CHAPTER ONE

INTRODUCTION

1.1 Soil microbiology

Soil microbiology is a science field that comprises of soil microorganisms related studies and the activities they have, in the soil, which is among the most dynamic environments for biological interactions in the nature. The soil is known to be the outer, loose material of land surface, which supports plant growth. From the agricultural aspect, soil is the part that supports the growth of plants by providing the required nutrients and the environment in which happens a major part of the biological, biochemical, and physical reactions, regarding organic materials' decomposition. Bacteria preserve the fertility of soil where some decompose waste materials and recycle them into nutrients and others fix nitrogen to make it available for plants.

On the immediate surface of the earth, the loose and unstratified mineral and natural material is called soil and it is known as an organic means of land plants' growth. Main compartments of a typical soil are illustrated in the below figure 1.

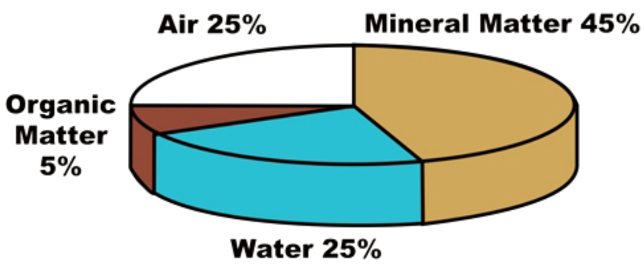

Figure 1: Main compartments of a typical soil. (Kincheloe, Efficient Fertilizer Use)

Two or more soil layers, named horizons, make the soil profile (Figure 2). The highest layer in the soil profile is Horizon A, or the surface soil, and it is rich in organic materials such as bacteria, fungi, plant roots, and small animals. Horizon B, also known as subsoil, lies between Horizon A and Horizon C, therefore this layer owns some features of both horizons C and A. In this layer, there are fewer living organisms compared to Horizon A; and more common compared to Horizon C. Between layer A and layer C, the color is also convertible. Actually this is usually higher in clay, in comparison to any of the other horizons. The deepest layer is Horizon C, in which the materials for the mineral section of the soil are formed.

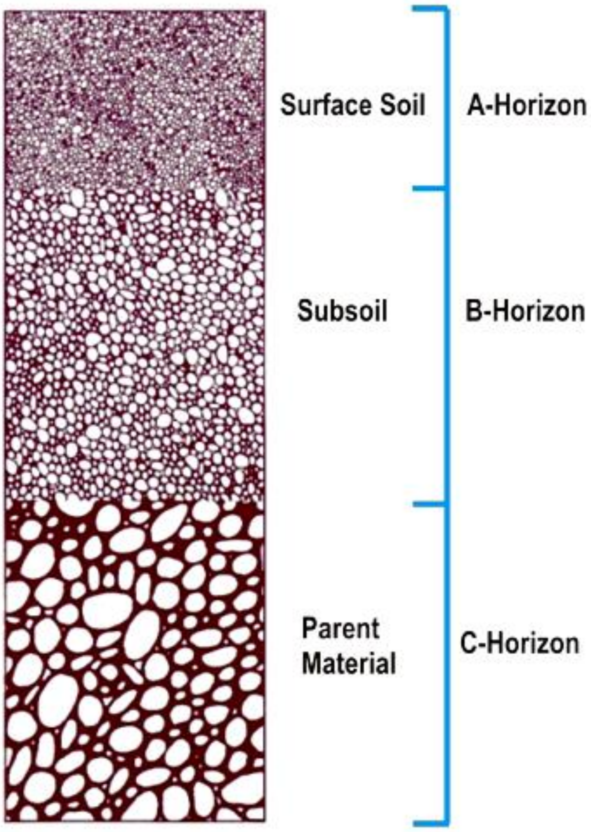

Figure 2: Soil profile showing the three major horizon divisions (Kincheloe, Efficient Fertilizer Use)

