# CHAPTER FOUR

# DISCUSSION AND CONCLUSIONS

The increased use of chemical fertilizers and some organic fertilizers in agriculture helped the country in achieving self sufficiency in food grain production. However, it has also polluted the environment and caused slow deterioration of soil health. The chemical residues in the food product are also causing injury to human beings and cattle population. To combat these problems and in the light of sustainable agriculture, green technology is now being greatly used (Singh et al., 2004). Indigenous microorganisms (IMO's) and green manures act as reserve source for all nutrients. It adds organic matter to the soil and this increases soil fertility. The importance of green manuring had been recognized as early as 5000 BC in India. IMO's inoculated plants exhibit an increased plant growth, high nutrient status including that of phosphorus besides offering resistance to pathogenic and disease causing microorganism. IMO's suspension contains a wide range of naturally chelated plant nutrients and trace elements, carbohydrates, amino acids and their growth promoting substances and these can help as a soil conditioner by stimulating microbial activity in the soil which results in improved air-water relationships in soil, improved fertility and makes soil less prone to compaction and erosion. IMO's are organisms that enrich the nutrient quality of soil.

Microorganisms have many properties included the ability to degrade waste products. Soil bacteria has been an incredibly productive source of antibiotics, anticancer compounds (chemotherapeutic doxorubicin hydrochloride, bleomycin,

daunorubicin and mitomycin), immunosuppressive agents, insecticides and other molecules that are important in nature and useful to humans (Handlesman et al., 1998; Omura, 1992; Pettit, 2004). As a result, the genetic and biodiversity of microorganisms is an important area in research. The use of IMO as a soil enhancer was started in Malaysia by the Department of Agriculture in 2001 through the Asian Productivity Organization (Zakaria, 2006). It also was applied as odor removal against animal wastes (personel communication with Puan Mariam). According to Puan Mariam who was working in Serdang Agricultural Center, when they applied IMO4 soil to the smelly cow den's floor, there was no fly around it and the floor turn to white after scrubbing. However, this technique is lacking understanding of the basic microbiology in treating the odor from animal wastes. According to Liao and Bundy (1994) and Barrington (1994), microbial digestive additives contain bacteria or enzymes that eliminate odors and suppress gaseous pollutants by their biochemical digestive processes. Therefore understanding the characteristics of the microflora present in IMO soil in the treatment of animal waste is essential for developing effective odor control techniques. IMO soil contain biosurfactant activity due to its microbial activity in the soil. Microbial biosurfactants production, applications and future potential in environmental, industrial and biomedical has been described (Banat, 2010).

Therefore, it's of a great interest to isolate the IMOs soil project in finding the potential bacterial producing biosurfactant. Biosurfactants are widely used in industries like cosmetics, specialty chemicals, food, pharmaceutics, agriculture, cleansers, and oil recovery (Kim et al., 1999; Demin et al., 1999; Mulligan et al., 2001; Daoshan et al., 2004; Yeh et al., 2005; Joshi et al., 2008). In this recent year, the biosurfactants have been placed on the environmental impacts of chemical surfactants and new surfactants

for use in any field. The aim of this study is to preliminary screen and isolate indigenous (IMO) microorganism from formulated soil collected at Serdang Agricultural Center, Selangor, towards future purpose application in environmental odor removal and oil remediation.

Hence, preliminary screening of the bacterial isolates from IMO4 soil was performed to isolate soil bacteria using dilution streaking on BHI blood agar medium for colony morphological analysis and Gram staining to provide a basis for differentiating strains. It was then further tested for preliminary screening biosurfactant activity among the colonies produced via haemolytic and drop collapsing with emulsification activities. Antibacterial sensitivity, mice lethality test and presence of plasmid were determined too in this study.

## 4.1 Colony morphology and characterization of IMO4 soil bacteria

The isolation process is a procedure of isolating the mixture of colonies to a single colony. This process was done by using streaking method to obtain pure cultures. They also grew well on other types of agar and broth medium such as nutrient, Mueller-Hinton and LB medium with optimum temperature of 37ºC. From the observation, various colonies were formed on BHI blood plate medium; some with haemolysis and some non haemolysis; some small colonies and smooth whereas others big and rough colonies; minority were mucoid type. BHI medium was used for enrichment of any fastidious microorganisms from the agricultural soil and blood was used to ensure its reproducibility of the haemolytic activity and its colonial morphology. All colonies on BHI blood plate and broth grown at optimum temperature of 37ºC and for the haemolytic colonies, zone inhibition were shown within 8-12 hours with β-haemolytic

activity. There was no α-haemolytic was observed among the 139 unknown isolates and neither fungal colonies on all the plates used.

