CHAPTER 1

INTRODUCTION

1.1 Introduction

Generally, development of new industrial parks, plantations, highways, townships as well as new housing estates is parallel with cleaning of green land covered by highly diversified vegetations. One of the major challenges which always attracts greater attention is on how to conserve the slope areas. The main focus is to avoid soil erosion that has a potential to change the original landscapes as well as destructing the newly developed areas. Many techniques as well as materials has been tested to alleviate the problem originated from soil erosion.

For centuries, living plants and wood were the only materials adapted for hill and slope stabilization works. Now, various types of plants and grasses are used for hillside and slope stabilizing. Dry-seeding and hydroseeding are popular techniques, in which the uncovered areas of seed is protected with a combination straw and bitumen or meshes of wire and jute. There are numerous methods for slope and hillside stabilization which use plants in combination with constructions of stone, wire and wood such as planted pole walls, live wooden crib walls, live slope grids, vegetated gabions and vegetated stone walls (Florineth and Gerstgraser, 1996). Nowadays, the practice of using vegetation to prevent and control erosion for slopes stabilizing is well accepted all over the world. This technique is low maintenance, environmental friendly, low cost and much easier to establish as well (Mitsch, 1998).

Basically, plants and grasses are used in the slope stabilization work as they naturally grip the soil which is aided by roots. Previous studies have shown the significant contribution of vegetation cover for slope stabilizing (Mokhtar *et al*., 2006). Despite taking part in its original function to supply nutrients to plants and grasses, roots also play an important role as soil-root reinforcer in order to avoid them from being flushed away by soil erosion. The stems and root systems of grasses tend to trap fine particles of soil, thus inhibiting the migration of these particles (Mokhtar *et al*., 2006). Hence, this specific character of roots will indirectly maintain the topography of the area where they are grown. Study has shown at stable slopes that are covered by high density vegetation would exibit high root length density (RLD) which eventually resulted in a lower soil water content (SWC) and vice versa. (Normaniza and Barakbah, 2006). Concomitant to this low SWC, the slope becomes more stable due to low saturation level of the soil.

Currently, various types of grasses have been used in slope stabilization works. All plants and grasses require relatively large amounts of nitrogen (N) for proper growth and development. Generally, slope grasses are grown at isolated areas with extremely lower or virtually ‘zero’ amount of nitrogen and without systematic farming system and fertilization. Hence, nitrogen supply as well as other nutrients required for the plants growth should come from natural sources such as nitrogen fixing bacteria of the slope grasses. Previous study had shown that there was a significant relationship between nitrogen fixation and grasses (Stoltzfus *et al.*, 1998). Plant growth shows significant influence on bacterial communities.

Biological nitrogen fixation involves the reduction of atmospheric nitrogen gas to ammonia or nitrate. Prokaryotes that fix nitrogen are classified into two main groups, symbiotic and free-living nitrogen fixing bacteria. In farming system, it is estimated that biological nitrogen fixation contribute 180 x 10^6 metric tons/year globally (Postgate, 1998), of which 80% comes from symbiotic associations and the rest from free-living or associative systems (Graham, 1988). However, non-symbiotic nitrogen fixation is also known to have a great influence on plants growth. For example, in an intensive wheat rotation at Avon, South Australia, non-symbiotic nitrogen fixation contributed 20 kilograms per hectare per year, which met 30–50% of nitrogen needs of this system for a long-term (Vadakattu and Paterson, 2006). In this study, high diversity of free-living nitrogen fixing bacteria is anticipated to enhance the soil quality and thus influence the stability of slope.

1.2 Objectives of study

1. To study the relationship between the slope profiles and the population number of free-living nitrogen fixing bacteria.
2. To assess the diversity of free-living nitrogen fixing bacteria from the slope grasses.

CHAPTER 2

LITERATURE REVIEW

2.1 Nitrogen fixation

Nitrogen is widely known as an essential nutrient for all living organisms on earth (Sylvia *et al*., 1999). It is one of the major components in amino-acid, proteins and nucleic acids. Animals, plants and microorganisms can die of nitrogen deficiency. In nature, nitrogen compromises 78% of atmospheric gasses and become the most abundant in the atmosphere (Lindemann, 2008). Unfortunately, nitrogen has a triple covalent bond which higher plants cannot break for their uses as plants use a reduced form of nitrogen such as nitrate or ammonia for growth (Triplett, 2000). Hence, nitrogen gas is useless for living organisms unless they have a mechanism for reducing the nitrogen into a consumable form.

