficial effect on cell viability (Figure 4.7).

4.3.4 Viability of UPM 39B3 Cells in Various Formulations with Sunlight Exposure

The benefits of formulative materials in sustaining cell viability were clearly observed when the formulated cells were exposed to sunlight. The mean viable cell count of FC formulation decreased significantly from 5.99 $\log_{10}$ CFU/ ml to 1.82 $\log_{10}$ CFU/ ml (Figure 4.8) after 6 hours of sunlight exposure while cell viability of B, BP, BNP, BSP, KS and KSNP formulations was not significantly different between exposing and not exposing to sunlight (Table 4.4). Among clay-based formulations which were exposed to sunlight, bentonite-based formulations (B, BN, BP, BS, BNP, BSP and BNSP) produced higher cell viability when compared with kaolin-based formulations (K, KN, KP, KS, KNP, KSP and KNSP), with collective mean viable cell count of 2.24 $\log_{10}$ CFU/ ml and 0.10 $\log_{10}$ CFU/ ml, respectively. Similar to UTAR EPA2, bentonite-based formulations were more suitable in protecting the cells from sunlight exposure if compared with FC formulation and kaolin-based formulations.

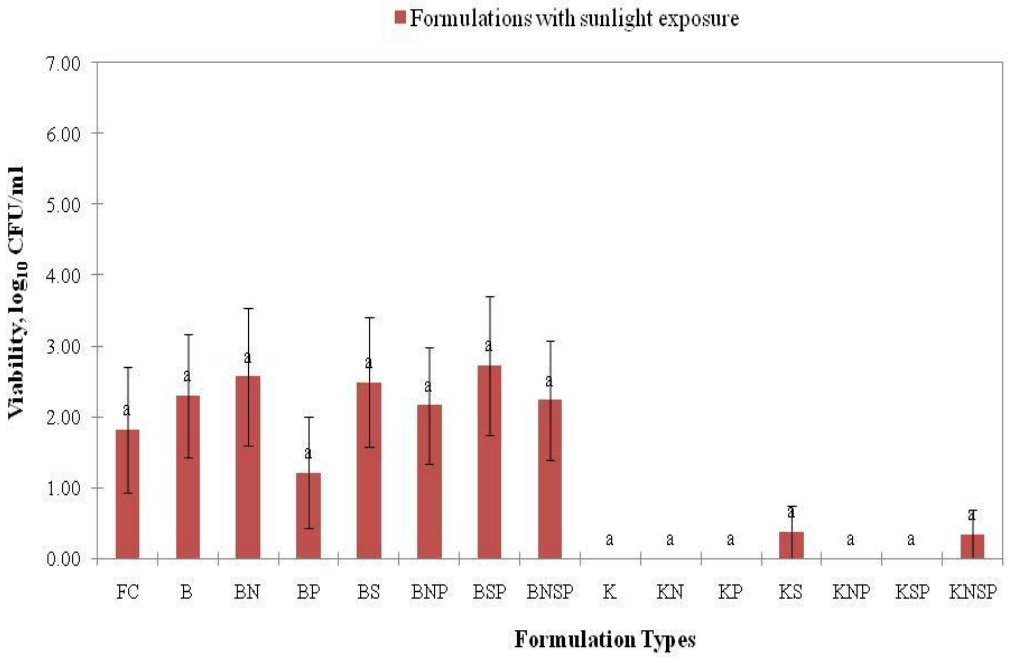

Figure 4.8: The mean viable cell count of UPM 39B3 cells in various formulations with exposure to 6 h of sunlight. Means with the same letters and same captions are not significantly different ($HSD_{(0.05)}$). The bars on top of each column represent the standard error of the mean. (Note: B: bentonite; FC: free-cell formulation; K: kaolin; N: non-fat skim milk; P: *p*-amino benzoic acid and S: sucrose).

Among bentonite-based formulations which were exposed to sunlight, the mean viable cell counts for all bentonite-based formulations (except BP) were higher than kaolin-based formulations and FC but were not significantly different (Figure 4.8). The addition of enrichment materials in the bentonite-based formulations produced higher cell viability as observed in BN, BS, BNP, BSP and BNSP formulations while addition of *p*-amino benzoic acid showed detrimental effect on cell viability as observed in BP (Figure 4.8). Similarly, the addition of sucrose to kaolin-based formulations showed beneficial effect and produced viable cells as recorded in KS and KNSP formulations if compared with other kaolin-based formulations (Figure 4.8).

Table 4.4: Results of T-test for the comparison of the UPM 39B3 cell viability between the formulations exposed and without exposed to sunlight.

T-test	t Value	Pr > |t|
FC	4.920	0.003*
B	1.710	0.138
BN	2.650	0.038*
BP	0.200	0.851
BS	2.700	0.035*
BNP	2.400	0.053
BSP	2.140	0.077
BNSP	2.520	0.046*
K	0.000	0.000
KN	0.000	0.000
KP	0.000	0.000
KS	1.000	0.356
KNP	0.000	0.000
KSP	0.000	0.000
KNSP	1.000	0.356

Note: FC: Free-cell formulation; B: Bentonite clay; N: Non-fat skim milk; S: Sucrose; P: *p*-amino benzoic acid.

*: Statistically different ($p \leq 0.05$).

Comparisons between the viable cell counts after sunlight exposure and without sunlight exposure, B, BP, BNP, BSP, KS and KNSP formulations showed that the formulations were able to maintain cell viability. The viable cell counts after and before sunlight exposure was not significantly different for these formulations (Table 4.4). The FC, BN, BS and BNSP formulations recorded statistically significant different viable cell count values (Table 4.4), which indicating a significant reduction in the number of viable cells recovered from formulations exposed to sunlight.

The FC formulation of UPM 39B3 without sunlight exposure showed the highest cell viability. However, cells from FC formulation were unable to sustain and maintain cell viability upon sunlight exposure. The UPM 39B3

formulated with bentonite clay and enrichment materials (BN and BS) were suitable in sustaining cell viability after 6 hours of sunlight exposure and higher viable cell count was produced throughout the experimental period.

4.4 Efficacy Assessments

The efficacy results of the formulated UTAR EPA2 isolate with or without sunlight exposure in degrading toluene, petrol and mineral oil were recorded. Similarly, the effect of various bentonite- and kaolin-based formulations of UPM 39B3 on inhibiting *Fusarium oxysporum* f. sp. *cubense* race 4 (FocR4) were studied and recorded.

4.4.1 Efficacy of Formulated UTAR EPA2 in Degrading Various Hydrocarbon Substrates

The colour changes of formulations with and without sunlight exposure was observed after they were added into the aqueous solution which contains hydrocarbon substrates (toluene, petrol and mineral oil). The visible changes of formulations when they were added into these mixed solutions were recorded. Generally, the presence of *p*-amino benzoic acid and non-fat skim milk in the formulations turned the aqueous solution to yellowish or brownish. The bentonite clay in the bentonite-based formulations with or without sunlight exposure formed a grout during all the hydrocarbon substrates degradation investigation. On the other hand, the kaolin clay in the kaolin-based formulations with or without sunlight exposure eventually sinks down as sediment during all the hydrocarbon substrates degradation investigation. There was no significant changes observed for free-cell-, kaolin- and

bentonite-based formulations with or without sunlight exposure for the toluene and petrol samples.

The bentonite clay in all the formulations turned greyish/black during incubation for mineral oil samples while free-cell and kaolin-based formulations did not show obvious colour changes (Figure 4.9 A & B). The cause of the colour change observed in the bentonite clay formulations for the mineral oil sample was unknown and only recorded as additional information.

Toluene itself was used as the key compound to quantify the amount of toluene which has been degraded (Appendix C). Ethylbenzene and xylene were used as the key compounds to quantify the amount of petrol which has been degraded (Appendix C). While for mineral oil, nanodecane, octadecane, hexadecane and dodecane were used as the key compounds to quantify the amount of mineral oil which has been degraded (Appendix C).

Figure 4.9: The changes of bentonite-based formulations (A) (from left to right: BC, B, BN, BP, BS, BNP, BSP, BNSP, FC and negative control) and kaolin-based formulations (B) (from left to right: KC, K, KN, KP, KS, KNP, KSP, KNSP, FC and negative control) observed during degradation investigation for the mineral oil sample.

4.4.1.1 Degradation of Toluene by Formulated UTAR EPA2 without Sunlight Exposure

Toluene (methylbenzene) is a simple aromatic hydrocarbon which is one of the commonly used commercial chemical in solvent applications. The ability of formulated UTAR EPA2 to degrade toluene was studied using GCMS analysis. The toluene degradation percentage recorded in this study was the mean of six months toluene degradation percentage of a formulation. Generally, free-cell- (FC), bentonite- (B, BN, BP, BS, BNP, BSP and BNSP) and kaolin-based formulations (K, KN, KP, KS, KNP, KSP and KNSP) were able to degrade toluene effectively. The collective toluene degradation percentage of the bentonite- or kaolin-based formulations was calculated as the average mean of the toluene degradation percentages from all the bentonite-based formulations or kaolin-based formulations. The efficacy of UTAR EPA2 cells in degrading toluene was slightly higher for kaolin-based formulations when compared to bentonite-based formulations and FC. The collective toluene degradation percentages for kaolin-based, bentonite-based and FC formulations were 90.22%, 85.29% and 88.34%, respectively. However, the percentage of toluene degradation from each formulation was not significantly different (Figure 4.10).

The kaolin-based formulations with addition of sucrose (KS, KSP and KNSP) showed higher degradation efficacy when compared to FC and bentonite-based formulations. The highest degradation efficacy for toluene was obtained by using KNSP formulation followed by KS and KSP formulations, with the mean degradation percentage of 95.39%, 91.76% and 90.73%, respectively (Figure 4.10). Similarly, among bentonite-based formulations, incorporation

of sucrose showed higher degradation efficacy, in which the highest degradation efficacy was obtained by using BNSP formulation and followed by BSP and BS formulations, with the degradation percentage of 87.90%, 86.58% and 86.03%, respectively (Figure 4.10).

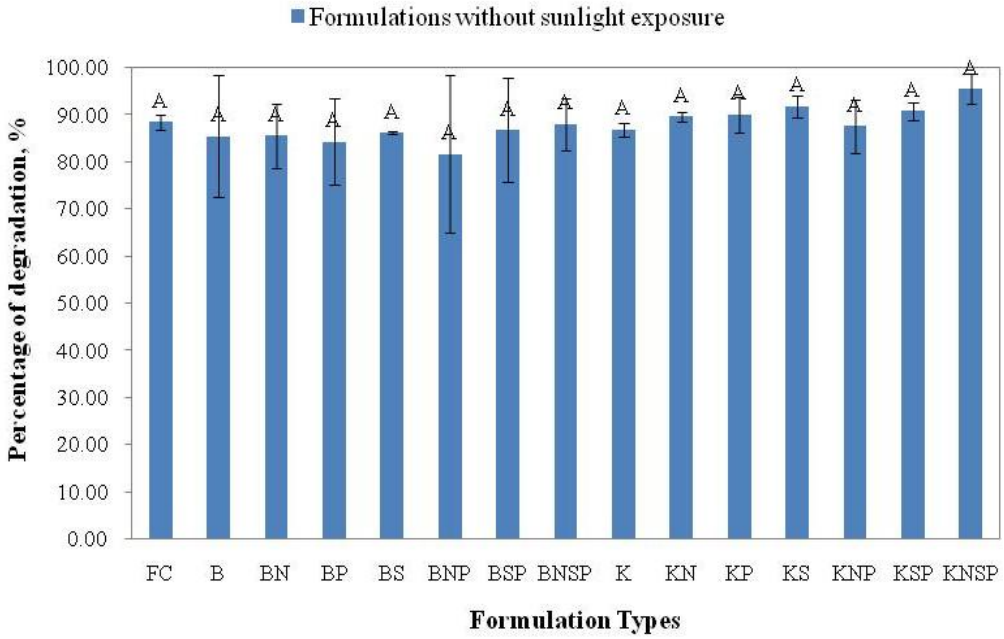

Figure 4.10: The percentage of toluene degradation by UTAR EPA2 cells in various formulations without exposure to 6 h of sunlight. Means with the same letters and captions are not significantly different ($HSD_{(0.05)}$). The bars on top of each column represent the standard error of the mean. (Note: B: bentonite; FC: free-cell formulation; K: kaolin; N: non-fat skim milk; P: *p*-amino benzoic acid and S: sucrose).

Generally, better efficacy was observed from both clay-based formulations incorporated with enrichment materials (KN, KS, KSP, KNSP, BN, BS, BSP and BNSP). In contrast, the addition of *p*-amino benzoic acid in the formulation had slightly lowered the degradation efficacy as observed in KP, BP and BNP formulations. Nevertheless, the addition of sucrose, non-fat skim

milk and *p*-amino benzoic acid in various combinations of kaolin- and bentonite-based formulations did not significantly enhance or inhibit the degradation activity of the UTAR EPA2 cells (Figure 4.10).

4.4.1.2 Degradation of Toluene by Formulated UTAR EPA2 with Sunlight Exposure

All formulations with sunlight exposure were able to produce higher degradation efficacy compared to formulations without sunlight exposure. In this assessment, the toluene degradation percentage is the mean of three months toluene degradation percentage of the formulation with sunlight exposure. Generally, the FC formulation produced the highest collective degradation efficacy, 98.18%, followed by kaolin-based formulations (K, KN, KP, KS, KNP, KSP and KNSP) (96.78%) and bentonite-based formulations (B, BN, BP, BS, BNP, BSP and BNSP) (90.42%). The collective mean toluene degradation percentage was calculated as the average of the toluene degradation percentages from all the bentonite-based formulations or kaolin-based formulations with sunlight exposure.

Among clay-based formulations after sunlight exposure, the highest degradation efficacy was obtained from BS formulation followed by KN and K formulations, with the degradation percentage of 99.40%, 98.54% and 98.19%, respectively (Figure 4.11). Addition of sucrose in BS formulation benefited cells especially after sunlight exposure as significant higher degradation efficacy was observed, 93.68% (without sunlight exposure) to 99.40% (with sunlight exposure) (Table 4.5). However, the addition of non-fat skim milk and *p*-amino benzoic acid in various combinations to kaolin- and

bentonite-based formulations after sunlight exposure did not significantly enhance or reduce the degradation activity by the UTAR EPA2 cells (Figure 4.11 & Table 4.5). *P*-amino benzoic acid was also not particularly useful in rendering benefits as UV protectant. The degradation efficacy (based on 3 months' results) between sunlight and non-sunlight exposed formulations using the same formulation was not significantly different (Table 4.5).

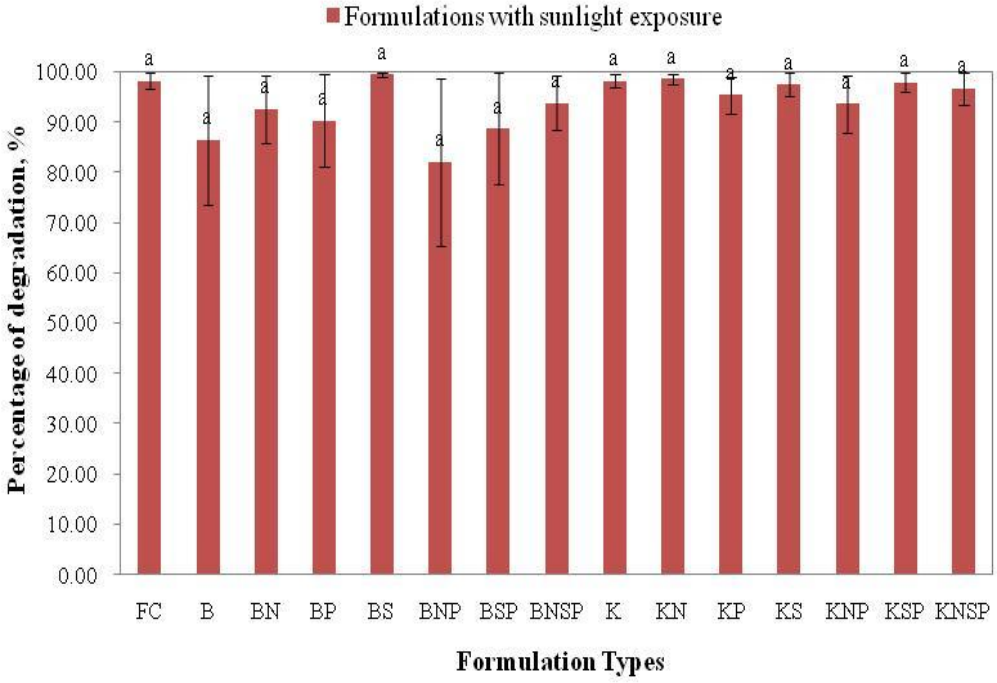

Figure 4.11: The percentage of toluene degradation by UTAR EPA2 cells in various formulations with exposure to 6 h of sunlight. Means with the same letters and captions are not significantly different ($HSD_{(0.05)}$). The bars on top of each column represent the standard error of the mean. (Note: B: bentonite; FC: free-cell formulation; K: kaolin; N: non-fat skim milk; P: *p*-amino benzoic acid and S: sucrose).

Table 4.5: Results of T-test for the comparison of the UTAR EPA2 efficacy in toluene degradation between the formulations exposed and without exposed to sunlight.

T-test	t Value	Pr > |t|
FC	-1.280**	0.330
B	0.200	0.858
BN	-0.230**	0.842
BP	-0.250**	0.823
BS	-4.480**	0.046*
BNP	0.400	0.726
BSP	0.130	0.906
BNSP	-0.340**	0.766
K	-1.900**	0.198
KN	-1.760**	0.221
KP	-1.040**	0.408
KS	-1.990**	0.185
KNP	-0.730**	0.539
KSP	-2.120**	0.168
KNSP	-2.210**	0.158

Note: FC: Free-cell formulation; B: Bentonite clay; N: Non-fat skim milk; S: Sucrose; P: *p*-amino benzoic acid.