Interestingly the soil samples diluted mixed with only distilled water which kept for more than 2 years at room temperature had shown no fungal contamination occurred neither in the soil samples nor on BHI blood agar plate, which this might due to antimicrobial activities of soil bacteria against a fungal strain. In addition, the bacterial colonies in this study were still alive when subcultured routinely on BHI blood agar medium. This is very interesting discovery since the microorganisms are still surviving without any addition of nutrition in the sterile distilled water-containing soil samples (3-5 ml in the bijou bottles in triplicate) for more than 2 years. All the results were recorded systematically and carefully in order to further test for biosurfactant activity. All the colonies obtained were maintained on BHI slant agar as a stock culture for further used in future. The purified colony was maintained on BHI blood agar medium as a working culture plate. A single colony of unknown bacterial culture was prepared in BHI broth medium and incubated overnight at $37^{o}$C.

## 4.2 Bacterial gram differentiation using Gram staining, cell lysis and aminopeptidase-strip test

The Gram staining method is one of the most important staining techniques used in microbiology. It is the first test performed for the identification of bacteria, those bacteria that are stained (purple colour) by the Gram method are commonly classified as Gram-positive, whereas those that are not stained (pink colour) are referred to as Gram-negative. There are four basic steps involved in Gram staining, which includes the

application of a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture, followed by the addition of a mordant (Gram's iodine), rapid de-colorization step with alcohol or acetone, and counterstaining with safranin or basic fuchsin. Gram staining is based on the ability of bacterial cell wall to retain the crystal violet dye used in the procedure. Gram-positive microorganisms have a thicker peptidoglycan layer in their cell walls which retains the primary crystal violet stain and appears purple while the Gram-negative cell wall contains a thinner layer of peptidoglycan that will appear pink when it is counterstained with safranin. The length of the de-colorization step is critical in differentiating the Gram-positive bacteria from the Gram-negative ones. A prolonged exposure to the de-colorizing agent will remove all the stain from both types of bacteria. Some Gram-positive bacteria may lose the stain easily and therefore appear as a mixture of Gram-positive and Gram-negative bacteria (Gram-variable). Thus, the arising ambiguity may pose a problem in obtaining accurate results. A commercial test kit based on the aminopeptidase activity detection is currently available and is also widely used to distinguish between Gram-positive and Gram-negative bacteria.

Cell lysis is a cell biology method that normally used for the release of cells' content including DNA, RNA, organelles, proteins and lipids. This lysis solution (alkaline lysis solution) contains sodium hydroxide (0.2N NaOH) that helps in breaking down the cell wall and Sodium Dodecyl (lauryl) Sulfate (1% SDS) that solubilize the cell membrane. Here we report the use of lysis solution on bacterial cells for the purpose of differentiating Gram-positive and Gram-negative bacteria. This study shows an application of lysis solution to a series of bacteria that can distinguish between Gram-positive and Gram-negative groups based on the ability of cell wall disruption. The results were compared with the Gram stain reaction and aminopeptidase-strip test

to confirm the efficacy of our preliminary in-house method for Gram-positive and Gram-negative bacterial classification.

Previously a total of six hundred and ninety one (691) bacterial isolates from various sources maintained in our laboratory, were subjected to Gram staining, aminopeptidase strip test and an in-house alkaline lysis method. This simple, rapid method involves breakage of the cell wall by introducing equal volume of alkaline lysis solution (0.1N NaOH, 1% SDS) into overnight grown bacterial broth culture. A clear viscous bacterial lysate was observed within 2 minutes at $4^{o}C$ for all Gram-negative bacterial isolates but not for any of the Gram-positive isolates. The standard aminopeptidase strip test was used to confirm our observation. There was a 100% correlation between the present in-house method and the aminopeptidase strip test and the results were consistent and reproducible in all of our studies which carried out in laboratory. This rapid technique is very economical and useful in the classification and preliminary differentiation of bacteria isolated from various sources including clinical, soil, animal and industrial waste.