1.2 Soil microorganism

Soil microorganism is a group of living and breathing organisms. There is a competition between them and the plants on different nutrients, such as Phosphorus, Nitrogen, and Potassium and also micronutrients. As well, they use up vitamins, amino acids, and other components of the soil. They get their required nutrients from the natural materials upon which they feed (Carrow et al., 2001). Soil contains several distinct groups of microorganisms which can be grouped into two categories as soil microflora (bacteria, fungi, actinomycetes, algae) and soil microfauna (protozoa, nematodes, earthworms, moles, ants, rodents) (Franzluebbers 2004). The number of the bacteria is much higher than microorganisms of other sorts, and their diversity varies depending on the type of the soil and the ecosystem (Nannipieri et al., 2003). The composition of bacteria can determine the types of plants growth in the soil (Ma´rcia et al., 2010). The amount of the microbial population is in correlation with the depth of soil. Every organism or a bunch of organisms are in charge of a special alteration in the soil (James and Rafiq, 2010).

1.3 Soil bacteriology

The period 1890 – 1910 has been one of notable progress in soil bacteriology. Robert Koch, in 1882, built the technique of gelatin plate/streak plate, for the aim of isolating a special kind of soil bacteria. Beijerinck and Winogradsky, in 1890, came up with the idea of enrichment culture, for soil organisms' isolation, whereby they are believed to be pioneers of soil bacteriology. In 1893, S. N. Winogradsky was given the title of "Father of Soil Microbiology" for discovery of bacteria's autotrophic mode of life and the establishment of the transformation of sulphur and nitrogen, microbiologically. In 1918, "direct soil examination" was established by Conn for the study of soil microorganisms. Winogradsky (1925) categorized soil microorganisms into two general classifications that are the indigenous species of Autochnotus and the fermentative Zymogenous. By adding a special substrate to the soil, the population of Zymogenous bacteria increases gradually. Nitrogen utilizing bacteria, cellulose decomposers, and ammonifiers are part of this category. According to the Bergey's Manual of Systematic Bacteriology, there are three orders in the class of Schizomycetes which are Pseudomonadales, Eubacteriales and Actinomycetales (Sneath et al., 2002).

The common bacteria found in soil go under the genera *Arthrobacter, Enterobacter Clostridium Achromobacter, Pseudomonas* and *Sarcina*. The other group of bacteria is the Myxobacteria, which comprises from genera *Archangium, Chondrococcus, Cyptophaga, Micrococcus* and*Polyangium*,.

Table 1: Degrading capabilities of various soil bacteria (webpage citation, http://agriinfo.in)

Cellulose	***Hemicelluloses***	***Lignin***	***Proteins***
Pseudomonas	*Bacillus*	*Pseudomonas*	*Clostridium*
Cytophaga	*Vibrio*	*Micrococcus*	*Proteus*
Spirillum	*Pseudomonas*	*Flavobacteriumm*	*Pseudomonas*
Actinomycetes	*Erwinia*	*Xanthomonas*	*Bacillus*
Cellulomonas		*Streptomyces*	

1.4 Soil bacteria

There are several environments where bacteria live, including soil water, the film of moisture which surrounds soil particles. Some bacteria can swim by the aid of flagella. Most of the useful bacteria require oxygen (as a result they are called aerobic bacteria), while those which do not need air, are termed anaerobic, and they result in decay of dead organic materials (Reid and Wong, 2005). Base on COG Organic Field Crop Handbook, in a moist soil and neutral soil pH, and wherever a lot of food is (e.g. micronutrients and carbohydrates from organic materials) available Aerobic bacteria are believed to be most active (Reid and Wong, 2005). Bacteria will not die completely, in hostile conditions; but their growth will be stopped and they get into an inactive stage, and those active may fight better in the new conditions. Some Gram positive bacteria make non-vegetative stage (spore) for waiting for more desirable conditions and Gram negative bacteria gets into a "non-culturable" stage (Matheson and Reid, 1992).

There are four functional groups for soil bacteria. The most common decomposers are the ones that use simple carbon compounds so that the bacteria can change the energy in soil organic matter into forms which are of use to other organisms as a food cycle (Alexandra and José, 2005). They are able to break down the pesticides and pollutants in soil and this is important in retaining or immobilizing nutrients to prevent loss of nutrients, from the plant's roots (Elaine and Ingham, 1999). Mutual bacteria are the second category which forms partnership with plants where they are nitrogen-fixing bacteria (Kahindi et al., 1997). The third batch of bacteria is the bacteria cause disease in plants. These pathogens are Agrobacterium, Erwinia and Xymomonas species. The last category is chemoautotrophs or lithotrophs where these bacteria gain energy from hydrogen, iron, nitrogen, sulfur and carbon compounds (Elaine and Ingham, 1999).