*: Statistically different ($p \leq 0.05$).

**: A negative t-value indicates a reversal in the directionality of the effect, which has no bearing on the significance of the difference between groups.

4.4.1.3 Degradation of Petrol by Formulated UTAR EPA2 without Sunlight Exposure

Petrol is a yellow-tinted liquid mixture derived from petroleum. It is a mix of mostly aliphatic hydrocarbons from petroleum distillation and containing other additives for better engine performance and reducing harmful exhaust emissions. In this study, ethylbenzene and xylene were used as the key compounds to quantify the amount of petrol which has been degraded. Benzene, toluene, ethylbenzene and xylene (BTEX) are commonly found in petroleum hydrocarbon such as petrol (Leusch & Bartkow, 2010).

The petrol degradation percentage was the mean of six months petrol degradation percentage of a formulation without sunlight exposure. Generally, the collective degradation efficacy of UTAR EPA2 cells was relatively higher for cells in FC formulation (95.78%) if compared with kaolin- (86.22%) and bentonite-based formulations (79.95%). The collective petrol degradation percentage was calculated as the average of the mean petrol degradation percentages from all the bentonite-based formulations or kaolin-based formulations without sunlight exposure. Comparisons between the two clay-based formulations indicated that most of the kaolin-based formulations had higher mean of petrol degradation.

Kaolin-based formulations, K, KS and KNSP, were able to produce good degradation efficacy with 86.85%, 86.62% and 83.72%, respectively (Figure 4.12). Among bentonite-based formulations, the formulations with only the bentonite clay (B), with sucrose (BS) and with non-fat skim milk (BN) were able to produce good degradation efficacy with 77.82%, 88.42% and 80.62%, respectively (Figure 4.12). The addition of sucrose, non-fat skim milk and *p*-amino benzoic acid in various combinations to kaolin- and bentonite-based formulations did not significantly enhance or reduce the degradation activity by the UTAR EPA2 cells (Figure 4.12).

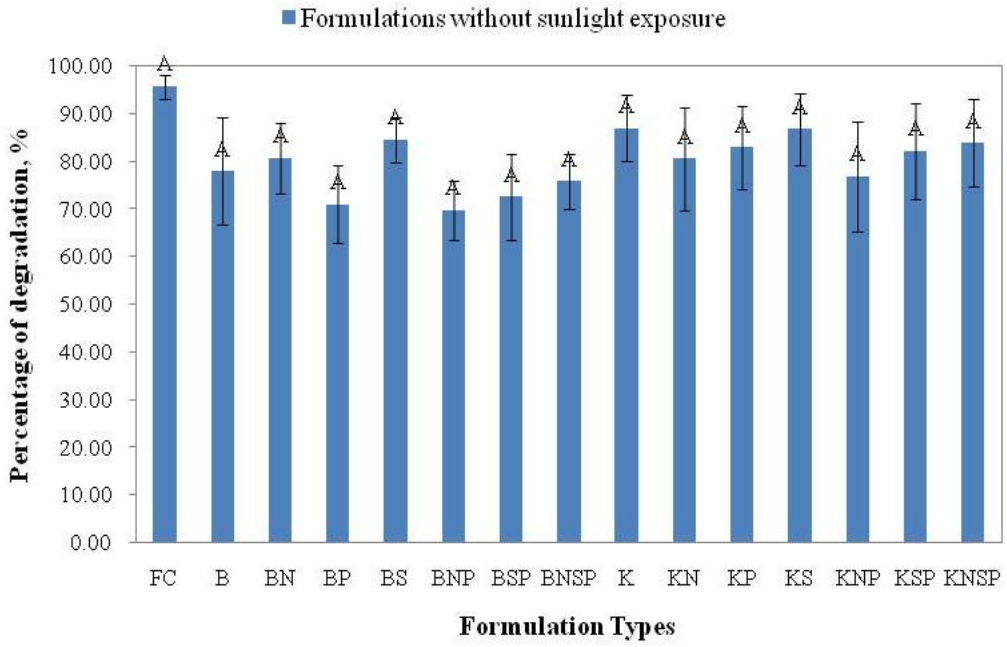

Figure 4.12: The percentage of petrol degradation by UTAR EPA2 cells in various formulations without exposure to 6 h of sunlight. Means with the same letters and captions are not significantly different ($HSD_{(0.05)}$). The bars on top of each column represent the standard error of the mean. (Note: B: bentonite; FC: free-cell formulation; K: kaolin; N: non-fat skim milk; P: *p*-amino benzoic acid and S: sucrose).

4.4.1.4 Degradation of Petrol by Formulated UTAR EPA2 with Sunlight Exposure

The benefit of kaolin-based formulations in ensuring high petrol degradation was most evident when the formulations were exposed to sunlight. In this assessment, the petrol degradation percentage was the mean of three months petrol degradation percentage of the formulation with sunlight exposure. After sunlight exposure, the kaolin-based formulations (K, KN, KP, KS, KNP, KSP and KNSP) achieved a higher collective degradation efficacy (89.67%) than the bentonite-based formulations (B, BN, BP, BS, BNP, BSP and BNSP) which was 79.08%. Both clay-based formulations, however, had lower

degradation efficacy when compared to FC formulation (95.93%) after exposing to sunlight.

Generally, formulations containing only clay (B or K) after sunlight exposure showed good degradation efficacy. Among kaolin-based formulations after sunlight exposure, the highest degradation efficacy was obtained from K formulation (94.02%) and followed by KS (93.77%) and KN (93.57%) formulations (Figure 4.13). While among bentonite-based formulations, after sunlight exposure, the highest degradation efficacy was obtained from B formulations (93.22%), followed by BN (90.15%) and BNSP (88.83%) formulations (Figure 4.13).

The bentonite-based formulations incorporated with *P*-amino benzoic acid (BP and BNP) showed poor degradation efficacy if compared with the bentonite-based formulations added with sucrose (BSP and BNSP) and the kaolin-based formulations. This suggested that the clay-based carrier materials and sucrose might have more significant impact on petrol degradation. However, the addition of sucrose, non-fat skim milk and *p*-amino benzoic acid in various combinations to kaolin- and bentonite-based formulations did not significantly enhance or reduce the degradation activity by the UTAR EPA2 cells (Figure 4.13). *P*-amino benzoic acid as UV protectant did not improve petrol degradation, as the degradation efficacy between sunlight and non-sunlight exposed formulations within the same formulation was not significantly different (Table 4.6).

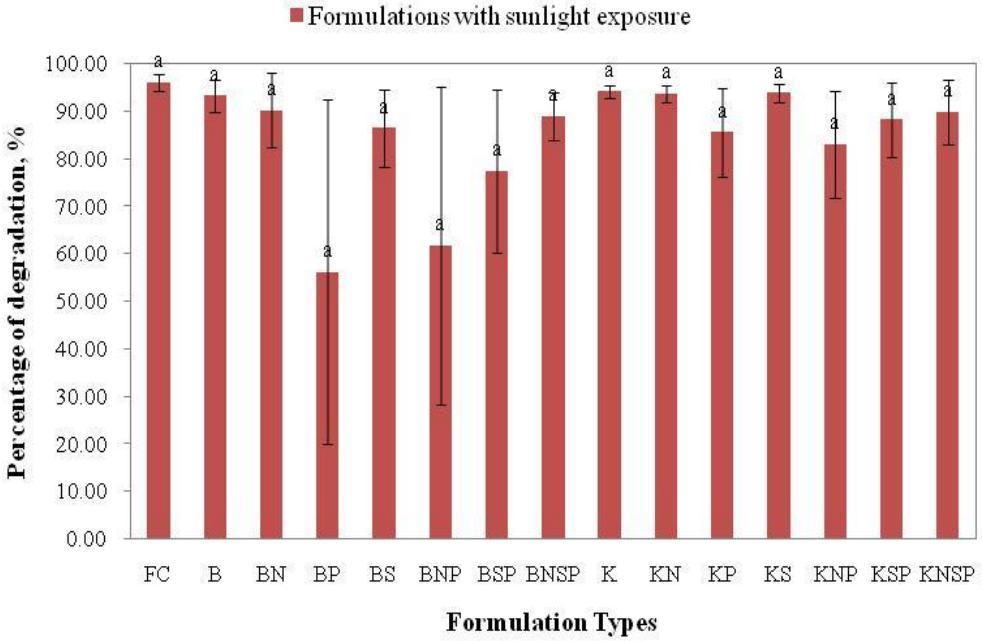

Figure 4.13: The percentage of petrol degradation by UTAR EPA2 cells in various formulations with exposure to 6 h of sunlight. Means with the same letters and captions are not significantly different ($HSD_{(0.05)}$). The bars on top of each column represent the standard error of the mean. (Note: B: bentonite; FC: free-cell formulation; K: kaolin; N: non-fat skim milk; P: *p*-amino benzoic acid and S: sucrose).

Table 4.6: Results of T-test for the comparison of the UTAR EPA2 efficacy in petrol degradation between the formulations exposed and without exposed to sunlight.

T-test	t Value	Pr > |t|
FC	-0.620**	0.597
B	-0.960**	0.439
BN	-1.630**	0.245
BP	1.080	0.392
BS	-0.070**	0.953
BNP	0.540	0.642
BSP	0.080	0.941
BNSP	-1.720**	0.228
K	-0.870**	0.476
KN	-0.950**	0.441
KP	-0.930**	0.449
KS	-0.900**	0.464
KNP	-1.010**	0.420
KSP	-0.930**	0.453
KNSP	-1.000**	0.424

Note: FC: Free-cell formulation; B: Bentonite clay; N: Non-fat skim milk; S: Sucrose; P: *p*-amino benzoic acid.

*: Statistically different ($p \leq 0.05$).

**: A negative t-value indicates a reversal in the directionality of the effect, which has no bearing on the significance of the difference between groups.

4.4.1.5 Degradation of Mineral Oil by Formulated UTAR EPA2 without Sunlight Exposure

Mineral oil is usually used to lubricate contact surfaces, dissipate heat, and remove ground materials from the contact site. Alkanes represent a majority of the mass in a typical mineral oil (Konrad, Biedermann, Caramanschi, & Pacciarelli, 1991). Alkanes such as nanodecane, octadecane, hexadecane and dodecane were used as the key compounds to quantify the amount of mineral oil which has been degraded in this study.

The mineral oil degradation percentage was the mean of six months mineral oil degradation percentage of a formulation without sunlight exposure. The

highest degradation efficacy was obtained from FC with the degradation percentage of 66.60 %. However, UTAR EPA2 in bentonite- (B, BN, BP, BS, BNP, BSP and BNSP) and kaolin-based formulations (K, KN, KP, KS, KNP, KSP and KNSP) showed no significant degradation efficacy to that of the cells in FC. The degradation efficacy of UTAR EPA2 cells was higher for cells in kaolin-based formulations if compared with the bentonite-based formulations, with collective percentages of 62.96% and 60.96%, respectively.

For the clay-based formulations, formulations containing clay only (B and K) showed better degradation efficacy when compared with other combinations. Among kaolin-based formulations, K formulation was able to produce the highest degradation efficacy toward mineral oil (66.53%) and was followed by KN and KNSP formulations, with the degradation efficacy of 65.10% and 62.69%, respectively (Figure 4.14). While among the bentonite-based formulations, B formulation showed the highest degradation efficacy (63.28%) and was followed by BNSP and BNP formulations, with the degradation efficacy of 62.73% and 61.74%, respectively (Figure 4.14). In mineral oil degradation study, addition of non-fat skim milk in formulations showed slightly better performance if compared to formulations added with sucrose and *p*-amino benzoic acid. However, the addition of sucrose, non-fat skim milk and *p*-amino benzoic acid in various combinations to kaolin- and bentonite-based formulations did not significantly enhance or reduce the degradation activity by the UTAR EPA 2 cells (Figure 4.14).

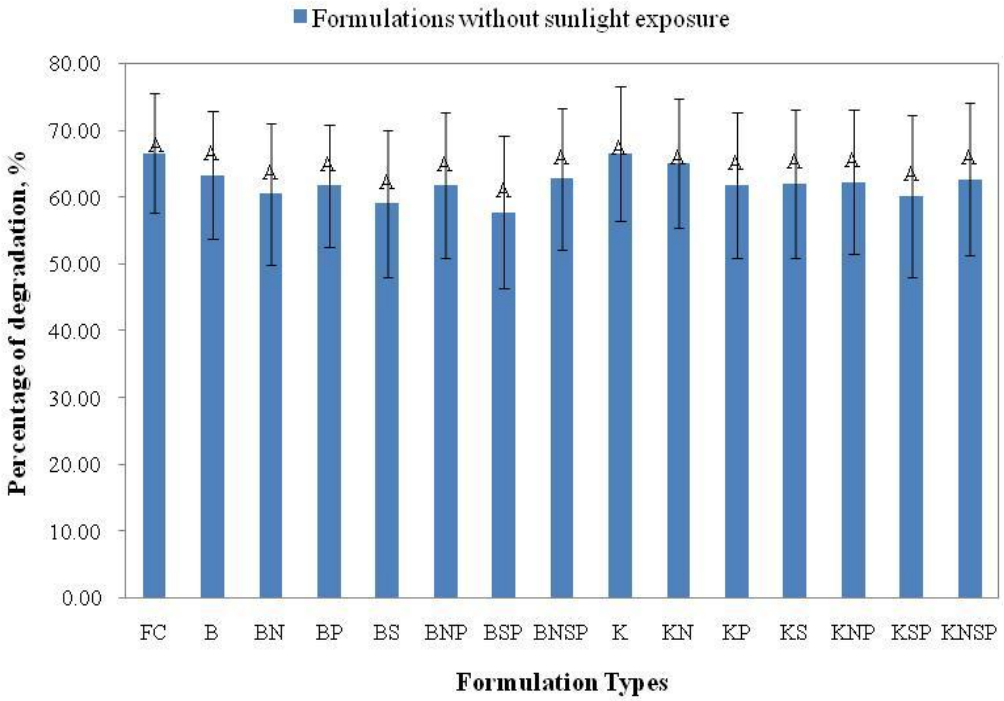

Figure 4.14: The percentage of mineral oil degradation by UTAR EPA2 cells in various formulations without exposure to 6 h of sunlight. Means with the same letters and captions are not significantly different ($HSD_{(0.05)}$). The bars on top of each column represent the standard error of the mean. (Note: B: bentonite; FC: free-cell formulation; K: kaolin; N: non-fat skim milk; P: *p*-amino benzoic acid and S: sucrose).

4.4.1.6 Degradation of Mineral Oil by Formulated UTAR EPA2 with Sunlight Exposure

In this assessment, the mineral oil degradation percentage was the mean of three months mineral oil degradation percentage of the formulation with sunlight exposure. The exposure of the formulations to the sunlight resulted in lower mineral oil degradation efficacy if compared with the formulations without sunlight exposure. Similarly, the FC formulation recorded the highest collective degradation percentage (56.79%) and was followed by kaolin-based formulations (48.57%) and bentonite-based formulations (42.17%). The collective degradation percentage was calculated as the average of the mean

mineral oil degradation percentages from all the bentonite-based formulations or kaolin-based formulation

Among the kaolin-based formulations, K formulation achieved the highest degradation efficacy (51.45%) and was followed by KN and KNP formulations, with 50.11% and 49.67% degradation efficacies, respectively (Figure 4.15). While for bentonite-based formulations, B formulation achieved the highest degradation efficacy (45.10%), followed by BNSP and BN formulations, with 43.94% and 43.53% degradation efficacies, respectively (Figure 4.15). Similar to the clay-based formulations without sunlight exposure, for the clay-based formulations after sunlight exposure, the formulations containing only clay showed better degradation efficacy compared with other combinations.

Addition of N in the formulations had shown slightly better performance if compared to the formulations containing sucrose and *p*-amino benzoic acid. However, the addition of sucrose, non-fat skim milk and *p*-amino benzoic acid in various combinations to kaolin- and bentonite-based formulations exposed to sunlight did not significantly enhance or reduce the degradation activity by the UTAR EPA2 cells (Figure 4.15). The use of *p*-amino benzoic acid did not improve the degradation efficacy after exposing the formulations to sunlight. The degradation efficacy between sunlight and non-sunlight exposed formulations for the same formulation was not significantly different (Table 4.7).