Nitrogen fixation is one stage in the nitrogen cycle steps which maintain the balance of this element in nature. It is the reduction of atmospheric nitrogen into nitrate and ammonia. Generally, nitrogen is fixed in 3 ways: spontaneously by lightening and photochemical reactions (10% of the nitrogen fixed in natural processes is fixed in this manner), industrially by using the “Haber process” which is an expensive process to produce fertilizers rich in nitrogen (Berkum and Bohlool, 1980) and biologically by specific nitrogen fixing bacteria which have a specific mechanism to reduce atmospheric nitrogen (Triplett, 2000). Currently, atmospheric nitrogen fixation undergone by nitrogen fixing bacteria is becoming an important topic among scientist in order to reduce consumption of inorganic fertilizer in agriculture.

2.2 Biological nitrogen fixation

Biological nitrogen fixation is one of the primary processes involved in the nitrogen cycle and is mediated in nature only by bacteria. It involves the reduction of dinitrogen (N_2) to ammonia (NH_3) via the activity of a complex metalloenzyme nitrogenase. This process is observed in species of more than 100 genera which is distributed among several of the major phylogenetic divisions of prokaryotes (Eubacteria and Archaea) (Young, 1992). The overall stoichiometry of the reaction is depicted as follows:

$$N_2 + 8H^+ + 8e- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16Pi$$

As shown above, the process is ATP-dependent and produces ammonia and hydrogen.

2.2.1 Mechanism of biological nitrogen reduction

Basically, the reduction process is catalyzed by the enzyme complex nitrogenase, which is ancient and widespread among bacteria. The nitrogenase complex consists of two separate proteins called dinitrogenase and dinitrogenase reductase. Both components contain iron and dinitrogenase contains molybdenum as well (Brock *et al*., 1994). Nitrogenase can use a variety of substrates to undergone the process. Nevertheless, nitrogen is the most important substrate in the natural nitrogen cycle for nitrogenase. The nitrogen fixation mechanism can be summarized as in Figure 2.1.

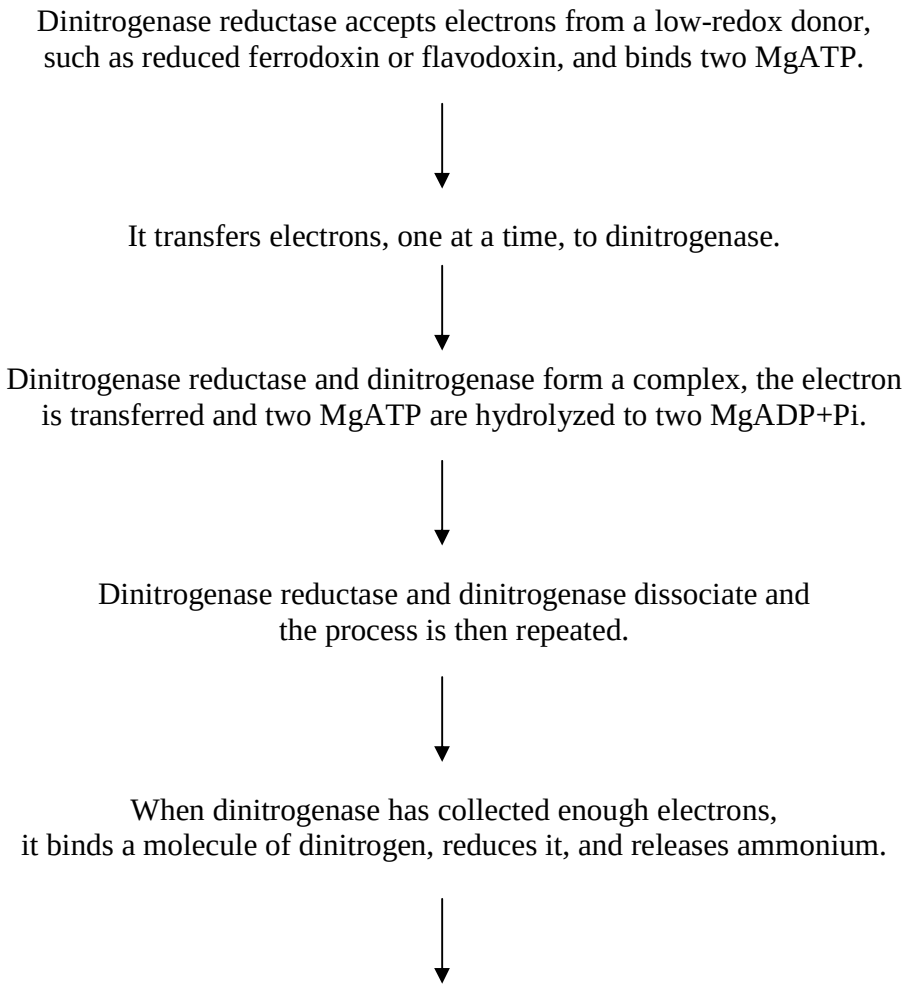


Figure 2.1 Summary of the biological nitrogen fixation mechanism (Sylvia *et al.*, 1999)

Despite many complex processes which make nitrogen fixation possible, the end products are ammonia (NH_3) and water. Nitrogenase, the vital ingredient which makes nitrogen fixation possible, is destroyed when it comes in contact with oxygen. So the process of fixation in nitrogen fixing bacteria occurs only in anaerobic conditions or the oxygen is neutralized by its combination with chemicals like leghemoglobin.