Bacteria play an important role in the carbon cycle, since by fixation (photosynthesis) and decomposition; they give carbon to the system. In grassland environments, bacteria are significant decomposers (Davidson and Janssens, 2006). There is a particular effect in Actinomycetes where they breakdown materials such as cellulose and chitin under difficult circumstances, such as high pH in the soil (Stirling, 2001). There can be a change in the dominance of decomposers from bacterial to fungal, by doing changing nutrient levels in the soil. Upon the dominance of one group, where it shouldn't be dominant, there also appears a change in the rest of the system. The conditions supporting weed invasions, on range lands, can improve with the shift from bacterial to fungal dominance. Atmospheric nitrogen is fixed and added to the soil nitrogen pool, by free-living bacteria (Franche et al., 2009). Associations with the roots of leguminous plants, such as lupine, clover, alfalfa, and milkvetches are formed by other nitrogen-fixing bacteria (Stirling, 2001). Actinomycetes create coalitions, with some non-leguminous plants and fix nitrogen, which is then useable to microorganisms and plants. The nitrogen fixing bacteria will convert the unusable nitrogen in the soil that can be used by other plants. Some bacteria produce sticky materials which helps the soil granules to bind and form small aggregates of soil. This will help to improve water penetration, water-holding volume, constancy and ventilation in the soil. In term of bioremediation, bacteria are able to breakdown toxic materials in the soil and water in the ground to cleanse the soil system. This includes herbicides, heavy metals and oil products. The soil bacteria are capable to change toxic compounds to non-toxic compounds by the process called 'biodegradation' (McGuinness and Dowling, 2009).

1.5 Indigenous microorganisms (IMO)

IMO are beneficial soil microorganisms that can be collected from non-cultivated soil and are often found in high concentrations with the presence of earthworm castings and under bamboo trees. Nutrient uptake by plants is significantly boosted when the diversity and activity of these beneficial microorganisms is increased stimulating the decomposition process (Jensen et al., 2006). IMO is becoming increasingly popular among farmers and it has successfully applied in agricultural system. Previously it has been used for bad odors removing from animal wastes, soil composting, and fertilizing to crops.

Indigenous microorganism (IMO) provides natural fertilizer for the plants and burrow deep to loosen soil providing a no-till environment. Indigenous microorganisms (IMO) live in harmony with the soil and aid in establishing the very environment in which they live. IMO are responsible for much of the vitality in the region. They have the ability to withstand any weather conditions, and given the right environment, will function with great vitality (Pett-Ridge and Firestone, 2005). They also easily adapt to fluctuations in the environment. Indigenous microorganism gives strong input on soil and plant condition (Prell, 2010). These microorganisms can be cultivated and collected from bamboo tree area or nearby jungle using container filled with steamed rice (Prell, 2010). The collected microorganisms are survived and adapted to that local place for many generation and this microbes can be saved and grow for agricultural purpose (Park and DuPonte, 2008). IMO purify the soil water and provide various nutrients. IMO have the ability to decompose when complex organic materials such as plants, animals, excrements, and organic fertilizers enter the soil, IMO break these down into simpler compounds or elements that can undergo ionic interactions (Rodelio et al., 2011). Even the diverse inorganic matters decomposed by IMO increase in their effectiveness, and are converted to a form readily absorbable by plants. They catalyze the chemical processes in the soil by producing numerous enzymes, antibiotics, organic acids and various complexes. The majority of chemical reactions in the soil and plants are reliant on the enzymes which are catalysts. They also revitalize the ecosystem where when the soil environment is revitalized through the use of IMO, various bacteria and fungi appear first, followed by nematodes, earthworms, mole crickets, moles, etc. The use of IMO brings the ecosystem back to life in this manner (Rodelio et al., 2011). On the other hand, they can suppress diseases by circulating naturally active materials. IMO bring diversity back to soil in which the balance among the microbial population has been broken due to the abuse of chemicals. IMO are strong survivors that cannot be languid even in extreme conditions. The recovered diversity of microbes can, then, reduce the occurrence of diseases rapidly. IMO also have the ability to withstand any weather conditions, and given the right environment, will perform their function with great vitality. They are also easily adaptable to various changes in their living conditions.