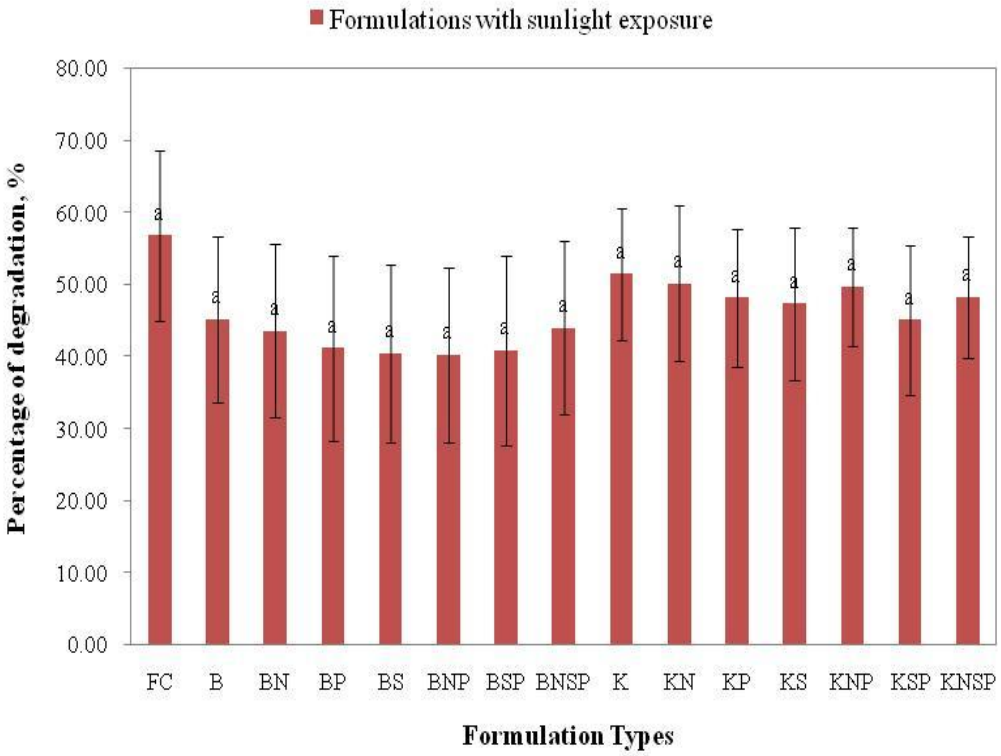

Figure 4.15: The percentage of mineral oil degradation by UTAR EPA2 cells in various formulations with exposure to 6 h of sunlight. Means with the same letters and captions are not significantly different ($HSD_{(0.05)}$). The bars on top of each column represent the standard error of the mean. (Note: B: bentonite; FC: free-cell formulation; K: kaolin; N: non-fat skim milk; P: *p*-amino benzoic acid and S: sucrose).

Table 4.7: Results of T-test for the comparison of the UTAR EPA2 efficacy in mineral oil degradation between the formulations exposed and without exposed to sunlight.

T-test	t Value	Pr > |t|
FC	-0.230**	0.841
B	2.250	0.154
BN	1.530	0.265
BP	1.460	0.282
BS	1.550	0.261
BNP	2.230	0.156
BSP	0.190	0.868
BNSP	0.160	0.889
K	-0.110**	0.920
KN	0.180	0.877
KP	-0.200**	0.857
KS	-0.360**	0.755
KNP	-0.490**	0.675
KSP	-0.350**	0.762
KNSP	-0.170**	0.882

Note: FC: Free-cell formulation; B: Bentonite clay; N: Non-fat skim milk; S: Sucrose; P: *p*-amino benzoic acid.

*: Statistically different ($p \leq 0.05$).

**: A negative t-value indicates a reversal in the directionality of the effect, which has no bearing on the significance of the difference between groups.

4.4.1.7 Efficacies of Formulated UTAR EPA2 on Degradation of Hydrocarbon Substrates

Based on the results obtained from sections 4.4.1.1 to 4.4.1.6, generally, toluene and petrol were more susceptible to degradation by UTAR EPA2 if compared to mineral oil. The degradation efficacy of each formulation in degrading hydrocarbon substrates was not significantly different among all the formulations investigated. Free-cell, bentonite-based and kaolin-based formulations were able to degrade hydrocarbon substrates with similar efficiency. In general, the degradation efficacy of formulated UTAR EPA2 between sunlight and non-sunlight exposed formulations was not significantly

different. This showed that exposing to the sunlight for 6 h might not affect the bacterial ability to degrade hydrocarbons.

Based on the results obtained, the formulations containing kaolin clay and enrichment materials (K, KN and KS) showed good efficacy hydrocarbon degradation for petrol and mineral oil samples if compared with the formulations containing *p*-amino benzoic acid (BP, BNP, BSP and KNP). The K formulation was the most suitable formulation for degrading petrol and mineral oil and BS was a potential formulation for toluene degradation.

4.4.2 Efficacy of Formulated UPM 39B3 in Inhibiting the Growth of *Fusarium oxysporum* f. sp. *cubense* race 4 (FocR4)

The appearance of formulations with or without sunlight exposure was recorded after they were added in the aqueous solution which containing FocR4. The visible changes of microbial formulations when they were used to treat the fungal pathogen were observed. Generally, the presence of *p*-amino benzoic acid and non-fat skim milk caused the mixed solution to turn yellowish or brownish in colour. For formulations containing bentonite clay, bentonite clay tends to form a grout after absorbing water (Figure 4.16 A) while kaolin-based formulations were precipitated (Figure 4.12 B). All bentonite-based formulations and KN formulation turned greyish (Figure 4.16). Certain parts of the tubes containing the B, BP and BS turned black (Figure 4.16 A). However, the reason for the colour changes of bentonite clay observed during the FocR4 growth inhibition study was unknown. For the tubes containing KN, KS and KSP formulations, a layer of fungal growth was observed on top of the solution (Figure 4.16 B).

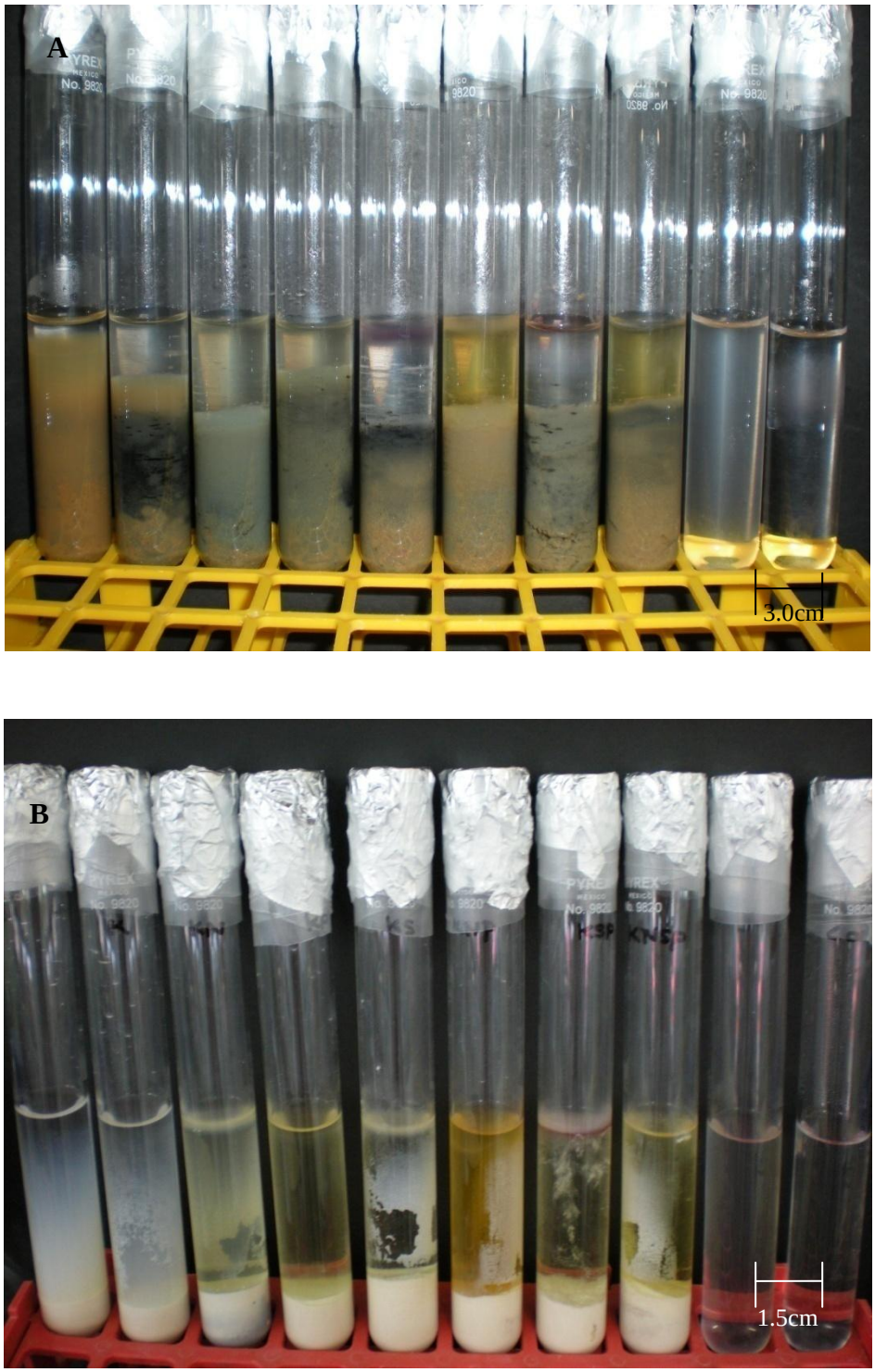

Figure 4.16: Physical appearance or changes of bentonite-based formulation (A) (from left to right: BC, B, BN, BP, BS, BNP, BSP, BNSP, FC and negative control) and kaolin-based formulation (B) (from left to right: KC, K, KN, KP, KS, KNP, KSP, KNSP, FC and negative control) during FocR4 inhibition.

In this assessment, the inhibition percentage of UPM 39B3 formulation toward FocR4 was the mean of six months inhibition percentages of the formulation without sunlight exposure. The inhibition percentage of UPM 39B3 formulation toward FocR4 was the mean of three months inhibition percentages of the formulation with sunlight exposure.

4.4.2.1 FocR4 Treated with UPM 39B3 Formulations without Sunlight Exposure

Based on the results obtained, FC formulation showed higher percentage of FocR4 inhibition, 19.41%, if compared with most of the clay-based formulations. Among clay-based formulations, bentonite-based formulations (B, BN, BP, BS, BNP, BSP and BNSP) showed higher percentage of FocR4 inhibition if compared with kaolin-based formulations (K, KN, KP, KS, KNP, KSP and KNSP) with collective inhibition percentage of 13.68% and 9.07%, respectively. Among kaolin-based formulations, KP formulation which contained *p*-amino benzoic acid showed the highest inhibition percentage (25.60%) and was followed by single kaolin (K) formulation (35.66%) (Figure 4.17). Similar with kaolin-based formulations, the BP formulation showed higher inhibition percentage (20.34%) and was followed by the B formulation (22.95%) which contained bentonite only (Figure 4.17).

Generally, cells formulated with only the carrier and additive materials (*p*-amino benzoic acid) in both clay-based formulations showed higher efficacy in inhibiting the growth of FocR4. In contrast, formulations containing enrichment materials such as non-fat skim milk or/and sucrose were less

effective in inhibiting the growth of FocR4. The inclination in inhibiting efficacy toward FocR4 was more obvious in kaolin-based formulations containing sucrose. In fact, higher number of FocR4 viable cells was recovered from KS, KSP and KNSP formulations which suggested that no inhibition occurred. In contrast, these formulations might stimulate the growth of FocR4 (Figure 4.17).

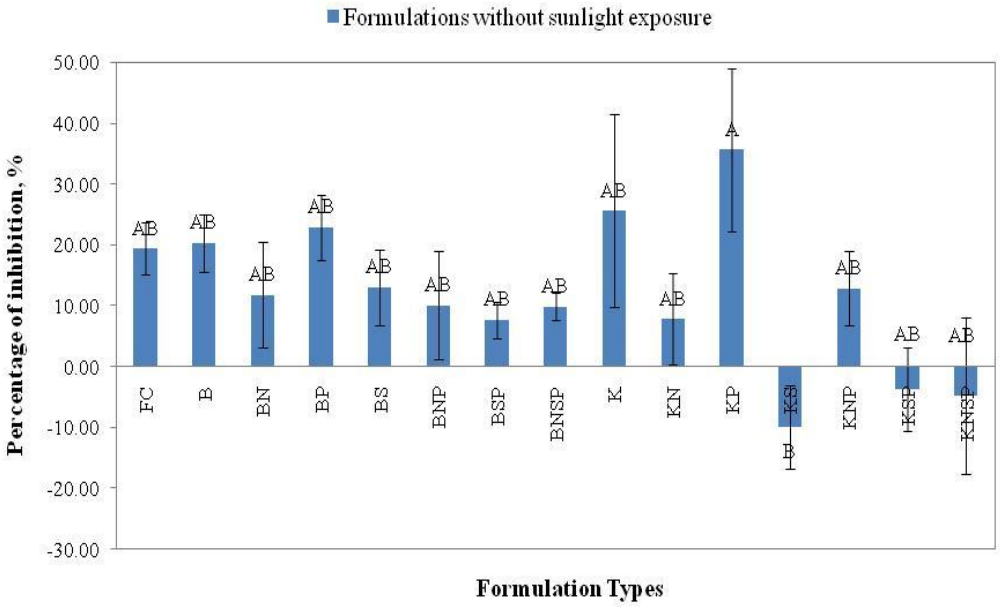

Figure 4.17: The percentage of FocR4 inhibition by UPM 39B3 in various formulations without exposure to 6 h sunlight. Means with the same letters and same captions are not significantly different ($HSD_{(0.05)}$). The bars on top of each column represent the standard error of the mean. (Note: B: bentonite; FC: free-cell formulation; K: kaolin; N: non-fat skim milk; P: *p*-amino benzoic acid and S: sucrose).

4.4.2.2 FocR4 Treated with UPM 39B3 Formulations with Sunlight Exposure

Formulated cells of UPM 39B3 recorded varying percentages of inhibition towards FocR4 and lower inhibition percentage were recorded from the

formulations with sunlight exposure (Figure 4.18). Collectively, for formulations after sunlight exposure, FC formulation showed higher inhibition efficacy (7.11%) if compared to all the clay-based formulations. Upon exposure to sunlight, kaolin-based formulations were more effective when compared to bentonite-based formulations, achieved 3.00% of collective inhibition and 0.01% of collective pathogen growth, respectively.

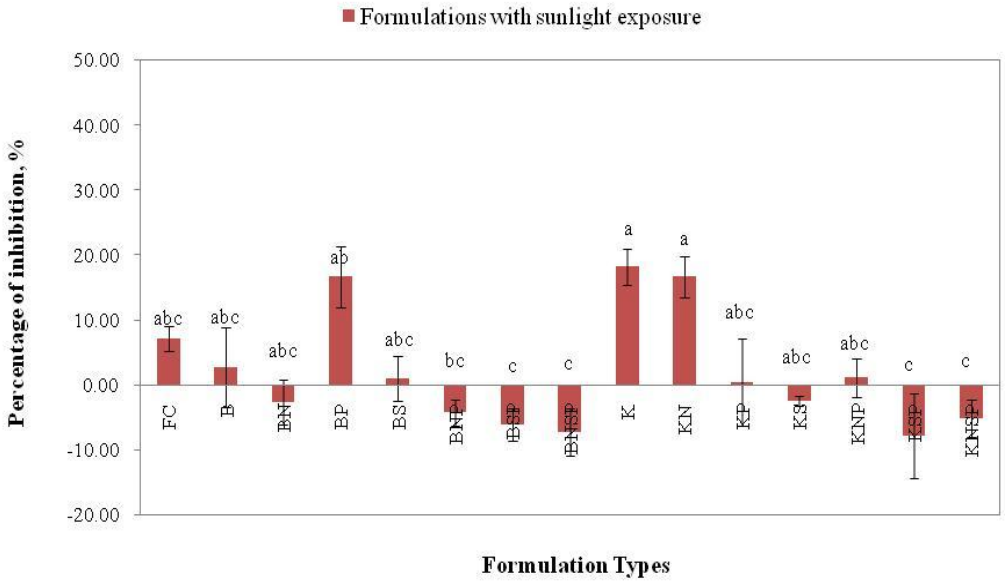

Figure 4.18: The percentage of FocR4 inhibition by UPM 39B3 in various formulations with exposure to 6 h of sunlight. Means with the same letters and same captions are not significantly different ($HSD_{(0.05)}$). The bars on top of each column represent the standard error of the mean. (Note: B: bentonite; FC: free-cell formulation; K: kaolin; N: non-fat skim milk; P: *p*-amino benzoic acid and S: sucrose).

The poor inhibition towards FocR4 by all the formulations investigated was more evident after exposure to sunlight for 6 hours, except KN formulation. Among the formulations with exposure to sunlight investigated, FC, B, BP,

BS, K, KN, KP and KNP formulations were able to inhibit FocR4 (Figure 4.18). The formulated cells with kaolin clay only (K) showed the highest efficacy (18.16%) in FocR4 inhibition and was followed by KN and BP formulations, with the inhibition percentage of 16.67% and 16.64%, respectively (Figure 4.18).

Comparisons between the inhibition percentages towards FocR4 for the formulations with and without exposure to sunlight, for the same formulation type, the results obtained showed that they were able to produce similar percentages of inhibition regardless to either with or without exposure to sunlight (Table 4.8). This suggested that the incorporation of *p*-amino benzoic acid in all the formulations, did not significantly improve the efficacy of formulated cells in inhibiting FocR4 upon UV exposure. However, incorporation of *p*-amino benzoic acid in formulation without enrichment materials showed better FocR4 inhibition as observed in BP and KP formulations. Besides, incorporation of enrichment materials, N and sucrose, were observed to enhance the growth of FocR4.

Irrespective to sunlight exposure (UV radiation), FC, B, BP, BS, K, KN, KP and KNP formulations were able to inhibit the growth of FocR4. Among them, BP, K and KN formulations produced higher inhibition efficacy regardless of with or without sunlight exposure treatments.

Table 4.8: Results of T-test for the comparison of the UPM 39B3 inhibition efficacy between the formulations exposed and without exposed to sunlight.