Therefore cell lysis technique was applied in this study to confirm the bacterial gram of unknown soil isolates. All unknown soil bacterial isolates (100%) of Gram-negative could be lysed by the alkaline lysis solution and showed results positive for aminopeptidase activity. In case of Gram-positive bacteria, all isolates (100%) were not lysed by the alkaline lysis solution and did not show aminopeptidase activity. Aminopeptidase enzyme is only present in Gram-negative isolates. Sometime the strip test produces a very weak activity or signal against Gram-positive and a delay in colorization for Gram-negative. Furthermore, the growth medium from which the colonies were taken from should not contain any dyes or indicators and it is not recommended to take any pigmented colonies. The Gram staining and aminopeptidase

test strips methods are both color dependant-test kits which is affected by time exposure of the dye agents thus producing false-positive results. These confusing and inconsistent results affected the reproducibility of the test strip efficacy. However, it does not replace the Gram-staining, as it cannot show morphology. Of all the methods used so far, alkaline lysis was found to be the most simple, definite, economical, rapid and accurate method. It can also be used as an alternative method in addition to Gram staining for a rapid differentiation for a large number of bacterial isolates from soil or any other source. Bacterial identification and characterization of unknown soil isolates were not further analysed in this study. Therefore, rather than describing the bacterial genus and species, we emphasized on the biosurfactant screening in order to determine the potential biosurfactant-producing microorganisms for further study. In addition to investigate the potential compounds for odor treatment against animal wastes by using the indigenous bacteria, a further examination on individual soil isolate is needed.

## 4.3 Screening for biosurfactant activity

Biosurfactants are surface active compounds produced by microorganisms and their screening are generally carried out by monitoring parameters that estimate surface activity, such as surface tension, interfacial tension and the ability to emulsify oils or hydrocarbons (Cooper & Zajic 1980). Biosurfactants have received considerable attention in the field of environmental remediation processes such as biodegradation, soil washing and soil flushing. Biosurfactants influence these processes because of their efficacy as dispersion and remediation agents and their environmentally friendly characteristics such as low toxicity and high biodegradability (Mulligan, 2005; Das et al., 2009; Sivapathasekaran et al., 2010; Kiran et al., 2010; Satpute et al., 2010). There are many reports describing the soil indigenous microbial biosurfactants treatment in

polluted crude oil (Abalos et al., 2004; Owsianiak et al., 2009). Biosurfactants are potential antimicrobial agents to specific microbes and thus can be exploited for its antimicrobial applications against plant, animal and human microbial pathogens (Van-Hamme et al., 2006). The enormous diversity of biosurfactants makes them an interesting group of materials for application in many areas such as agriculture, public health, food, health care, waste utilization, and environmental pollution control such as in degradation of hydrocarbons present in soil.

### 4.3.1 Haemolysis assay

Different selection criteria have been assayed to detect potential biosurfactant producers. Persson & Molin (1987) used the spreading of a water droplet over the surface of a microscope slide after the addition of the microbial culture. Haemolytic activity was also investigated, as a potential predictor of surfactant production and Passeri et al. (1988) had tested the haemolytic capacity of the strains. Hemolysis on blood agar has been widely used to screen biosurfactant producing bacteria and for preliminary identification of many types of clinically important bacteria (Mulligan et al., 1984). At a very beginning of our work, the idea use BHI blood agar as enrichment medium was for enhancement growth of the fastidious soil bacteria in agricultural soil of IMO4. On the other hand, this criterion was applied for biosurfactant screening and was first discovered by Bernheimer and Avigad (1970) reported that the production of biosurfactant (surfactin) by *B. subtilis* may cause the red blood cells to lysis.

Nowadays, many researchers have used this technique to screen for biosurfactant production by new isolates (Carrillo et al., 1996; Yonebayashi et al., 2000). Hemolytic reactions are generally classified as alpha (α), beta (β) or gamma (ɣ) according to the appearance of zones around the isolated colonies growing on blood

agar (Pape et al., 1987). The hemolytic reaction is called alpha hemolysis when the colony is surrounded by a zone of intact caused by the decolorisation of which appear as a greenish zone. This appearance is generally due to the action of peroxide (peroxidases) produced by the bacteria capable of decolorisation. Beta hemolysis indicates a zone of clearing in the blood agar in the area surrounding a bacterial colony. If there is no change in the medium around the colony, which is no hemolysis occurred on the blood agar, the reaction is called gamma hemolysis.