1.6 Indigenous microorganism cultivation

IMO can be categorized into 4 levels; IMO1, IMO2, IMO3 and IMO4. To prepare the IMO1 inoculum, a teacup of rice is placed in a container before tightly covered with white paper .The container is kept undisturbed near bamboo tree for few days until the fungal hyphae observed on the rice. This will formed IMO1 mixture which will be used in the preparation of IMO2. Equal volume of IMO1 on brown sugar is mixed before kept in the container and covered with white paper for one week to produce IMO2. To prepare IMO3, 10 g of IMO2 are added to 1000ml of water and mixed well before pouring it into 8 kg of rice. The container is covered firmly and kept for five days. IMO4 is performed by mixing the IMO3 with an equal volume of soil. This mixture of IMO4 is ready to be used by the farmers for odor removal in animal waste and as bio-fertilizer in crop plantation. (Hoom Park and Michael W. DuPonte., 2008) (Figure 3 & 4).

1.7 Indigenous microorganisms in Natural Farming

The application of IMO in Natural Farming has been applied in Asia and Korea for many years. These beneficial soil microbes enhance soil fertility and plant nutrient uptake. The pioneer of Natural Farming was Mr. Han Kyu Cho who has travelled many countries to educate from many small scale and commercial farmers about Natural Farming. IMO soil is practically applied in natural farming and it is most important to achieve the right soil conditions, i.e. invigorating the life of the soil. For this, the optimal living environment for microbes and other small animals must be provided for them to live and prosper. The goal of Natural Farming is to revitalize the earth with growing increasingly desolate by reinstating these organisms back to their original form. Natural Farming is meant to be practiced by individual farmers because the ingredients and indigenous microbes are cheap and easily available to each locale or farm. Natural Farming base on bacteria without adding any chemical compounds. The advantage of natural farming methods includes lower costs to the farmer and healthier and better quality. By using IMO4 in natural farming it control the pest and disease. The farmers only used natural materials without using chemical soil in the market including the pesticides because pesticides do not only kill insects and they stay in the soil and fruit. When we eat the fruit, it can cause diseases. However natural farming method used earthworms and microorganisms instead of using machines.

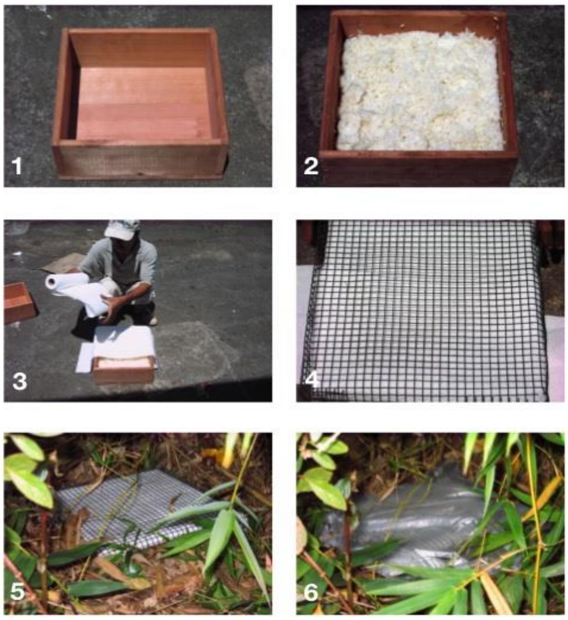

Figure 3: IMO cultivation preparation steps 1- 1; a container 2; steamed rice, 3; white paper 4; a plastic to cover container 5; located under a bamboo tree, 6; left for one week

Figure 4: IMO cultivation preparation steps II- 7; Molded rice formed after 5 days of incubation, 8; removing the molded rice in to the bowl into the bowl 9; brown sugar added equally 10; sugar and rice are mixed consistently, 11; the rice/ sugar mixture are covered, 12; the mixture are placed in clay pot before futher stored under direct sunlight for 7 days.(Hoom Park and Michael W. DuPonte., 2008)