T-test	t Value	Pr > |t|
FC	0.740	0.536
B	4.480	0.046
BN	0.520	0.658
BP	-0.470**	0.687
BS	0.100	0.929
BNP	0.530	0.649
BSP	3.420	0.076
BNSP	4.080	0.055
K	0.390	0.735
KN	-0.700**	0.558
KP	1.520	0.268
KS	-0.810**	0.505
KNP	0.660	0.579
KSP	-0.530**	0.651
KNSP	-0.860**	0.481

Note: FC: Free-cell formulation; B: Bentonite clay; N: Non-fat skim milk; S: Sucrose; P: *p*-amino benzoic acid.

*: Statistically different ($p \leq 0.05$).

**: A negative t-value indicates a reversal in the directionality of the effect, which has no bearing on the significance of the difference between groups.

4.4.3 The Impact of Cell Viability on Cell Efficacy

In this study, the impact of various formulations with or without sunlight exposure on the cell viability and cell efficacy of isolates towards hydrocarbon degradation and FocR4 inhibition were investigated. The bacterial cell viability of formulation with and without sunlight exposure did not relate with its efficacy in most cases. Higher cell viability obtained from formulations with and without sunlight exposure may not necessary indicate that good efficacy may occur. For example, UTAR EPA2 formulated with bentonite clay only (B) produced significantly higher viability while UTAR EPA2 formulated with kaolin and *p*-amino benzoic acid (KP) produced significantly lower viability if compared with other formulations. However,

both formulations (B and KP) showed similar hydrocarbons degradation efficacy with other formulations. Besides, kaolin formulation of UPM 39B3 incorporated with *p*-amino benzoic acid (KP) had no viable cell recovered throughout this study. However, significantly higher percentage of FocR4 growth inhibition was observed using KP formulation.

Higher cell viability recorded from formulations exposed to sunlight may not necessarily yield better degradation or control efficacy. Generally, kaolin-based formulations which had significantly lower viable cell count for UPM 39B3 compared to bentonite-based formulations but had shown relatively higher degradation activity and inhibition efficiency towards FocR4 in this study. Addition of *p*-amino benzoic acid in the formulation caused reduction of cell viability in both UTAR EPA2 and UPM 39B3 isolates, however, did not affect much in their degradation activity and inhibition efficiency.

For UTAR EPA2, kaolin-based formulations without sunlight exposure (K, KN, KP, KS, KNP, KSP and KNSP) showed low collective mean cell viability of 3.15 $\log_{10}$ CFU/ ml, but recorded higher collective mean degradation activity (based on 3 months' results) for toluene, petrol and mineral oil, 94.89%, 78.35% and 47.07%, respectively (Table 4.9). While bentonite-based formulations without sunlight exposure (B, BN, BP, BS, BNP, BSP and BNSP) showed high collective mean cell viability of 5.51 $\log_{10}$ CFU/ml, but recorded a lower degradation activity (based on 3 months' results), 90.40%, 80.27% and 46.02%, for toluene, petrol and mineral oil degradation, respectively (Table 4.9).

Table 4.9: The collective mean viable cell counts of UTAR EPA2 in free-cell, bentonite- and kaolin-based formulations with and without sunlight exposure and their efficacy (based on 3 months' results) in degrading toluene, petrol and mineral oil.

	Free-cell Formulation		***Bentonite-based Formulations**		****Kaolin-based Formulations**	
	[a]Without	**[b]With**	**[a]Without**	**[b]With**	**[a]Without**	**[b]With**
Viable cell count, log_{10} CFU/ ml	6.97	2.70	5.51	4.44	3.15	2.57
Toluene degradation efficacy, %	96.65	98.18	90.40	90.42	94.89	96.78
Petrol degradation efficacy, %	93.81	95.93	80.27	79.08	78.35	89.67
Mineral oil degradation efficacy, %	54.20	56.79	46.02	42.17	47.07	48.57

Note: [a]Without: Without sunlight exposure; [b]With: with sunlight exposure
*: The collective mean value of B, BN, BP, BS, BNP, BSP and BNSP formulations.
**: The collective mean value of K, KN, KP, KS, KNP, KSP and KNSP formulations.

Upon sunlight exposure, lower viability was obtained from the formulations however better degradation efficacy was recorded. For kaolin-based formulations with sunlight exposure, only 2.57 log_{10} CFU/ ml was recorded but 96.78, 89.67% and 48.57% collective mean degradation activity for toluene, petrol and mineral oil were achieved (Table 4.9). On the other hand, the bentonite-based formulations with sunlight exposure recorded a lower collective mean degradation activity for toluene, petrol and mineral oil degradation, 90.42%, 79.08% and 42.17%, respectively (Table 4.9) even though collective mean cell viability was higher at 4.44 log_{10} CFU/ml. Free-cell formulation (FC) with sunlight exposure had good degradation activity, although the number of viable cells was significantly reduced from 6.97 log_{10}

CFU/ ml (without sunlight exposure) to 2.70 $\log_{10}$ CFU/ ml (with sunlight exposure) (Table 4.9). Similarly, kaolin-based formulations containing *p*-amino benzoic acid with sunlight exposure showed poor viability, yet good degradation efficacy was observed, as in KP, KNP, KSP and KNSP formulations with sunlight exposure (Figure 4.8 and Table 4.10).

Table 4.10: The mean toluene, petrol and mineral oil degradation percentages (based on 3 months' results) of UTAR EPA2 formulations with and without sunlight exposure and their mean viable cell counts.

Formulation	Mean Viability (log_{10} CFU/ ml)		Hydrocarbon Substrates	Mean Degradation Percentage (%)	
	[a]Without	[b]With		[a]Without	[b]With
FC	6.97	2.70	Toluene	96.65	98.18
			Petrol	93.81	95.93
			Mineral Oil	54.20	56.79
B	7.35	6.58	Toluene	89.09	86.42
			Petrol	73.74	93.22
			Mineral Oil	53.42	45.10
BN	6.70	6.48	Toluene	91.00	92.50
			Petrol	88.41	90.15
			Mineral Oil	47.85	43.53
BP	4.58	2.33	Toluene	88.66	90.19
			Petrol	80.45	56.04
			Mineral Oil	49.39	41.15
BS	6.60	5.98	Toluene	93.68	99.40
			Petrol	86.09	86.37
			Mineral Oil	42.83	40.39
BNP	3.34	3.29	Toluene	88.03	81.99
			Petrol	74.64	61.64
			Mineral Oil	42.83	40.23
BSP	5.24	2.53	Toluene	90.22	88.63
			Petrol	78.69	77.34
			Mineral Oil	41.23	40.84
BNSP	4.77	3.89	Toluene	92.12	93.79
			Petrol	79.83	88.83
			Mineral Oil	44.42	43.94

Note: B: bentonite; FC: free-cell formulation; N: non-fat skim milk; P: *p*-amino benzoic acid, S: sucrose, [a]Without: without sunlight exposure and [b]With: with sunlight exposure

Table 4.10 (continued): The mean toluene, petrol and mineral oil degradation percentages (based on 3 months' results) of UTAR EPA2 formulations with and without sunlight exposure and their mean viable cell counts.

Formulation	Mean Viability log_{10} CFU/ ml		Hydrocarbon Substrates	Mean Degradation Percentage, %	
	[a]Without	[b]With		[a]Without	[b]With
K	5.91	4.98	Toluene	94.29	98.19
			Petrol	85.58	94.02
			Mineral Oil	50.42	51.45
KN	6.04	4.92	Toluene	95.13	98.54
			Petrol	74.35	93.57
			Mineral Oil	51.19	50.11
KP	0.00	0.61	Toluene	94.15	95.34
			Petrol	78.35	85.46
			Mineral Oil	46.52	48.15
KS	6.88	5.84	Toluene	95.64	97.38
			Petrol	82.42	93.77
			Mineral Oil	45.50	47.31
KNP	1.57	0.53	Toluene	93.09	93.60
			Petrol	71.86	82.95
			Mineral Oil	46.40	49.67
KSP	1.62	1.08	Toluene	96.33	97.88
			Petrol	77.68	88.11
			Mineral Oil	42.67	45.03
KNSP	0.00	0.00	Toluene	95.59	96.55
			Petrol	78.18	89.82
			Mineral Oil	46.81	48.25

Note: B: bentonite; FC: free-cell formulation; N: non-fat skim milk; P: *p*-amino benzoic acid, S: sucrose, [a]Without: without sunlight exposure and [b]With: with sunlight exposure

Similarly, for isolate UPM 39B3, higher cell viability may not necessary lead to higher FocR4 inhibition. Results in the efficacy assessment also indicated that the incorporation of enrichment materials especially sucrose into all formulations with sunlight exposure resulted in the increase of FocR4 viable cells instead of an inhibition. This clearly showed that sucrose might be beneficial for the growth of both UPM 39B3 and FocR4. The results also showed that *p*-amino benzoic acid exhibited antimicrobial effect to both bacterial and fungal isolates. Clay-based formulations with *p*-amino benzoic acid (BP and KP) recorded lower cell viability but higher inhibiting efficacy (Table 4.11).

The formulative materials were advantages in protecting the formulated cells from sunlight exposure as higher cell viability was observed if compared to FC formulation. However, their role in enhancing the inhibition efficacy towards FocR4 was not significant. Higher viability of cells remained from formulations upon sunlight exposure was not necessarily efficient in inhibiting FocR4 (Table 4.11). There were no clear improvements on the inhibitory effect towards FocR4 upon the introduction of the formulated cells if compared to FC formulation in both conditions, with or without sunlight exposure (Table 4.11). FC formulation showed good inhibition efficacy with or without exposure to sunlight, 7.11% and 19.41%, respectively (Table 4.11).

Table 4.11: The mean FocR4 inhibition percentage (based on 3 months' results) of UPM 39B3 in each formulation without and with sunlight exposure and their mean viable cell counts.

Formulation	Sunlight Exposure	Mean Viable Cell Counts, $\log_{10}$ CFU/ ml	FocR4 Inhibition Percentage, %
FC	Without	5.99	13.34
	With	1.82	7.11
B	Without	3.13	18.02
	With	2.30	2.66
BN	Without	4.38	5.55.
	With	2.57	-2.67*
BP	Without	1.43	14.92
	With	1.21	16.64
BS	Without	3.98	1.99
	With	2.49	1.04
BNP	Without	2.67	4.06
	With	2.16	-4.30*
BSP	Without	4.23	6.71
	With	2.73	-6.11*
BNSP	Without	3.67	8.08
	With	2.23	-7.30*
K	Without	0.00	30.78
	With	0.00	18.16
KN	Without	0.00	5.73
	With	0.00	16.67
KP	Without	0.00	48.43
	With	0.00	0.42
KS	Without	0.56	-13.94*
	With	0.37	-2.40*
KNP	Without	0.00	7.30
	With	0.00	1.14
KSP	Without	0.00	-9.83*
	With	0.00	-7.80*
KNSP	Without	0.54	-15.21*
	With	0.34	-5.21*

Note: B: bentonite; FC: free-cell formulation; K: kaolin; N: non-fat skim milk; P: *p*-amino benzoic acid and S: sucrose

*: enhancement of FocR4 growth

CHAPTER 5

DISCUSSION

5.1 Identification of Beneficial Isolates UTAR EPA2 and UPM 39B3

Isolates UTAR EPA2 and UPM 39B3 used in this study were previously isolated from hydrocarbons polluted environments (Tan, 2007) and wild banana (Ting et al., 2004), respectively. Through colony morphology, BIOLOG system and molecular tools, both isolates were identified. These three methods are commonly used to identify unknown bacterial isolates. For example, the colony morphology, 16S rRNA gene sequencing and BIOLOG analysis were also used to identify a *Pseudomonas* strain which was capable of utilising carbendazim as the sole source of carbon and energy (Fang, Wang, Gao, Yan, Dong, & Yu, 2010).

As different types of bacteria have similar colony morphology, colony morphological observation alone is not a reliable way to identify bacteria (Leboffe & Pierce, 2010). However, the special and unique colony visible characteristics are able to provide information that aids in bacterial identification. For example, *Serratia. plymuthica*, *Serratia rubidaea* and *Serratia marcescens* are able to produce water insoluble red pigment, prodigiosin (Grimont, Grimont, Dulong De Rosnay, & Sneath, 1977; Stratton, 1983). Besides, the ability to produce water-soluble and diffusible pigments is a primary characteristic used in identification of *Pseudomonas* species. For example, *P. aeruginosa* produces a bluish-green pigment consists of blue

pyocyanine (Howard & Dedman, 1964). The red pigmentation of UPM 39B3 colonies and green-pigmented UTAR EPA2 colonies observed in this study did provide preliminary information for identifying these isolates.

Another bacterial identification method based on the carbon source utilisation, BIOLOG system, was also employed in this study because this system is easy to use, update, and customize for specific needs (Klingler, Stowe, Obenhuber, Groves, Mishra, & Pierson, 1992). The BIOLOG identification system is a bacterial identification method that establishes identification based on the exchange of electrons generated during respiration, leading to a subsequent tetrazolium-based color change (Miller & Rhoden, 1991). This system tests the ability of a microorganism to oxidize a panel of 95 different carbon sources (Miller & Rhoden, 1991). Generally, BIOLOG identification is accepted as correct if the similarity index of the genus and species is 0.750 or greater at 4 h of incubation or 0.500 or greater at 24 h of incubation (Klingler et al., 1992). Among three common phenotypic identification methods, BIOLOG identification proved more effective than fatty acid methyl ester (FAME) profile and analytical profile index (API) identification in identifying isolates of *Serratia* species where positive identification is obtained regularly (Ashelford, Fry, Bailey, & Day, 2002).

As the species metabolic activities may also exhibit variability, genotypic identification such as 16S rRNA gene sequencing was used to complement the phenotypic methods (Tang, Ellis, Hopkins, Smith, Dodge, & Persing, 1998). In this study, a species-specific primer pair was used to amplify the 16S rRNA

gene of *Serratia marcescens* (Wilson et al., 1999). The amplified sequence was then aligned with other bacterial sequences that deposited in Genbank by using BLASTN program. Similar procedure was also used to characterize isolates of *Lactobacillus* and *Bifidobacterium* which were isolated from processed milk. Species-specific primers for *Lactobacillus* and *Bifidobacterium* were designed to amplify the 16S rRNA gene as a conserved region in the bacterial DNA (Hashem, Sabit, Amin, Tawakkol, & Shamseldin, 2010). Results of BIOLOG and molecular identification obtained in this study both showed that isolate UPM 39B3 was *Serratia marcescens.*

However, there are cases where preliminary result from BIOLOG system deviated from molecular identification as closely related isolate may show similar carbon source utilisation characteristics. Molecular identification showed isolate UTAR EPA2 was *Pseudomonas aeruginosa* in which the BIOLOG analysis showed that UTAR EPA2 was *Pseudomonas oryzihabitans* (formerly known as *Flavimonas oryzihabitans*). Conventional methods are based on the recognition of differences in morphology, growth, enzymatic activity, and metabolism to define genera and species (Petti, Polage, & Schreckenberger, 2005). However, these characteristics are not static and can change with stress or evolution (Ochman, Lerat, & Daubin, 2005). The 16S rRNA gene sequencing (molecular identification), in contrast, is a highly accurate, consistent and resourceful method for identification of bacteria to the species level if compared with traditional identification methods (Tang et al., 1998). Due to higher accuracy than BIOLOG system, UTAR EPA2 was identified as *Pseudomonas aeruginosa*. In addition, the morphology of the

bacterial colony observed for UTAR EPA2 was also similar with these characteristic of *Pseudomonas aeruginosa*.

5.2 Formulation of Microbial Isolates

In this study, although both clay-based formulations were prepared using similar methods, the amount of bentonite clay and kaolin clay (carrier materials) used to formulate bacterial isolates was different. Fifty percent lesser bentonite clay was needed to formulate the bentonite-based formulations than the kaolin clay used to formulate kaolin-based formulations. However, bentonite-based formulations had better mechanical strength if compared with kaolin-based formulation. Higher mechanical strength of a solid formulation has higher ability to withstand an applied stress.

Kaolin-based formulations, which can be easily crushed into powder, is less preferred as it needs careful handling and possess hazard to human inhalation system if compared to granule. This showed that kaolin clay used was a weaker material to provide enough mechanical strength for the kaolin-based formulations. For further improvement, incorporation of binders such as gums, molasses, polyvinyl pyrrolidone (PVP) or resins in the formulation might aid in binding the particulates in kaolin granules together (Teera-Arunsiri, Suphantharika, & Kentunuti, 2003).

Bentonite is known to be a suspending and colloidal agent for various manufacture industries (Ash & Ash, 2004). The viscosity of an aqueous medium is increased substantially when a small amount of bentonite is added

(Clem & Doehler, 1961). The ability of the bentonite to absorb a large quantity of water makes it easy to prepare the formulation with a small amount (Adamis et al., 2005). This made bentonite clay a cost effective carrier material for many applications as less bentonite is required in producing a formulation with sufficient mechanical strength.

5.3 Viability of Formulated Cells in Various Formulations

5.3.1 Influence of Carrier Materials

In this study, addition of bentonite and kaolin clays in both UTAR EPA2 and UPM 39B3 formulations without sunlight exposure, except B formulation of UTAR EPA2, did not showed significant enhancement of cell viability if compared with the free-cell formulation without sunlight exposure. Without sunlight exposure, FC formulation of both isolates showed similar or higher cell viability when compared to most of the clay-based formulations. However, after 6 hours of sunlight exposure, FC formulation of both isolates recorded significant decrease in cell viability while most of the clay-based formulations were able to showed similar cell viability with or without sunlight exposure. Generally, addition of both carrier and enrichment materials especially bentonite clay and sucrose into formulations did aid in maintaining the cell viability when the formulations were exposed to sunlight. It is important to maintain viability of the formulated beneficial microbes exposed to sunlight because they might be used for environmental applications.