Carrillo et al. (1996) had proved the efficiency of this method in screening of biosurfactant-producing bacteria. They found an association between hemolytic activity and surfactant production. However, there were limitations of using this method to screen the biosurfactant producer. Not all biosurfactants have a hemolytic activity and compounds other than biosurfactant might cause hemolysis. On the other hand in this study, nonhaemolytic isolates also produced biosurfactant activity ($H^-DC^+$) which it shows the biosurfactant activity not only caused by lytic enzymes because it may also be associated with the presence of other microbial products such as virulence (Carrillo et al., 1996) that could influence the results. The absence of hemolytic activity does not mean the isolate is not a biosurfactant producer; it could be due to diffusion restriction of the surfactant through the blood agar (Jain et al., 1991). However, in some reports isolates with positive hemolytic activity were found negative for biosurfactant production (Youssef et al., 2004) and similarly we had found it in 2 of our isolates. In addition, not all biosurfactants have hemolytic activity and this has shown in our study where 54 of 139 soil bacterial isolates were haemolytic negative and produced biosurfactant ($HA^-DC^+$).

Even though blood agar is already known as a rich growth medium for many organisms, however the method has some limitations. First, the method is not specific,

as lytic enzymes can also lead to clearing zones. Second, hydrophobic substrates cannot be included as sole carbon source in this assay. Third, diffusion restriction of the surfactant can inhibit the formation of clearing zones. In addition, (Schulz et al., 1991) showed that some biosurfactants do not show any hemolytic activity at all. (Youssef et al., 2004) and (Plaza et al., 2006) also confirmed the poor specificity of this method. It can give a lot of false negative and false positive results. (Mulligan et al., 1984) recommend the blood agar method as a preliminary screening method which should be supported by other techniques based on surface activity measurements.

Hence, in the present study drop collapse test and emulsification assay were included to confirm biosurfactant production among 139 unknown soil bacterial isolates. In this experiment cell free culture broth was used as the biosurfactant source.

### 4.3.2 Drop collapse assay and emulsification ability of soil bacteria

The drop collapse test together with emulsifying activity with crude oil was included to confirm biosurfactant production. It was based on the ability of destabilization of oil droplet to liquid droplets containing biosurfactants followed with emulsification ability (Figure 19). Youssef et al. (2004) have found that drop collapse method was correlated with the ability of the cultures to reduce surface tension. There were strong correlation between the drop collapse method and surface tension reduction, which cultures showing a greater degree of collapse had low surface tension values. The amount of surfactant required to cause drop collapse was found dependent on the ability of the surfactant to reduce surface and interfacial tension (Bodour and Miller-Maier, 1998). The more potent the surfactant, the smaller the quantity and time required to cause drop collapse. This method was therefore, very specific since only organisms which produced significant surface-active compound, will cause collapse of

aqueous drops on oily surfaces (Bodour and Miller-Maier, 1998). The drop collapse assay is rapid and easy to carry out, requires no specialized equipment and just a small volume of sample. The test volume required in this technique is much smaller (5µl) than the volume required for the surface tension measurement (20ml) and the results were easy to determine visually. Furthermore, this technique was easy to perform, more reproducible and can be used to screen large number of isolates (Bodour et al., 2003). In addition, it can be performed in microplates (Tugrul and Cansunar, 2005). This assay has been applied several times for screening purposes (Batista et al 2006; Bodour et al., 2003; Plaza et al., 2006; Youssef et al., 2004). But it displays a relative low sensitivity since a significant concentration of surface active compounds must be present in order to cause a collapse of the aqueous drops on the oil or glass surfaces. Drop collapse together with emulsifying activity methods are reliable methods to screen large number of samples for biosurfactant production because these methods require only 5-10 µl of biosurfactant solution. Persson and Molin (1987) described a similar assay using a glass surface instead of the oil coated surface.