1.8 Literature reviews

The indigenous microorganism can be found at any time during the year (Park and Duponte, 2008). The best place for collecting microorganisms is undistributed jungle or bamboo areas. Mixture of bacteria collected from various sites will probably leads to more robust culture. Incorporation of commercial chitosan into soil comprising indigenous microorganisms such as of *Paenibacillus macerans, B. pumilus* and *B. subtills* has increased growth of roots, shoot length and the yield (Vasudevan *et al.*, 2002). Indigenous microorganisms of some species in crude oil to enhanced oil recovery have been reported elsewhere. Singh and Lin (2007) showed that an uncontrolled release of chemical hazards of petroleum compounds into soil and groundwater cause serious dieses to human and animal health. These chemical hazards can be treated by using indigenous microorganisms to degrade the hydrocarbon-contaminated soil which has been proved as an efficient, economic, versatile and environmentally sound treatment. Singh and Lin (2007) isolated ten indigenous microorganisms from contaminated soils and 5 of them identified as *Acinetobacter calcoaceticus* (LT1 and ETS2), *Acinetobacter* sp. (LT1A), *Citrobacter freundii* (MRC3) and *Bacillus pumilus* (JLB). *B. pumilus* achieved 86.94% of diesel degradation in two weeks. An additional degradation assay was carried out in liquid media using three local commercial fertilizers (F1, F2 and F3) as nutrient supplements in comparison with the Bushnell-Haas (BH) media. The results show that the addition of fertilizer F1stimulated diesel degradation by all isolates especially *B. pumilus* while the addition of fertilizer F3 seemed to strongly inhibit the bacterial ability of diesel degradation. The inoculation with the consortia did not show a higher degradation potential than the individual isolate. The results strongly indicate that environmental conditions of the contaminated sites play a crucial role in the degradation even though additional diesel-degrader has been introduced into the contaminated site.

Dutta et al., (2003) presented data on comparative studies of unknown mixed cultures of indigenous microorganisms versus single cultures isolated from PCB-contaminated soil samples containing varying concentrations of PCBs (polychlorinated biphenyls). Remediation of PCBs was studied using isolated indigenous mixed cultures, by indigenous single colony cultures, in presence or absence of the leguminous alfalfa (*Medicago sativa*) plant. The congener 2′, 3, 4-PCB was used as known standard to spike the mixture of clean soil and vermiculite. Analytical methods such as HPLC (high performance liquid chromatography) and GC (gas chromatography) were used for PCB degradation quantification. The plant experiments were carried out in a controlled growth chamber environment with a 16 hours fluorescent and incandescent light period which mimicked day light. Results obtained suggest that higher the PCB concentration in soil, the more efficient the indigenous soil bacteria particularly in presence of plants. Mixed indigenous cultures were 2 –3 times more efficient than single cultures. When compared to known PCB co-metabolizing bacteria such as *Comamonas testosteroni* and *Rhodococcus* sp., indigenous bacteria showed not only higher amounts of PCB degradation, but also significantly better growth as shown by the CFU (colony forming units) counts. When the alfalfa plant was present, the indigenous mixed cultures were even more effective than known and unknown single cultures. These findings suggest that a combination of endemic microorganisms utilized with alfalfa plant is a promising approach for bioremediation of PCB contaminated soils.

1.9 Indigenous microorganisms soil research in Malaysia

In 2010 a study entitled: ‘Discovery of Novel Properties from Bacterial Culture Supernatant via A Rapid and Simple Method towards Developing Anti-Quorum Sensing and Anti-Biofilm Drugs’ has been reported by Rahmat Omar (2010), where the novelty of this research lies in the discovery of novel anti-biofilm properties from IMO4 soil supernatant (future diagnostic kit). It develops an economical screening method for biofilm detection among clinical samples using Light Microscope. The Advantages are that it is a naturally-produced anti-biofilm compound(s) obtained from IMO4 soil bacteria. It has the ability to yield large quantities of the product by using simple culture.