From the results obtained, the kaolin clay did not able to improve cell viability when it was used to formulate both isolates especially isolate UPM 39B3. On

the other hand, bentonite clay was beneficial when it was used to formulate the bacterial cells especially isolate UTAR EPA2 as significantly higher cell viability was recovered. The B formulation of UTAR EPA2 without and with sunlight exposure recorded cell viability of 7.35 $\log_{10}$ CFU/ ml and 6.58 $\log_{10}$ CFU/ ml, respectively. For all the formulations investigated with or without sunlight exposure, cell viability was higher in the bentonite-based formulations than in the kaolin-based formulations. For example, both UTAR EPA2 and UPM 39B3 isolates had higher viability in B formulation than K formulation.

Bentonite clay has higher cation exchange capacity and absorption efficacy compared with kaolin clay (Adamis et al., 2005). High cation exchange capacity might enhance bacterial metabolic activity (Beveridge, 1988) leading to higher viability. High cation exchange capacity of clay particles allows binding and exchanging of biologically important substances such as nutrients and toxic metabolic waste products via cation and ligand exchange processes (Jakson, 1995). This interaction is especially helpful for attached bacteria as it aids in buffering and maintaining the system pH within the optimal range for bacterial growth (Stotzky, 1986; Beveridge, 1988). High adsorption ability enables the molecular binding of inorganic cations, organic cations and bio-organic particles. This might explain why higher cell viability was observed for cell formulated using bentonite clay (bentonite-based formulations) than the kaolin clay (kaolin-based formulations). The high adsorption efficacy of bentonite clay was not only to the surface but also between the layers of clay (Adamis et al., 2005), which might provide higher surface reactivities (Rong,

Huang, & Chen, 2007). In contrast, kaolin clay has lower adsorption efficacy which is limited to the surface of the particles (Adamis et al., 2005). As such, the cells, enrichment and additive materials added to kaolin-based formulations may not be held strongly between the layers of kaolin. This might have resulted in poor formulated cell viability especially for isolate UPM 39B3.

When formulations were exposed to sunlight, it was observed that there was a significant effect of the different types of carrier materials on the viability of tested bacterial isolates. The presence of clay-based carriers might have conferred protection to the cells. This could be observed when the formulations were exposed to sunlight. Both isolates (UTAR EPA2 and UPM 39B3) in free-cell formulation with sunlight exposure recorded a significant reduction of cell viability. Clays such as bentonite and kaolin show potential for UV protection through absorption or reflection of UV radiation (Hoang-Minh, Le, Kasbohm, & Giere, 2010). In addition, the clay itself is a good carbon source with various necessary minerals (Tu & Randall, 2005). Most of the clay-based formulations with sunlight exposure were able to maintain similar cell viability. It showed that bentonite clay and kaolin clay added in the formulation not only functioned as carriers but also as the potential UV-protection agents.

However, kaolin-based formulations with sunlight exposure were again less effective in sustaining the cell viability of isolate UPM 39B3. Bentonite clay was more suitable for maintaining the cell viability for both isolates

investigated in this study. Bentonite clay, which has high cation exchange and buffering capacities, might be an ideal carrier material that is able to provide protection for the cells against toxic compounds and adverse environmental conditions (Gentry et al., 2004).

Besides, bentonite clay is also known for its temperature and desiccation protectant effects (Vettori et al., 2000; Tu & Randall, 2005). Bentonite clay in the formulation acts as physical barrier and prevents the cells from dehydration when the microbial formulations were exposed to the sunlight. This might also due to the strong absorptive capability and large surface areas formed in bentonite clay which acts as an effective survival unit for microbial cells and the nutrient absorption when exposed to sunlight (Lunsdorf, Erb, Abraham, & Timmis, 2000). The cells entrapped in the bentonite-based carrier material were able to withstand the sunlight upon exposure. As a result, higher cell viability was recovered from bentonite-based formulations than the free-cell and kaolin-based formulations after exposure to sunlight.

In this study, addition of carrier materials in formulations with or without sunlight exposure showed more beneficial effect on cell viability of isolate UTAR EPA2. Besides, bentonite clay added in the formulations had significantly enhanced the cell viability of isolate UTAR EPA2 in the formulations either with or without sunlight exposure. The isolate UTAR EPA2 had better viability than isolate UPM 39B3, in the same type of clay, might due to different bacterial isolates showed different degree of tolerance and preference toward the type of clay used as the carrier material (Ekschmitt

& Griffiths, 1998). The K formulation of UTAR EPA2 without and with sunlight exposure recorded cell viability of 5.91 $\log_{10}$ CFU/ ml and 4.98 $\log_{10}$ CFU/ ml, respectively. On the other hand, no viable cell was recovered from K formulation of UPM 39B3 with or without sunlight exposure.

5.3.2 Influence of Enrichment Materials

Sucrose and non-fat skim milk are common enrichment materials providing necessary nutrients especially carbon source to support the cell growth (Lee & Chang, 1993). Addition of both sucrose and non-fat skim milk in the formulations showed beneficial effect to UTAR EPA2 and UPM 39B3 cells as high viability was observed in BN, BS, KN and KS formulations. Generally, there was no significant benefit to formulate UTAR EPA 2 using non-fat skim milk and sucrose as bentonite clay only (B) formulation was able to achieve higher cell viability for isolate UTAR EPA2. However, sucrose showed better effect in enhancing cell viability of isolate UPM 39B3 for the formulations containing non-fat skim milk, especially for the kaolin-based formulations that contained non-fat skim milk.

When comparing with the cell viability between the formulation with or without sunlight exposure, similar cell viability was observed for both isolates formulated with enrichment materials. The enrichment material might provide beneficial effect to the formulated isolates UTAR EPA2 and UPM 39B3 that were exposed to sunlight by reducing the adverse effect of sunlight. Sucrose and non-fat skim milk are also known for their potential function as the rehydration medium in preventing severe dehydration of cells upon sunlight

exposure (Costa et al., 2000; Gouffi & Blanco, 2000). By exposing to sunlight, DNA damage and dehydration occurred due to UV radiation and the heat generated would have greatly affecting the viability of the cells. A study had shown that the effect of desiccation was more pronounced in bacterial cells in the presence of light because of photooxidative reactions catalyzed by residual water (Potts, 1994). Most bacteria such as *Pseudomonas* (Dickgiesser, 1978; Rokitko, Romanovs'ka, Malashenko, Iu, & Chorna, 2004) and *Serratia* species (Dickgiesser, 1978) are sensitive to increased temperature, drying and UV radiation when they are exposed to the sunlight. Thus, exposing the cells directly to sunlight would cause the reduction of cell viability and alteration of cell metabolism.

The membrane lipid bilayer is one of the primary cellular components affected by the hydration level. As water is removed from the biomembrane, the biomembrane is forced into the gel phase at room temperature. When rehydrated, the membrane undergoes a phase transition and as a result it can become leaky (Berner & Viernstein, 2006). Thus, the variations in the hydration level could cause changes in lipid packing that may have detrimental effects on cell viability (Scherber, Schottel, & Aksan,, 2009). The beneficial effect of rehydration medium such as N and sucrose is ascribed to the replacement of water at the membrane structure during dehydration (Crowe, Whittam, Chapman, & Crowe, 1984b). For example, when non-fat skim milk was used as the rehydration medium, highest survival of *Pantoea agglomerans* from spray-drying process was obtained (Costa et al., 2000). It showed that 1- 10% non-fat skim milk was able to rehydrate the injured cells

after spray drying and recovered 100% cell viability (Costa et al., 2000). A study also showed the non-reducing disaccharides sucrose was able to protect the cell membrane of *Escherichia coli* from dehydration damage by depressing the melting temperature of the phase transition; when water is removed from the phospholipid bilayer by forming hydrogen bounds with the polar headgroups (Crowe, Crowe, & Chapman 1984a). Thus, it maintains the dried membranes in a physical state similar to that of fully hydrated membranes. In addition, non-fat skim milk and sucrose are more economical materials than other complex and expensive rehydration medium such as trehalose.

5.3.3 Influence of Additive

Both isolates, UTAR EPA2 and UPM 39B3, formulated with *p*-amino benzoic acid (P) especially in BP and KP showed lower viable cell recovery. *P*-amino benzoic acid occurs naturally as an intermediate in the bacterial synthesis of folic acid and frequently served as UV protectant especially in biopesticides (Hadapada, Hirea, Vijayalakshmia, & Dongre, 2009). In contrast to the hypothesis that incorporation of additive with UV protection property aids in sustaining the cell viability upon sunlight exposure, incorporated of *p*-amino benzoic acid into formulations showed antimicrobial properties toward the tested isolates.

In this study, approximately 0.62- 0.83% (w/w) of *p*-amino benzoic acid added into the clay-based formulations showed antibacterial activity against UTAR EPA2 and UPM 39B3. Studies on the antibacterial activity of *p*-amino

benzoic acid against *Listeria monocytogenes*, *Salmonella enteritidis* and *Escherichia coli* also showed that *p*-amino benzoic acid had inhibitory effects on cell viability (Richards, Xing, & King, 1994). This might explain why no significant improvement of cell viability from the formulation containing *p*-amino benzoic acid upon sunlight exposure but lower cell viability was observed.

By comparing the kaolin-based and bentonite-based formulations containing *p*-amino benzoic acid with or without sunlight exposure, the detrimental effect of *p*-amino benzoic acid on the cell viability was less severe when added in bentonite-based formulations. This suggested the bentonite clay with high cation exchange capacity, buffering capability, adsorptive capability and surface area not only protect the cells from sunlight's UV radiation but might also play an important role in reducing the antibacterial activity of *p*-amino benzoic acid against the isolate cells. A study also showed clay can provide a barrier to contaminant migration (Peterson & Gee, 1985).

5.4 Efficacy of Formulated Cells in Various Formulations

5.4.1 Efficacy of Formulated UTAR EPA2 in Degrading Hydrocarbon Substrates

Results obtained in this study indicated that there was no significant difference between efficacies of free-cell formulation and formulated isolate UTAR EPA2 with or without sunlight exposure in degrading tested hydrocarbon substrates (toluene, petrol and mineral oil). The incorporation of sucrose, non-fat skim milk and *p*-amino benzoic acid in various combinations to kaolin- and bentonite-based formulations with or without sunlight exposure did not

significantly enhance or inhibit the isolate UTAR EPA2 hydrocarbons degradation activity. The addition of formulative materials did not improve the degradation efficacy of formulated isolate UTAR EPA2 cells for both the sunlight and non-sunlight exposed formulations except BS formulation in toluene degradation but the difference was not significant.

Toluene serves as one of the principal models for understanding the mechanisms of bacterial benzene ring metabolism (Wackett, 2000). Toluene can be degraded by microbes through aerobic or anaerobic pathways (Wackett, 2000). Many studies have been conducted on the degradation of toluene by *P. alcaligenes*, *P. putida*, *P. Mendocina* and *P. pickettii*, where intermediate metabolite produced vary from the *Pseudomonas* species and the pathway (Wackett, 2000). Although there was an enhancement of toluene degrading activity by bentonite formulation incorporated with sucrose (BS), kaolin formulations with sucrose did not give the same effect. This might suggest that the clay-based carrier materials might play a more important role in affecting the degradation process than the enrichment and additive materials incorporated. This might be due to the large surface area and the presence of exposed cations on the surface of the clay minerals that can enhance degradation and many studies used clays as the natural catalyst (Johns, 1979). For example, montmorillonite (bentonite) and kaolinite are commonly used to aid in the catalytic cracking and breaking of long hydrocarbon molecules into shorter hydrocarbons (Rong & Xiao, 2002; Adamis et al., 2005). The catalytic properties can be further improved by using acid-treated clays (Chitnis & Sharma, 1997).

In this study, all formulations with or without sunlight exposure of isolate UTAR EPA2, including free-cell formulation, produced good hydrocarbon degradation efficacy especially for toluene and petrol degradation. This might due to the fact that mineral oils are extremely complex mixtures of C_{20}–C_{50} hydrocarbons containing a range of linear alkanes, branched alkanes, cycloalkane, olefinic, and aromatic hydrocarbons (Salimon, Salih, & Yousif, 2010). In contrast, petrol consists mostly of C_5-C_{12} alkanes and smaller amounts of cycloalkane and aromatic compounds such as ethylebenzene, toluene, benzene and xylene (Nithya, 2010). Thus, it was expected that the petrol sample was easier to degrade if compared to mineral oil. In addition, the heteroatoms (mainly sulphur), longer cycloalkane and heavier aromatic hydrocarbons in mineral oil sample are more difficult to degrade if compared to simple aromatic (toluene and benzene) and short aliphatic hydrocarbons (Jobson, McLaughlin, Cook, & Westlake, 1974; Perry, 1984).

In this study, isolate UTAR EPA2 which was identified as *Pseudomonas aeruginosa* was known to be a good hydrocarbon degradation agent. The presence of different metabolic pathways in *Pseudomonas aeruginosa* is favourable for hydrocarbon bioremediation. *Pseudomonas aeruginosa* can assimilate a wide range of hydrocarbons, from simple n-alkanes and aromatics (toluene), polyaromatics (naphthalene) to a mixture of various hydrocarbons (crude oil) (Perez-Silva, Rodriguez, Gomez Montes de Oca, & Moreno, 2006). The metabolic pathways of mono and poly aromatics degradation by *Pseudomonas aeruginosa* are more complex, because different hydroxylated intermediates might form depend on the compounds present in the mixture of

hydrocarbons (Perez-Silva et al., 2006). The complete oxidation reaction produces carbon dioxide and water as the end products. However, under anaerobic conditions, the hydrocarbon substrates are converted to carbon dioxide and methane by the bacteria (Dunja & Vogel, 1987) and the rate of hydrocarbon degradation is lower by most of the aerobic bacterial strains under anaerobic conditions (Bauer & Capone, 1985). The isolate UTAR EPA2 (*Pseudomonas aeruginosa*, aerobic bacteria), is capable to degrade hydrocarbon more readily under aerobic conditions (Gibson, 1980) if compared with anaerobic conditions (Bauer & Capone, 1985). Oxygen is generally necessary for the initial breakdown of hydrocarbons, and subsequent reactions may also require direct incorporation of oxygen (Lee & Levy, 1989).

The biodegradation processes of hydrocarbons by pure culture are also limited by several factors. For example, the volatilization of hydrocarbon substrate and low aqueous solubility of hydrocarbons (Means, Ward, Hassett, & Banwart, 1980) have limited availability of hydrocarbon substrates to the microorganisms (Providenti, Flemming, Lee, & Trevors, 1995). Hydrocarbons are very insoluble in water and bacteria optimize their uptake by producing biosurfactants that help to emulsify the hydrocarbons, or by specific adhesion mechanisms that allow them to physically contact with the oil phase (Rosenberg & Ron, 1996; Bouchez-Naitali, Rakatozafy, Marchal, Leveau, & Vandecasteele, 1999). For example, some isolates of rhamnolipid-producing (surface active glycolipid) *P. aeruginosa* strain were able to synthesize a biosurfactant that emulsified the organic phase in the presence of alkanes (Yuste, Corbella, Turiegano, Karlson, Puyet, & Rojo, 2000). The isolate

UTAR EPA2, which was identified as *P. aeruginosa* might also be able to produce similar biosurfactant which assists the hydrocarbon degradation. Furthermore, better surface contact between the bacteria and hydrocarbon substrates might have improved through using formulated cells in this study.

Eventually, the formulations will be applied to the environment. Bentonite-based formulations might be preferred because they might be able to physically agglomerate the hydrocarbon which dispersed away by water movement. In most of the studies, hydrocarbon bioremediation are performed by applying formulated microbes with nutrients to the hydrocarbon polluted land or marine (Raghaven & Vivekanandan, 1999; Shome, Shome, & Bandyopadhyay, 1996) to sustain the growth of the microbes which helps in degrading the hydrocarbons (Thomas, Ward, Raymond, Wilson, & Loehr, 1992). However, the potential of using clay-based granular formulations in hydrocarbons bioremediation is not well studied. Thus, how the binding and adsorption properties of clay-based materials could be an advantage in hydrocarbon bioremediation is not clear.

Based on the observation from this study, hydrocarbons substrate floated and occupied the top layer of the aqueous solution if FC and kaolin-based formulations were added into the aqueous solution containing hydrocarbon substrate (toluene, petrol or mineral oil). In contrast, both hydrocarbon substrates and aqueous solution were absorbed by the bentonite-based formulations and formed a grout. Thus, there was a possibility that the volatile hydrocarbons in the samples might loss easier during the degradation process

in the free-cell and kaolin-based formulations. The bentonite clay has greater adsorption activity where volatile hydrocarbons might bind more effectively to the clay preventing them from evaporation. This might cause slightly higher hydrocarbon residues detected for the hydrocarbon substrates treated with bentonite-based formulations than the free-cell and kaolin-based formulations.

Results from this study also indicated that higher viability did not necessary correspond to better hydrocarbon degradation. High bacterial cell viability in a formulation did not directly result in a better hydrocarbon degradation efficacy in most cases. Hydrocarbon biodegradation is influenced and limited by several factors other than cell viability as discussed. Similar observation was also observed in the study of survival and biodegradation capability of freeze-dried and liquid-dried aerobic gram negative bacteria in degrading toluene, 2,4-dichlorophenoxyacetate, 2,2-dichloropropionate and 3-chlorobenzoate in the presence or absence of a protective agent (Lang & Malik, 1996). Lang and Malik (1996) reported that stability of the degradation potential was low although cell viability was high.