## 4.4 Other tests – Antibiotic resistance, plasmid presence and pathogenenicity of soil bacteria

One hundred and ten (110) of 138 isolates had shown various antibiotic resistances to soil bacteria at least more than one antibiotic whereas the other 22 isolates were sensitive to all antibiotics tested. When all the isolates were subjected to plasmid DNA screening, none of the isolate possessed plasmid. It shows that the antibiotics resistance in the isolates was not plasmid mediated. Furthermore all isolates are nonpathogenic and harmless when tested in mice even though they produced strong haemolytic activity. It shows here that the haemolytic property in the soil bacteria is not

virulent to mice. Biosurfactants may be involved in pathogenesis due to their surface activity; however, for security and regulatory reasons, production strains should be non-pathogenic (Walter et al., 2010) as discovered in this study.

The growing challenge of the emergence and spread of antibiotic resistance has been the subject of several investigations and reviews (Linton 1986; Bates et al., 1994; Jacobs-Reitsma et al., 1994; Piddock 1996; McDonald et al., 1997; Anonymous 1998; Levy 1998; Schnabel and Jones 1999). Antibiotics have been actively employed in human and veterinary medicine for the treatment of infectious diseases and as feed additives for about 50 years. It is estimated that more than 70% of the annual antibiotic out-put is fed to chicken, pigs and cows for non-therapeutic purposes and used in agriculture to protect plants and fruits (Anonymous, 2001). Research has consistently linked the use of antibiotics in food production to increased incidence of drug-resistant pathogens in animals and man (Bates et al., 1994; Piddock 1995; Blanco et al., 1997; McDonald et al., 1977; Al-Mustafa and Al-Ghamdi 2000) and animal gut flora is seen as a major reservoir of the R plasmid (Linton and Hinton 1984). Earlier studies on antibiotic resistance in aquatic habitat for instance, concentrated on its incidence in bacteria of fecal origin or those resistant to heavy metals (Niemi et al., 1983; Calomiris et al., 1984). Walter and Vennes (1985) among others have shown that waste effluents from hospitals contain higher levels of antibiotic-resistant enteric bacteria than waste effluents from other sources, but the effect of such releases on indigenous bacteria is poorly documented. In contrast, Jones et al. (1986) found that the incidence of resistance in indigenous bacteria was by far greater than that of introduced species and remained high even in undisturbed habitats.

## 4.5 Conclusions

Hundred and thirty nine (139) unknown bacteria were successfully isolated from agricultural IMO4 soil and purified using BHI blood agar medium. 49% of the isolates were smooth colonies, 42% of rough colonies and 9% of the mucoid colonies. Among 139 isolates, 78% were identified as Gram positive bacteria and 22% were Gram negative. Antibiotic resistant was discovered in 109 isolates with at least to one antibiotic tested. 29 antibiogram profiles were obtained from total isolates tested (139). All isolates (139) were plasmidless and nonpathogenic (harmless) in mice. Among the 139 soil isolates, 49% were haemolytic positive and 51% were nonhaemolytic. 86% of the total isolates produced biosurfactant activity and 14% were nonbiosurfactant producer. Among the 49% haemolytic isolates (68), 66 were biosurfactant positive ($HA^+CD^+$) and the other 2 were nonbiosurfactant producer ($HA^+CD^-$). Among 71 of the total nonhaemolytic isolates, 54 were biosurfactant positive ($HA^-CD^+$) and others 17 were nonbiosurfactant producer ($HA^-CD^-$).

In conclusion, the result of this preliminary study showed that IMO agricultural soil contains strong haemolytic microorganisms with potential biosurfactant activity and it need to be further investigated.

## 4.6 Future study

This study has contributed preliminary data that can be used for further investigation as follows:

1) **Analysis and Identification of Indigenous Microorganisms Isolated from Agricultural Soil:**
   a) Isolation, screening, characterisation and identification of biosurfactant-producing microorganisms from agricultural soil bacteria.
   b) Screening for biosurfactant activity.

2) **Extraction, Analysis and Identification of Bacterial Surface-Active Compounds (BSAC) – Biosurfactant:**
   a) Preparation of crude extract containing biosurfactant activity from soil bacteria.
   b) Analysis and characterisation of bacterial crude extract containing biosurfactant activity.
   c) Purification and characterisation of biosurfactant protein/compound.
   d) Identification and analysis of biosurfactant chemical structure.