There is a good demand for potential biofilm clearance in various fields especially in the clinical, industrial, and environmental fields. It is an economical and rapid method for detecting biofilm using Light Microscope rather than expensive and time consuming microscopic techniques. The product functions include IMO4 soil supernatant functions as a potential candidate to disrupt and break down the presence of microbial biofilms in various fields. An economical screening method for biofilm detection among clinical samples can be applied easily while surgery or treatment for the patients is taking place. This property also had shown its anti-biofilm activity in treating the chronic lung infection of the tested rat which was presented at BioMalaysia in 2010.
In the same year another study conducted entitled ‘Soil Derived Biocleaner Activity:
A novel Property of Bioactive Compounds from Indigenous Bacterial Soil’, was presented at Malaysian Technology Expo at Putra World Trade Center (PWTC) Kuala Lumpur had shown the discovery of biocleaner activity from soil bacteria type IMO4

On the other hand, Zakaria (2006) showed that high application of fertilizers has led to instability in plantation and yield production. Usage of soil enhancers could be known as one of the alternative which accounts in the form of composts, IMOs and enzymes from natural farming technology, effective microbes (EM) and arbuscular mycorrhizzal fungi (AMF). Increased plant growth, weight and sizes of plants using these soil-enhancing technologies have been reported in several states in Malaysia. Furthermore, farmers strongly are in the opinion that vegetables taste sweeter, are crisper, and have shinier skins than conventionally grown vegetables. The use of AMF is largely limited to the oil palm sector, particularly at the nursery stages. AMF also has a prophylactic impact on oil palms stricken with the soft rot fungi, Ganoderma. However, several thoughts such as enhancing the inoculum quality, research needs and usage protocols require to be explored to further advocate the use of these soil-enhancing technologies in Malaysia.

1.10 Biosurfactant

Biosurfactants (Microbial Surface Active Agents) have turned out as a significant product of biotechnology for industrial and medical applications in recent years. They can be practiced as emulsifiers, de-emulsifiers, wetting agents, spreading agents, foaming agents, functional food ingredients and detergents in various industrial sectors such as petroleum and petrochemicals, foods and beverages, organic chemicals, cosmetics and pharmaceuticals, agrochemicals and fertilizers, environmental control and management, etc. Surfactants are ampifilic compounds that diminish the free energy of the system by replacing the bulk molecules of higher energy at an interface (Mulligan and Gibbs, 2004). They encompass a hydrophobic moiety with little affinity for the bulk medium and a hydrophilic portion that is attracted to the bulk medium. Surfactants have been utilized industrially as adhesives, deemulsifiers, flocculating, wetting and forming agents, lubricants and penetrants (Mulligan and Gibbs, 1993). Surfactants are key ingredients implemented in detergents, shampoos, toothpaste, oil additives, and a number of other consumer and industrial products. They account for an imperative class of industrial chemicals broadly used in almost every sector of present industry. Biosurfactant is a structurally miscellaneous group of surface-active molecule synthesized by microorganisms. Their proficiency of decreasing surface and interfacial tension with low toxicity and high specificity and biodegradability, lead to an increasing interest on these microbial products as alternatives to chemical surfactants (Banat et al., 2000).

Based on Hester from the Technical Insights estimated that biosurfactants could capture 10% of the surfactant market by the year 2010 with sales of $US200 million (Sanket and Anjana, 2010). However, up to now, biosurfactants is still unable to compete with the chemically synthesized surfactants in the surfactant market. This could be due to their high production costs in relation to inefficient bioprocessing method available, poor strain productivity and the need to use expensive substrates (Cameotra and Makkar, 1998; Deleu and Paquot, 2004). The interest in biosurfactant has been steadily increasing in recent years due to the possibility of their production through fermentation and their potential applications in such areas as the environmental protection. Initial focus of industrial interest towards biosurfactants concentrates on the microbial production of surfactants, cosurfactants and so on for the application on microbial-enhanced oil recovery (Kosaric et al., 1987). The applications of biosurfactants however, are still currently remained at the developmental stage of industrial level. The development of biosurfactant application in industries has focused mainly on high biosurfactant production yield and the production of highly active biosurfactants with specific properties for specific applications. At present, biosurfactants plays an important application in petroleum-related industries which is use in enhanced oil recovery, cleaning oil spills, oil-contaminated tanker cleanup, viscosity control, oil emulsification and removal of crude oil from sludges (Bernheimer and Avigad, 1970). These industries are known to be the potential target for the application of these compounds. This is due to the ability of biosurfactant-producing microorganisms to use petroleum or its’ products as substrates as well as the properties of the biosurfactant which required less rigorous testing than chemical surfactant (Cooper, 1986).