Moreover, a study showed that the major limitation in bioremediation of hydrocarbon-contaminated soil and water is the availability of nutrients such as nitrogen, phosphorus and iron (Zaidi & Imam, 1999). Several studies have shown that an inadequate supply of these nutrients may result in a slow rate of biodegradation (Lee & Levy, 1989). Although the degrading substrates such as petroleum are rich in the carbon required by microorganisms, they are deficient in the mineral nutrients necessary to support microbial growth

(Atlas, 1988). In this study, carbon source (non-fat skim milk, sucrose and clay) present in the formulations did help in maintaining the cell viability but did not enhance degradation of hydrocarbon. Similarly, Zaidi and Imam (1999) reported that availability of an easily used carbon source did not enhance degradation of polycyclic aromatic hydrocarbon indicating that carbon was not a limiting factor in degradation. Higher recovery of cell viability with the incorporation of formulative materials rich in carbon source did not lead to higher degradation efficacy.

5.4.2 Efficacy of Formulated UPM 39B3 in Inhibiting the Growth of Pathogenic *Fusarium oxysporum* f. sp. *cubense* race 4 (FocR4)

In this study, plate count method was used to determine the efficacy of UPM 39B3 in inhibiting fungal growth inhibition. As bacterial cells were used as the active ingredient in the fungal growth inhibition assessment, streptomycin was added in PDA medium to inhibit the growth of the UPM 39B3 but allowed the growth of *Fusarium oxysporum* f. sp. *cubense* race 4 (FocR4). FocR4 is the causal agent of fusarium wilt (Panama disease) of banana. The pathogenic isolates of *F. oxysporum* cause fusarium wilt of several agricultural crops (Hawksworth, Kirk, Sutton, & Pegler, 1995). Cavendish banana varieties are highly susceptible to FocR4 which is one of the most destructive strains of the vascular wilt fungus *F. Oxysporum* (Fourie, Steenkamp, Gordon, & Viljoen, 2009).

Isolate UPM 39B3 was identified as *Serratia marcenscens* with potential in inhibiting the growth of FocR4. Biocontrol agents may use one or more types of mechanisms for diseases suppression (Weller, 1988). A primary

mechanism of pathogen inhibition is by producing secondary metabolites. The *in vitro* biocontrol by UPM 39B3 (*S. marcescens*) might be mediated by the combined effects of plural chitinases and antibiotic prodigiosin (Someya & Akutsu, 2006). Chitinolytic enzymes (chitinases) produced by *Serratia marcescens* caused degradation of the fungal cell walls which then inhibited the fungal growth (Someya, Kataoka, Komagata, Hibi, & Akutsu, 2000). The presence of chitin could increase chitinase production in *Serratia marcescens* (Suzuki, Suzuki, Taiyoji, Nikaidou, & Watanabe, 1998). The water insoluble red pigment (prodigiosin) has antifungal, antimicrobial, immunosuppressive and anti-proliferate activity (Khanafari, Assadi, & Fakhr, 2006; Moraes et al., 2009).

Although formulative materials were advantages in protecting the formulated cells from sunlight exposure, their role in enhancing the control efficacy towards FocR4 was less defined. In this study there was no clear improvement on the inhibitory effect towards FocR4 upon the introduction of the formulated cells compared with FC in both conditions, with or without sunlight exposure. UV irradiation from sunlight could cause mutations in the bacterial cell (Witkin, 1976). Mutations might affect the chitinase and prodigiosin gene expression. For example, mutations affecting any of metabolic pathways involved in prodigiosin production could result in loss of pigment production (Lim, 2003). The production of prodigiosin in *S. marcescens* is susceptible to temperature with maximum production at 28 ˚C and 30 ˚C and is substantially inhibited at temperatures higher than 37 ˚C (Tanaka, Yuasa, Baba, Tanikawa, Nakagawa, & Matsuyama, 2004; Giri,

Anandkumar, Muthukumuran, & Pennathur, 2004). Besides, prodigiosin is also sensitive to light (Casullo de Araujo, Fukushima, & Campos Takaki, 2010). Thus, upon sunlight exposure, most of the formulations including FC formulation showed poor inhibition efficacy.

Many studies demonstrated that the efficacy of the biocontrol agents including *S. marcescens* (Zeinat, Nagwa, El-Sayed, & Abd El-Wahab, 2010) was closely correlated with the cell viability (Racke & Sikora, 1992). Other researchers have also shown incorporating nutrient substrates such as chitin (Hsu & Lockwood, 1975), glucans (Beyer & Diekmann, 1985), skim milk (Bashan, 1986) and sucrose (Su, Tsou, & Liu, 2011) would increase the shelf-life and efficacy of the dry formulations of many biocontrol agents (Sabaratnam & Traquair, 2001). In this study, higher viability of cells derived from formulations with or without sunlight exposure did not show a better inhibition efficacy. The formulative materials might not only influence the inhibition activity of formulated UPM 39B3 but also the growth of the FocR4.

Formulative materials which improve the cell viability might also stimulate the growth of the fungal pathogen. Results showed that both isolate UPM 39B3 isolate and FocR4 had higher growth when the enrichment materials were present in the formulations. A layer of FocR4 was also observed on top of the UPM39B3- FocR4 solution when treated with KN, KS and KSP formulations during this study. FocR4 might utilise non-fat skim milk and sucrose as carbon sources for their growth. Similarly, *p*-amino benzoic acid also exhibited antimicrobial effect to FocR4 and thus good inhibition was

observed in KP and BP formulations although the UPM 39B3 cell viability was low. Studies had showed that *p*-amino benzoic acid functioned as a compound that inhibited and altered the yeast growth in the mineral medium when it was used in high doses (Reed, Schram, & Loveless, 1959; Surovtseva, 1969; Gientka, Gut, & Duszkiewicz-Reinhard, 2009). However, no inhibition of yeast growth was observed when enrichment medium such as molasses was added (Gientka et al., 2009). Similar to isolate UPM 39B3, medium rich in nutrients such as formulations containing non-fat skim milk, sucrose and clay might contain compounds that could eliminate the antibacterial effect of *p*-amino benzoic acid on FocR4. Thus, it is importance to carefully evaluate the use of formulative materials as part of the formulation process.

When comparing between isolate formulated with only the clay (B and K formulations), isolate formulated with kaolin clay showed better inhibition than bentonite clay. Montmorillonite (bentonite) has been shown to enhance the growth of microorganisms by maintaining the pH of the environment at a level suitable for sustaining the microbial growth (Stotzky, 1986; Beveridge, 1988). In contrast, kaolinte (kaolin) clay, is not able to buffer the pH changes to sustain microorganism growth due to lower cation exchange capability (Rosenzweig & Stotzky, 1979). It was reported that in the presence of 3% kaolin clay, the inhibition activity by *S. marcescens* was reduced and totally eliminated at higher concentrations (Rosenzweig & Stotzky, 1979). Similarly, under the unfavourable condition, the fungal growth was also inhibited. Starkey and South (2008) also reported that kaolin clay used in the root dip treatments did not support but inhibited the fungal growth if compared with

polyacrylamide hydrogel and cornstarch-based hydrogel. Only kaolin clay showed the inhibition potential in the growth of all the three soil-borne fungi tested (*Pythium sp., Fusarium sp., Rhizoctonia sp*). The type of clay used in the formulation affected the growth of microbial and similar effect was observed on the inhibition efficacy towards FocR4 in this study.

In addition, Campbell and Ephgrave (1983) reported that soluble toxins or inhibitors produced by biocontrol agents might be adsorbed or inactivated by the clay which indirectly would affect efficacy of the biocontrol agents. Thus, it might be due to that the better adsorption capability of clay-based formulations resulted in a poorer inhibition efficacy than the free-cell formulation towards FocR4 in this study. The clay used in this study might retard the liberation of inhibitors or toxins produced by the biocontrol agent used to inhibit the growth of FocR4 (Gerstl, Nasser, & Mingelgrin, 1998). However, in this study, the inhibition efficacy of FC formulation was not significantly different from other clay-based formulations. Overall, the clay-based formulations are preferred because it might help in adsorbing the volatile inhibitory substances produced by the isolate UPM 39B3 (Ting, 2005) when the formulations were applied into the soil.

CHAPTER 6

CONCLUSION

The morphology of colony, BIOLOG analysis and molecular methods were used to identify UTAR EPA2 and UPM 39B3 isolates. The identity of isolate UTAR EPA2 is *Pseudomonas aeruginosa* while isolate UPM 39B3 is *Serratia marcescens.* The identification was verified using PCR and DNA sequencing. Carrier materials (bentonite and kaolin) showed great impacts on the texture and physical appearance of the clay-based formulations. The quantity of bentonite used to formulate both isolates in bentonite-based formulations was 50% less than the quantity of kaolin which was used for kaolin-based formulations. Thus, preparation of bentonite-based formulation might be more cost effective. Bentonite-based formulations appeared to be brownish granules while kaolin-based formulations were creamy white powders.

This study showed that most of the clay-based formulations were better in sustaining viability of both UTAR EPA2 and UPM 39B3 bacterial cells especially for the bentonite-based formulations upon 6 hours of sunlight exposure than the free-cell (FC) formulation. This suggested formulating active cells was important as the formulated forms would at least maintain viability of cells. The number of viable cell count of isolate UTAR EPA2 in FC formulation decreased significantly upon 6 hours sunlight exposure, from 6.97 $\log_{10}$ CFU/ ml to 2.70 $\log_{10}$ CFU/ ml. Similarly, the number of viable cell count of isolate UPM 39B3 in FC formulation decreased significantly upon 6 hours sunlight exposure, from 5.99 $\log_{10}$ CFU/ ml to 1.82 $\log_{10}$ CFU/ ml.

Between bentonite and kaolin clays, bentonite clay showed better performance in sustaining both isolate UTAR EPA2 and UPM 39B3 cell viability during the experimental storage periods. No viable UPM 39B3 cell was recovered from most of the kaolin-based formulations except KS and KNSP formulations. In addition, influence of bentonite clay itself on UTAR EPA2 cell viability was significant where B formulation (with and without sunlight exposure) showed significantly higher cell viability. While the kaolin clay, enrichment materials and additive were not effective to sustain UTAR EPA2 cell viability.

This study also showed that enrichment materials were effective in sustaining cell viability of UPM 39B3 especially when bentonite clay was used as the carrier material in the formulation. Higher cell viability was obtained in BN, BS, BSP and BNSP formulations. However, inclusion of *p*-amino benzoic acid caused significant reduction in cell viability for both UTAR EPA2 and UPM 39B3 isolates as observed in BP and KP formulations.

In this study, the addition of formulative materials did not show beneficial effect in improving the efficacy of UTAR EPA2 in degrading hydrocarbon substrates tested. Both clay-based formulations and free-cell formulation with or without sunlight exposure showed similar degradation efficacy. Incorporation of formulative materials in UPM 39B3 formulations with and without sunlight exposure did affect the growth of the FocR4, where addition of enrichment materials showed enhancement of pathogen growth and

addition of *p*-amino benzoic acid showed inhibitory effect on pathogen growth.

To conclude, formulated UTAR EPA2 cells with bentonite clay only (B) was proposed as the most suitable formulation in this study as good viability and efficacy results were obtained. For isolate UPM 39B3, results indicated that bentonite clay only (B) and bentonite-based formulation with sucrose (BS) were able to produce both good cell viability and FocR4 inhibition efficacy. Thus, B and BS formulations might be more suitable to formulate UPM 39B3. Beneficial microbial cells are highly recommended for formulation as formulated cells can be protected from the sunlight exposure and remain viable during storage period.

Studies on the physico-chemicals aspects of the UTAR EPA2 clay-based formulations and their influence on hydrocarbon degradation are suggested for future studies. The understanding of biocontrol agent (UPM 39B3) and the inhibition mechanism in inhibiting the growth of pathogen is important before formulating the isolate. Thus, study on inhibition mechanisms used by the UPM 39B3 isolate and the characteristic of the extracellular compounds produced by UPM 39B3 need to be investigated. Optimization of the quantity of formulative materials used in the UPM 39B3 formulation to inhibit FocR4 is also recommended in future study.

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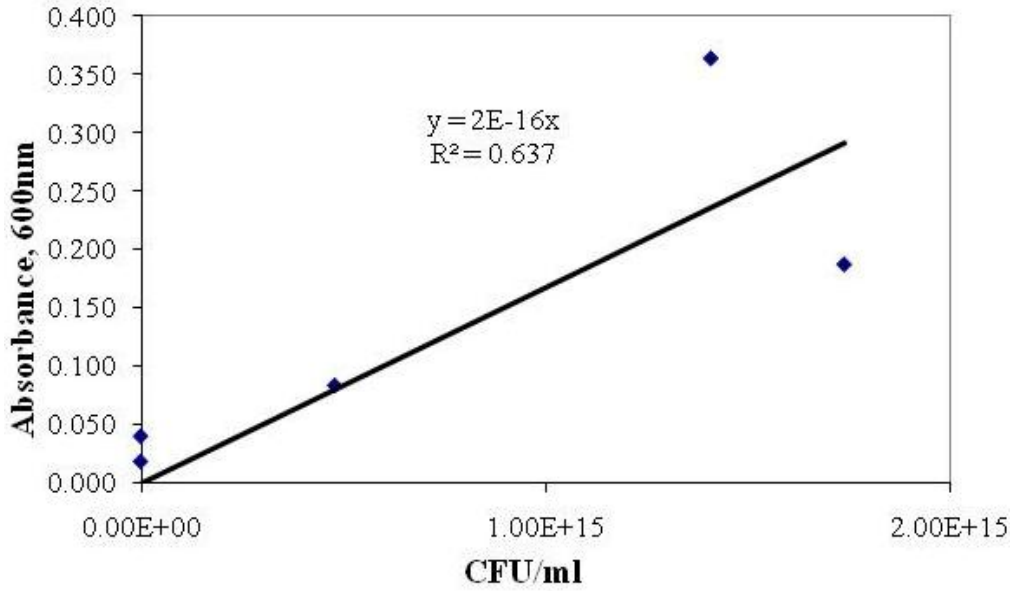
APPENDICES

Appendix A

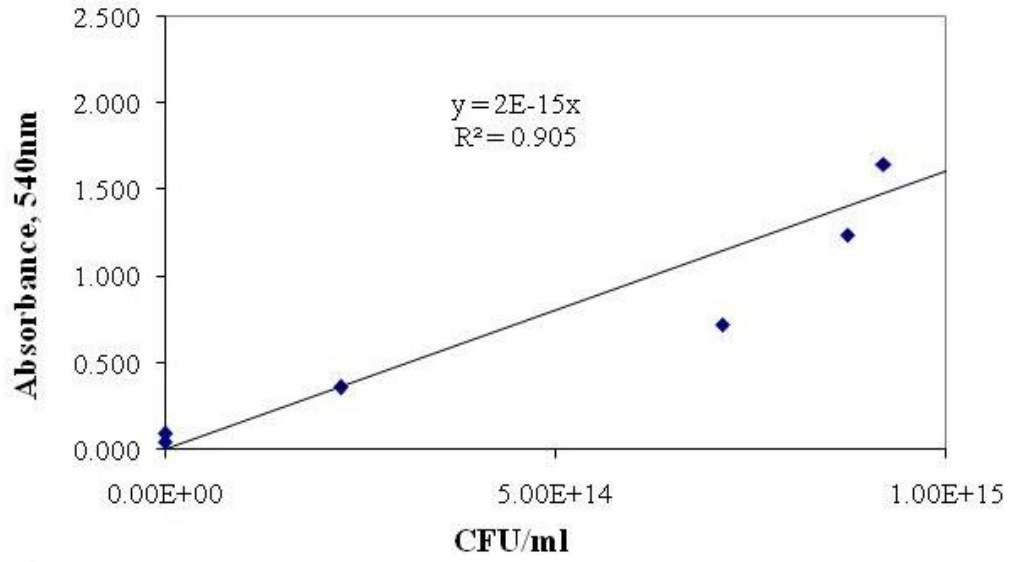
Standard Curve for Isolates

(1) Standard curve for isolate UTAR EPA 2



When y= 0.200; x= 1E+15

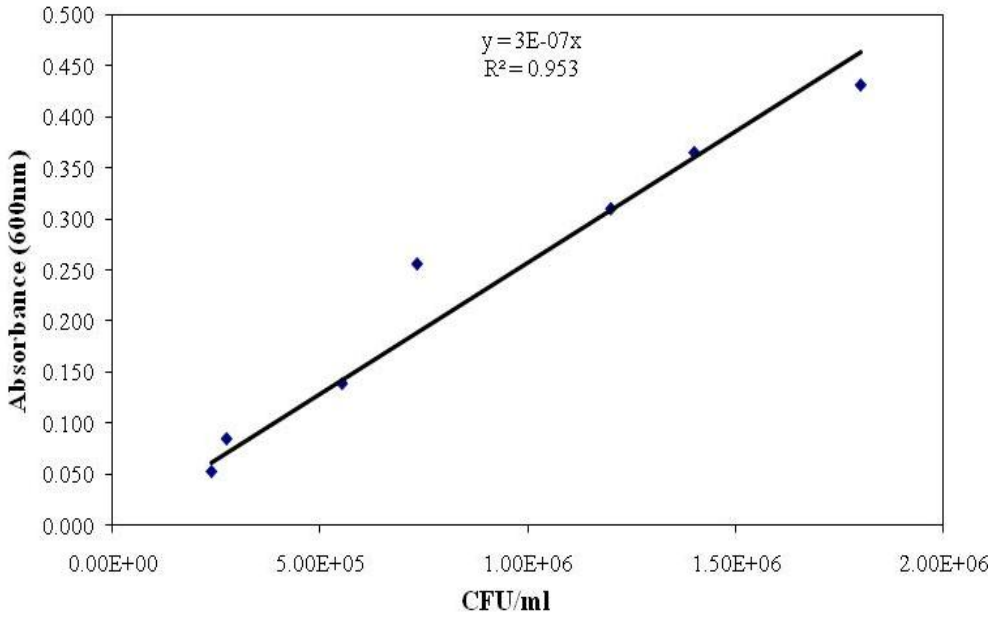
(2) Standard curve for isolate UPM 39B3



When y= 2.000; x= 1E+15

Appendix B

(1) Standard curve for fungal pathogen FocR4



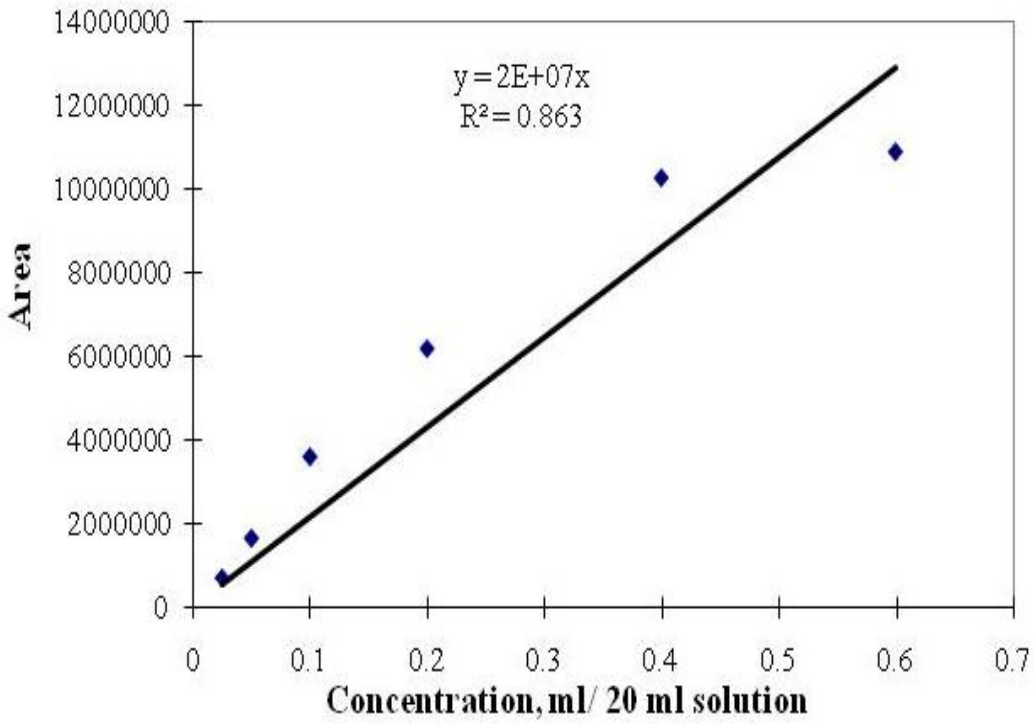
When y=0.980; x=3.26E+6

Appendix C

Standard Curve for Hydrocarbons

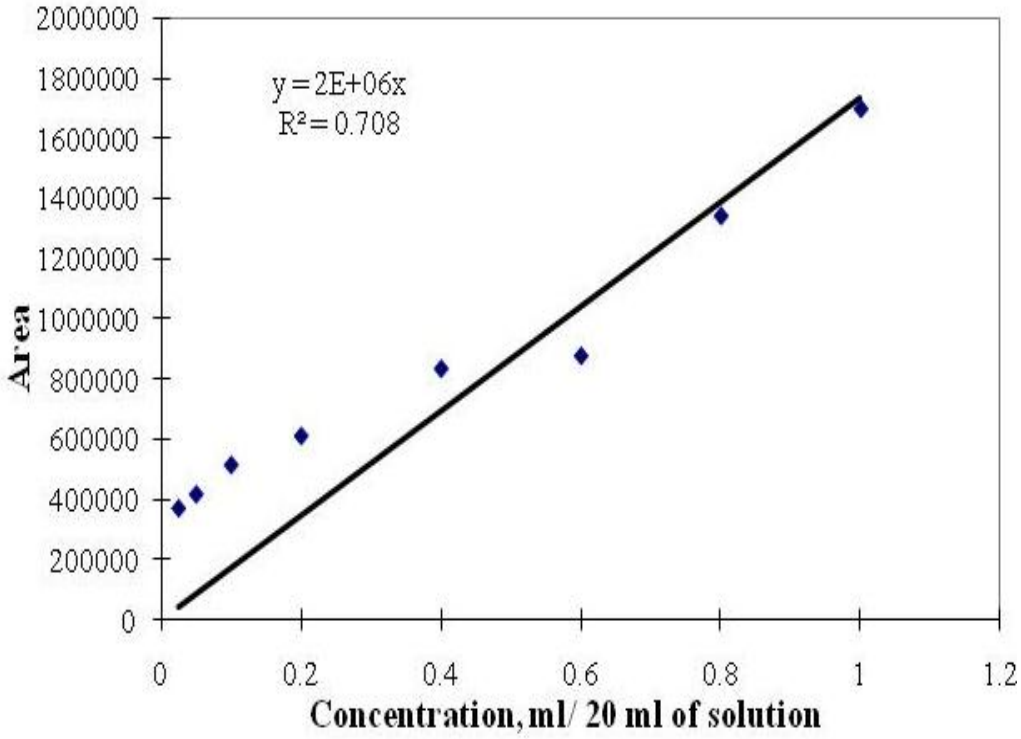
1) Standard curve for toluene using toluene as the key compound

a) 10 fold dilution



Note: The 10 fold dilution or the 100 fold dilution graph is adopted as standard curves corresponding to the concentration of the hydrocarbon residues after treatment. Samples with higher concentration are diluted to 100 fold hence the 100 fold dilution standard curve is used.

b) 100 fold dilution

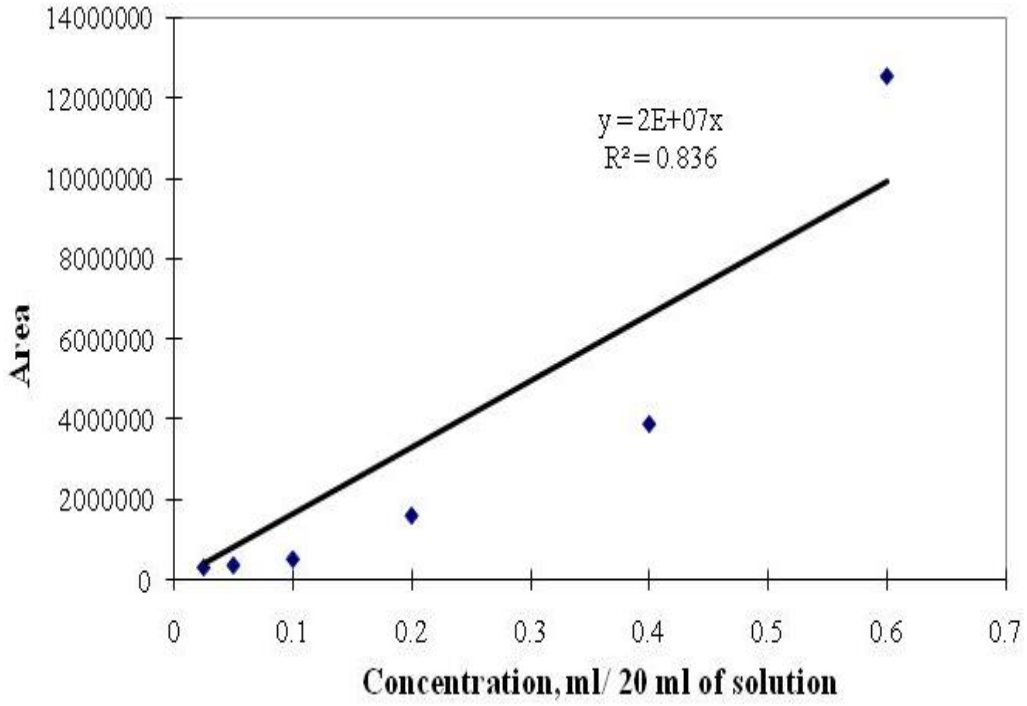


Note: The 10 fold dilution or the 100 fold dilution graph is adopted as standard curves corresponding to the concentration of the hydrocarbon residues after treatment. Samples with higher concentration are diluted to 100 fold hence the 100 fold dilution standard curve is used.

2) Standard curve for petrol by detecting different key compounds

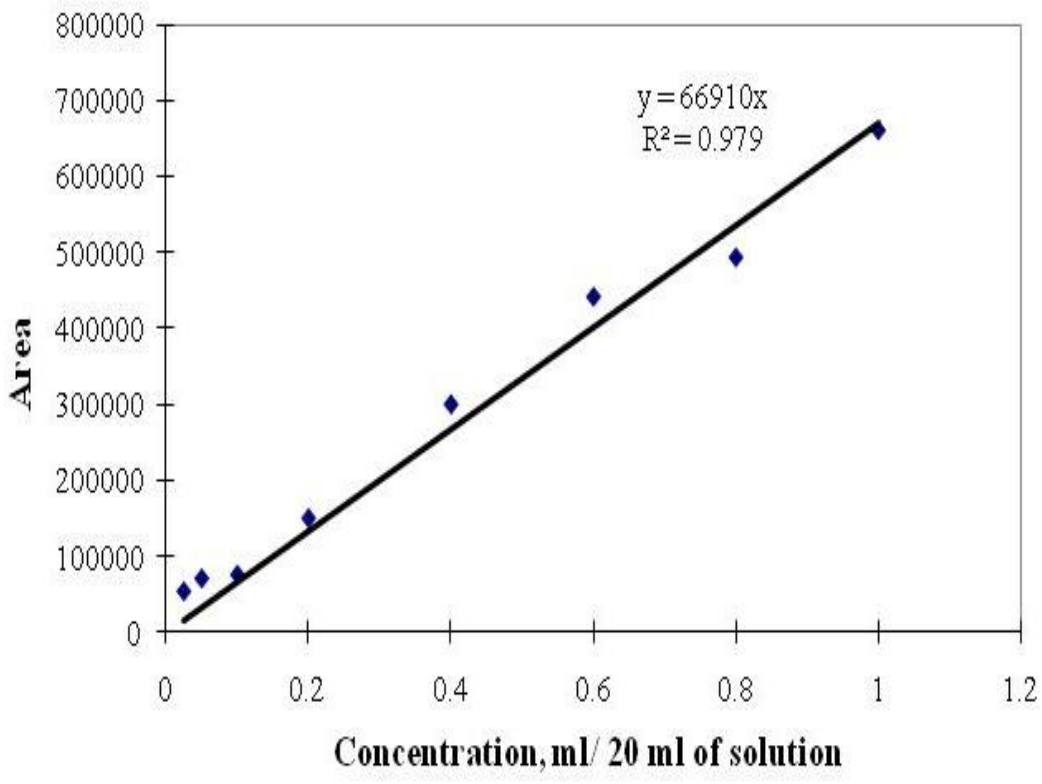
2.1) Ethylbenzene

a) Without dilution



Note: The without dilution or the 10 fold dilution graph is adopted as standard curves corresponding to the concentration of the hydrocarbon residues after treatment. Samples with higher concentration are diluted to 10 fold hence the 10 fold dilution standard curve is used.

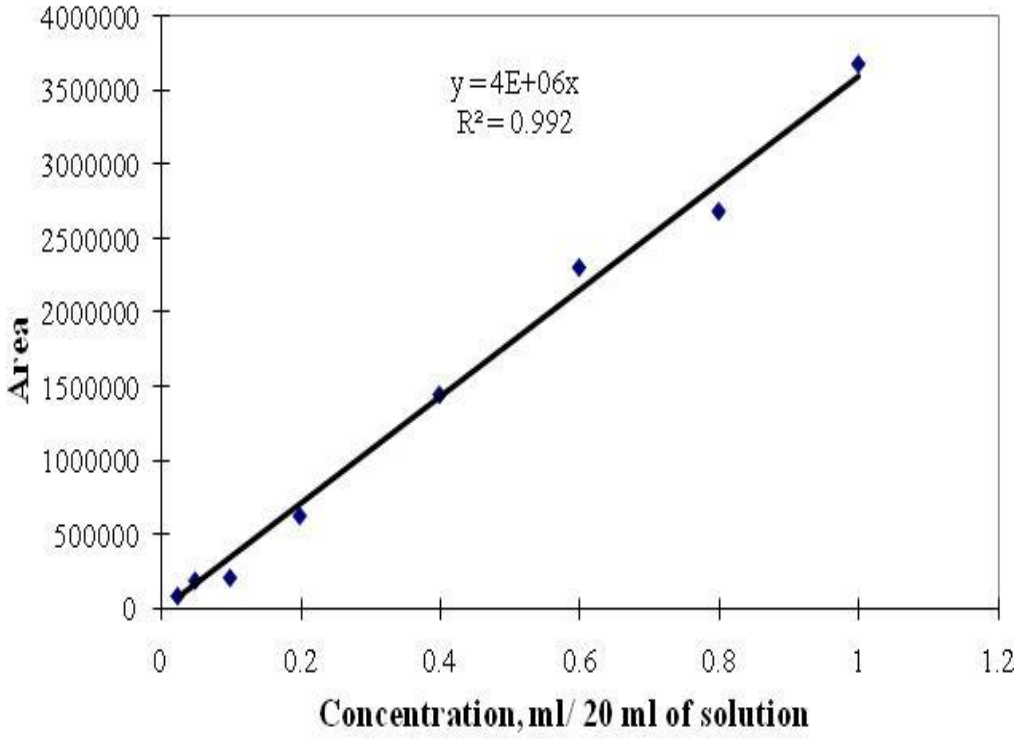
b) 10 Fold dilution



Note: The without dilution or the 10 fold dilution graph is adopted as standard curves corresponding to the concentration of the hydrocarbon residues after treatment. Samples with higher concentration are diluted to 10 fold hence the 10 fold dilution standard curve is used.

2.2) *p*-xylene

a) 10 fold dilution

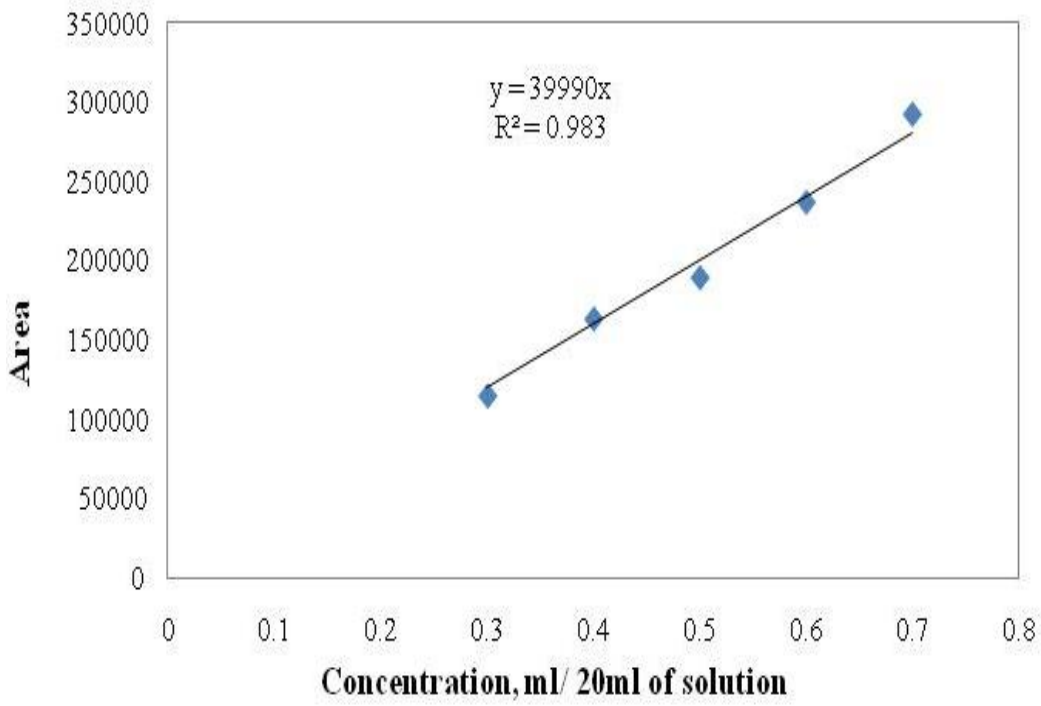


Note: The without dilution or the 10 fold dilution graph is adopted as standard curves corresponding to the concentration of the hydrocarbon residues after treatment. Samples with higher concentration are diluted to 10 fold hence the 10 fold dilution standard curve is used.