1.11 Problem statement and the importance of this study

From the agricultural point of view, longer persistence of pesticides leading to accumulation of residues in soil may result into the increased absorption of such toxic chemicals by plants to the level at which the consumption of plant products may prove deleterious hazardous to human beings as well as livestock's. There is a chronic problem of agricultural chemicals, having entered in food chain at highly inadmissible levels in India, Pakistan, Bangladesh and several other developing countries in the world. For example, intensive use of DDT to control insect pests and mercurial fungicides to control diseases in agriculture had been known to persist for longer period and thereby got accumulated in the food chain leading to food contamination and health hazards. DDT and mercurial fungicides has been banned to use in agriculture as well as in public health department. Therefore, a crucial study seems to be necessary for successful biodegradation to overcome this global problem.

. Another important aspect of this study is the air pollution problem (odors) which has become a center of public concern (Zhu, 2000). This is reflected in the increased frequency of odor-related complaints in areas where swine production facilities are more intensified. Odor management is currently impacting many aspects of the swine industry and there appears a potential that the sustainability, productivity, and profitability of swine producers will be dependent upon whether they can reduce the emission of offensive odorants from operating swine production units to a level which surrounding communities could tolerate. Therefore, there exists an acute need for effective methods of odor control, for if the swine industries are to coexist with their neighbors, such control measures will have to be put into operation. Microbial activities are normally considered to be responsible for the malodor generation from the stored swine manure slurry. As a matter of fact, microbes play a major role in both production and reduction of malodors. In odor generation, the odorous volatile organic compounds are the normal end products or intermediate products of fermentative degradation of fecal substances by anaerobic bacteria. In odor reduction, many odor control techniques that are being developed rely on the microbial properties in the swine manure. IMO4 is useful in removing bad odors from animal wastes. Since the malodor originates from microbial activities involving a variety of microbes, understanding the characteristics of the microflora present in swine manure is essential for developing effective odor control techniques.

1.12 Objectives of this study

The Natural Farming indigenous microorganisms Soil (IMOS) technology has been introduced by Korea and another technology brought from Japan is effective microbes (EM). The use of IMO as a soil enhancer was started in Malaysia by the Department of Agriculture in 2001 through the Asian Productivity Organization. Besides its use for enhancing soil fertility, it is more popular in livestock management for its odor-removing properties and as a floor-scrub cleaning (MARDI, 2006)Thus, it is our interest to investigate the bacterial activities of IMO soil bacteria such as haemolytic, emulsifying, antimicrobial susceptibility, toxicity in mice and plasmid presence. This study was a preliminary investigation into the application of biosurfactant. Therefore, the identification of bacterial types of colonies obtained is beyond the current study.

The main aim of this study was to screen and isolate the biosurfactant-producing microorganisms from IMO4 soil using hemolysis of red blood cell as suggested previously (Carrillo et al., 1996 and Fiebig et al., 1997). For strain characterization, the scope was only focused on the bacterial Gram differentiation methods (Gram staining, aminopeptidase strip test and an in-house alkaline lysis), cell and colony morphology. This is followed by the determination of antibiotic susceptibility test, mice lethality & virulence test and presence of plasmid DNA. The cell free-supernatant of the isolates were tested for the presence of biosurfactant (surface-active compounds) and emulsan using rapid method of qualitative drop collapsing (Youssef et al., 2004) with modification. Therefore the following experiments were designed:

1. To screen and isolate the bacteria from IMO soil.
2. To identify the soil bacteria against antibiotic, examine their resistance pattern and plasmid profile.
3. To screen biosurfactant activity of soil bacterial filtrates.
4. To determine the virulence of soil bacterial filtrates.