3) Standard curve for mineral oil by detecting different key compounds

3.1) Nonadecane

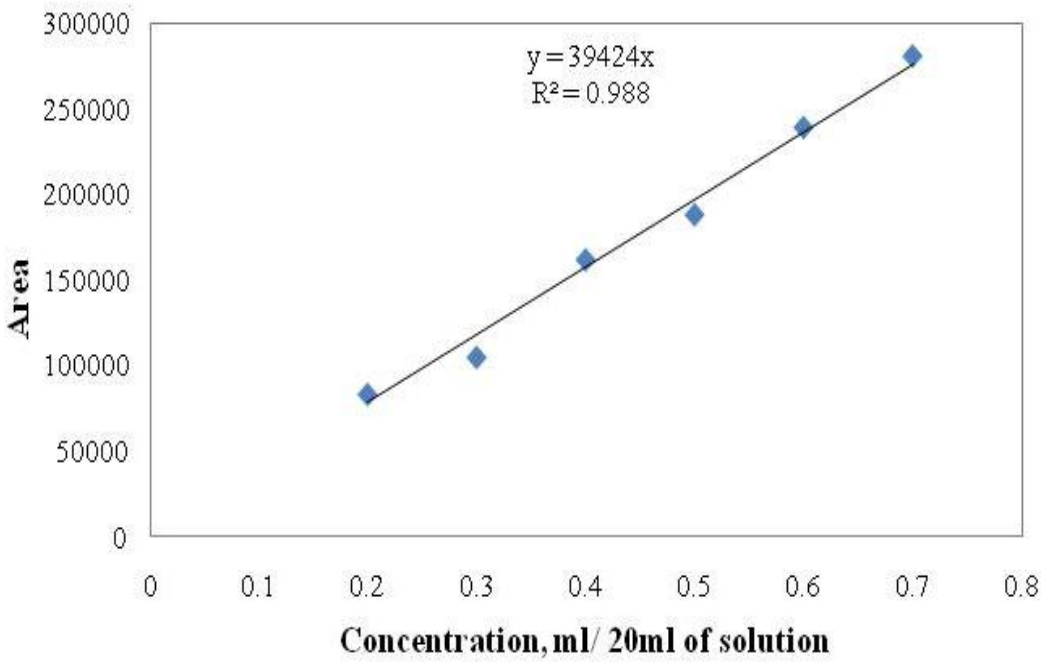
a) 10 fold dilution



Note: The without dilution or the 10 fold dilution graph is adopted as standard curves corresponding to the concentration of the hydrocarbon residues after treatment. Samples with higher concentration are diluted to 10 fold hence the 10 fold dilution standard curve is used.

3.2) Octadecane

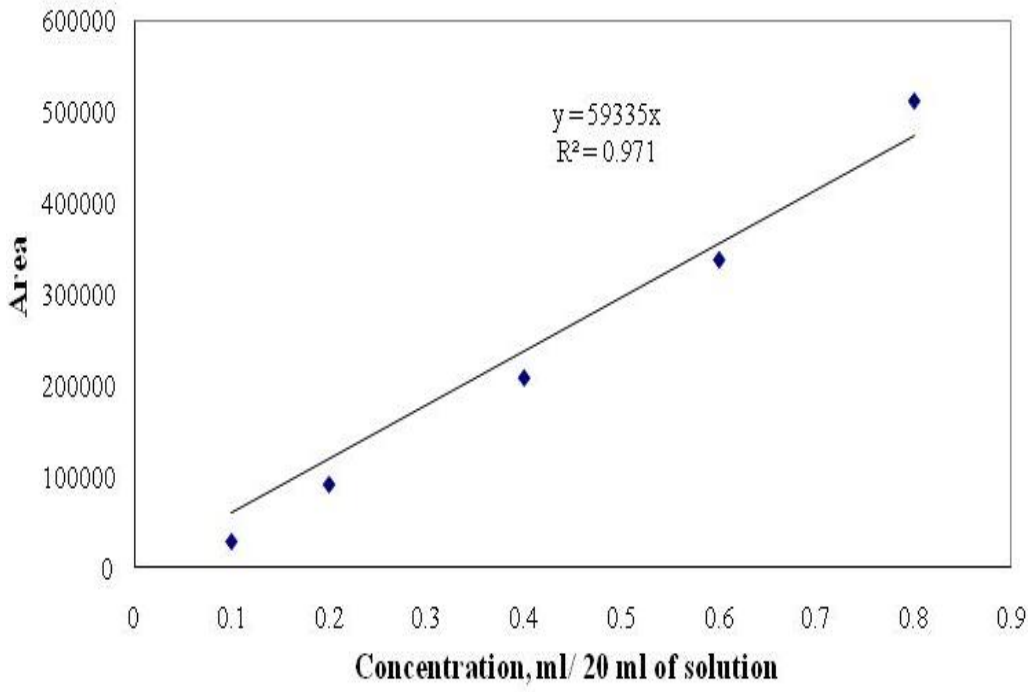
a) 10 fold dilution



Note: The without dilution or the 10 fold dilution graph is adopted as standard curves corresponding to the concentration of the hydrocarbon residues after treatment. Samples with higher concentration are diluted to 10 fold hence the 10 fold dilution standard curve is used.

3.3) Hexadecane

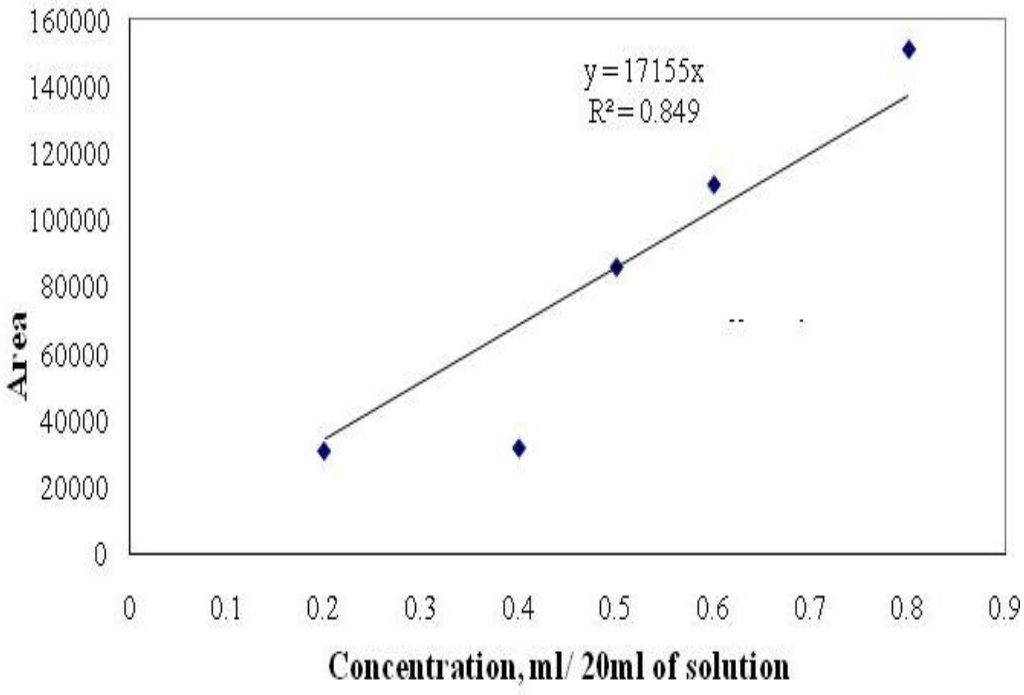
a) 10 fold dilution



Note: The without dilution or the 10 fold dilution graph is adopted as standard curves corresponding to the concentration of the hydrocarbon residues after treatment. Samples with higher concentration are diluted to 10 fold hence the 10 fold dilution standard curve is used.

3.4) Dodecane

a) 10 fold dilution



Note: The without dilution or the 10 fold dilution graph is adopted as standard curves corresponding to the concentration of the hydrocarbon residues after treatment. Samples with higher concentration are diluted to 10 fold hence the 10 fold dilution standard curve is used.

Appendix D

BIOLOG result

1) UTAR EPA2 isolate

Program	: MicroLog3 4.20.04
Read From File	: C:\Biolog420\UTAR.D4C
Save To File	:
Unrestricted Access?	: Yes
Read Time	: Mar 01 2008 12:35
Parent File	: Original Data Record
Plate Number	: 1
Incubation Time	: 16-24
Sample Number	: EPA2 Plate Type: GN2
Strain Type	: GN-ALL
Strain Number	:
Strain Name	:
Other	:
Data Input Mode	: File
590/750 Filters Used	: 6 / 5
Threshold Mode	: Automatic: Color: 23/48
Number +/b/- Reactions	: 11 / 10 / 75
Database To Search	: MicroLog
Data Base(s) Searched	: C:\Biolog420\Databases\GN601.KID

Key : <X>: positive; <X: mismatched positive; X: negative; X+: mismatched negative
{X}: borderline; -X: less than A1 well

Color	1	2	3	4	5	6	7	8	9	10	11	12
A	0	2	6	8	{ 41}	{ 27}	-1	7	-1	< 635>	-5	-25
B	1	< 121>	< 57>	< 155>	19	< 166>	1	0	0	-2	11	-16
C	3	1	{ 31}	2	3	2	0	14	11	-3	14	-2
D	9	6	< 64>	{ 48}	-0	{ 35}	< 124>	-0	{ 25}	4	1	-0
E	< 133>	14	13	< 63>	22	{ 35}	-0	{ 43}	-1	-3	-2	-4
F	1	-2	11	19	4	21	-22	< 415>	{ 30}	< 60>	-1	-6
G	-0	6	3	13	1	-7	8	-5	-8	-7	-3	{ 34}
H	-1	2	2	1	2	-20	9	1	15	6	-2	1

=> Species ID: Flavimonas oryzihabitans <=

	Species	PROB	SIM	DIST	TYPE
=>1)	Flavimonas oryzihabitans	89	0.58	5.30	GN-NENT OXI-
2)	Pseudomonas taetrolens	4	0.02	6.38	GN-NENT OXI+
3)	Chryseomonas luteola	2	0.01	6.54	GN-NENT OXI-
4)	Pseudomonas fluorescens biotype G	2	0.01	6.68	GN-NENT
5)	Pseudomonas corrugata	1	0.01	6.70	GN-NENT OXI+
6)	Pseudomonas marginalis	1	0.01	6.85	GN-NENT OXI+
7)	Pseudomonas asplenii	0	0.00	7.11	GN-NENT OXI+
8)	Pseudomonas syringae pv sesami	0	0.00	7.18	GN-NENT
9)	Pseudomonas fluorescens biotype F	0	0.00	7.22	GN-NENT
10)	Pseudomonas viridiflava (syringae)	0	0.00	7.28	GN-NENT OXI-
Other)					

Print Time = Mar 01 2008 12:55 Page 1 of 1 pages

Dr. Lum Keng Yeang
CAB INTERNATIONAL
SE Asia Regional Centre
Glass House No 2
(Opp. Block G). MARDI
43400 Serdang Selangor
Malaysia.

2) UPM 39B3 isolate

Program	: MicroLog3 4.20.04
Save To File	: C:\Biolog420\UTAR.D4C
Unrestricted Access?	: Yes
Read Time	: Mar 21 2008 12:40
Parent File	: Original Data Record
Plate Number	: 1
Incubation Time	: 16-24
Sample Number	: Plate Type: GN2
Strain Type	: GN-ALL
Strain Number	:
Strain Name	:
Other	:
Data Input Mode	: Manual
Number +/b/- Reactions	: 45 / 2 / 49
Database To Search	: MicroLog
Data Base(s) Searched	: C:\Biolog420\Databases\GN601.KID

Key : <>: positive; <>-: mismatched positive; X: negative; X+: mismatched negative
{X}: borderline; -X: less than A1 well

Color	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	<+>	-	-	-	<+>	<+>	<+>	-	-	-
B	<+>	<+>	<+>	<+>	-	<+>	<+>	-	-	<+>	<+>	<+>
C	-	<+>	<+>	-	-	<+>	<+>	<+>	-	<+>	<+>	<+>
D	-	<+>	<+>	<+>	-	-	<+>	-	-	-	-	-
E	-+	-	-	<+>	-	<+>	-	-	-	-	-	<+>
F	<+>	-	-	-	-+	<+>	<+>	<+>	<+>	{/}	-+	<+>
G	-+	<+>	-	-	-	<+>	-	<+>	<+>	-	-	-
H	-	<+>	<+>	<+>	-	{/}	-	-	<+>	<+>	<+>	<+>

=> Species ID: Serratia marcescens <=

	Species	PROB	SIM	DIST	TYPE
=>1)	Serratia marcescens	100	0.64	5.49	GN-ENT
2)	Serratia rubidaea	0	0.00	13.95	GN-ENT
3)	Serratia liquefaciens/grimesii	0	0.00	14.21	GN-ENT
4)	Serratia ficaria	0	0.00	14.32	GN-ENT
5)	Cedecea neteri	0	0.00	14.85	GN-ENT
6)	Raoultella planticola	0	0.00	14.96	GN-ENT
7)	Raoultella planticola/ornithinolytica	0	0.00	15.20	GN-ENT
8)	Citrobacter gillenii	0	0.00	15.23	GN-ENT
9)	Serratia odorifera	0	0.00	15.54	GN-ENT
10)	Serratia entomophila	0	0.00	15.75	GN-ENT
Other)					

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Appendix E

Blast Result of 16s rDNA sequences for Isolate UTAR EPA2 and UPM 39B3

1) Isolate UTAR EPA2

>gb|GU339238.1| *Pseudomonas aeruginosa* strain EH8 16S ribosomal RNA gene, partial sequence
Length=1498

Score = 1716 bits (929), Expect = 0.0
Identities = 941/946 (99%), Gaps = 3/946 (0%)
Strand=Plus/Plus

```
*s CGG - ATT- CTGGGCGT – AAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCG
   |||||||||||| |||||||||||||||||||||||||||||||||||||||||||||||||||||
*QCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCG

*s GGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGA
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
*Q GGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGA

*s ATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGAC
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
*Q ATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGAC

*s CACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
*Q CACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC

*s CCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTG
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
*Q CCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTG

*s GCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAA
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
*Q GCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAA

*s TGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
*Q TGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA

*s GAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGA
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
*Q GAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGA

*s ACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
*Q ACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT

*s CCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGA
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
*Q CCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGA

*s GACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACG
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
*Q GACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACG

*s GCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGA
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
*Q GCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGA
```

```
*S GCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGT
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
*Q GCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGT

*S CGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTAC
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
*Q CGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTAC

*S ACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTACCCGCAAGG
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
*Q ACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTAACCGCAAGG

*S GGGACGGTTACAACGGAGTGATTCATGACTGGGGTGAAGTCGTACA
   ||||||||||||||||||||||||||||||||||||||||||||
*Q GGGACGGTTACCACGGAGTGATTCATGACTGGGGTGAAGTCGTACA
```

*S :Subject
*Q :Query

2) Isolate UPM 39B3

a) UPM 39B3 amplified fragment sequences (215 bp) generated using smr16snr primer (Wilson *et al*.,1999)

>gb|HQ123483.1| *Serratia marcescens* strain XJalt12.1 16S ribosomal RNA gene, partial sequence
Length=1393

Score = 298 bits (161), Expect = 9e-78
Identities = 169/172 (98%), Gaps = 3/172 (2%)
Strand=Plus/Minus

```
*S CAGG- TGAG - CCGGGGATTTC - CATCTGACTTAACAAACCGCCTGCGTGCGCTTTACGCC
   ||||||| |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
*Q CAGGTTGAGCCCGGGGATTTCACATCTGACTTAACAAACCGCCTGCGTGCGCTTTACGCC

*S CAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAG
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
*Q CAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAG

*S CCGGTGCTTCTTCTGCGAGTAACGTCAATTGATGAGCGTATTAAGTTCACCA
   ||||||||||||||||||||||||||||||||||||||||||||||||||||
*Q CCGGTGCTTCTTCTGCGAGTAACGTCAATTGATGAGCGTATTAAGTTCACCA
```

*S :Subject
*Q :Query

b) UPM 39B3 amplified fragment sequences (215 bp) generated using smr16sm primer (Wilson *et al*.,1999)

>gb|JF718272.1| *Serratia marcescens* strain HS-L5 16S ribosomal RNA gene, partial sequence
Length=1415

Score = 289 bits (156), Expect = 5e-75
Identities = 163/166 (98%), Gaps = 1/166 (1%)
Strand=Plus/Plus

```
*S CGGCT-ACTCCGTGCCAGCAGCCGCGGTAATACCGAGGGTGCAAGCGTTAATCGGAATTA
   ||||| ||||||||||||||||||||||||||| ||||||||||||||||||||||||||
*Q CGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTA

*S CTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAAC
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
*Q CTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAAC

*S CTGGGAACTGCATTTGAAACTGGCACGCTAGAGTCTCGTAGAGGGG
   ||||||||||||||||||||||||| ||||||||||||||||||||
*Q CTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTCGTAGAGGGG
```

*S :Subject
*Q :Query

Appendix F

List of Publications

A) Conference proceedings

Ting, A. S. Y., **Fang, M. T.** and Tee C. S. 2009. Characterization and identification of the beneficial endobacterium *Serratia marcescens* isolated from wild bananas. Poster presentation. Proceedings in the Malaysian Biological Symposium: Harnessing the Potential of Biodiversity, (pp. 47-49). 17-18th November 2009. Bangi, Selangor (shortlisted as paper in PERTANIKA-pending)

B) Journal papers

Ting, A. S. Y., **Fang, M. T.** and Tee C. S. (2009). Assessment on the effect of formulative materials on the viability and efficacy of *Serratia marcescens*-a biocontrol agent against *Fusarium oxysporum* f.sp. *cubense* race 4. American *Journal of Agricultural and Biological Sciences* 4 (4), 283-288.

Ting, A. S. Y., **Fang, M. T.** and Tee, C. S. (2010). An *in vitro* Assessment on the Efficacy of Clay-Based Formulated Cells of *Pseudomonas* Isolate UTAR EPA2 for Petrol Degradation. *American Journal of Applied Sciences* 7 (2), 178-184.

Ting, A. S. Y., **Fang, M. T**. and Tee, C. S. (2010). Evaluation on the Efficacy of Formulated *Pseudomonas* Cells in Degrading the Aromatic Hydrocarbon Toluene. *Australian Journal of Basic and Applied Sciences*, 4 (8), 3122-3127.

Ting, A. S. Y., **Fang, M. T.** and Tee, C. S. (2011). Efficacy of Clay-Based Formulated *Serratia* in Reducing Inoculum of *Fusarium oxysporum* f.sp. *cubense* race 4. Acta Horticulturae, 897, 421-426.