2.2.2 Regulation of nitrogen fixation

Among issues arise on nitrogen fixation are oxygen sensitivity of nitrogenase, energy demands of the fixation process, supply of metalloenzymes and utilize other sources of fixed nitrogen before fixing atmospheric nitrogen. This regulation operates at the level of transcription of the nitrogen fixation (*nif*) genes in all organisms. Such regulation is usually effected a general nitrogen control system that co-ordinates cellular nitrogen metabolism and a *nif*-specific mechanism that facilitates regulation in response to particular signals. Moreover, a number of organisms have evolved special mechanisms that allow very rapid short-term regulation of the activity of the nitrogenase enzyme in response to fluctuations in availability of fixed nitrogen (Merrick, 2004).

2.3 Nitrogen fixing bacteria

The primary function of nitrogen fixing bacteria is 'survival' and in their efforts to survive, they enter into a symbiotic relationship with leguminous plants or some survive on their own (free living). As a part of their metabolic cycle, they fix nitrogen. The nitrogen fixing bacteria and other microorganisms that fix nitrogen are collectively called ‘diazotrophs’. There are many strains of nitrogen fixing bacteria in soil which do this task. They are important agents in the nitrogen cycle. All the different types of diazotrophs have a nitrogen fixing system based on iron-molybdenum nitrogenase. Prokaryotes that fix

nitrogen are classified in two main groups which are symbiotic and free-living nitrogen fixing bacteria

2.3.1 Symbiotic

In legumes and a few other plants, the bacteria live in small growths on the roots called nodules. Some plants benefit from nitrogen-fixing bacteria when the bacteria die and release nitrogen to the environment or when the bacteria live in close association with the plant. Nitrogen fixation is done within these nodules by the bacteria and the NH_3 which is produced is absorbed by the plant. Nitrogen fixation by legumes is a partnership between a bacterium and a plant (Lindemann, 2008). Nevertheless, the nitrogen produced by this activity is not 'free'. The bacteria need a significant amount of energy and other nutritional factors that must be contributed by the plant. A soybean plant may divert 20-30 percent of its photosynthate to the nodule instead of to other plant functions when the nodule is actively fixing nitrogen (Lindemann, 2008). This type of nitrogen fixing bacteria range from loose associations, such as associative symbiosis, to complex symbiotic associations in which the bacterium and host plant communicate on an exquisite molecular level and share physiological functions (Sylvia *et al*., 1999).

The bacteria belonging to the genus *Rhizobia* are rod shaped and motile bacteria. They are primarily found in soil and survive by their symbiotic relationship with legume plants of the *Fabaceae* family. Their nitrogen fixation process cannot be executed without the help of their symbiotic partners which are the legume plants. The bacteria belonging to the genus *Frankia* sp. survive through their symbiotic relationship with *Actinorhizal* plants which are similar to leguminous plants. These bacteria form nodules in the roots of these plants. They wholly contribute the nitrogen needs of these plants and indirectly enrich the

soil with nitrogen compounds. Some Cyanobacteria show symbiotic behavior by their association with lichens, liverworts, a type of fern plant and cycad plant. One example of this type of symbiotic nitrogen fixing bacteria is *Anabaena* sp.

2.3.2 Free living (non-symbiotic)

Nitrogen fixing microbes can also exist as independent, free living organisms. The heterotrophic diazotroph depend on carbon (e.g. from straw) for energy whereas the autotrophic bacteria derive their energy from photosynthesis. From Table 2.1 (Brock *et al.*, 1994), they can either be aerobic and anaerobic. Associative nitrogen fixing bacteria colonizing nonlegumes can be classified into three groups: (1) rhizosphere organisms, such as *Azotobacter paspali*; (2) facultative endophytes that colonize the rhizosphere or root anterior of forage grasses and cereals, such as *Azospirillium* spp.; and (3) obligate endophytes that occur within plant tissues, such as *Gluconacetobacter diazotrophicus* (formerly known as *Acetobacter diazotrophicus*), *Azoarcus* spp., *Herbaspirillum* spp. and *Bulkhoideria* sp.(Kennedy, 2005).

Table 2.1: Some of nitrogen-fixing organisms (Brock *et al*., 1994)

Free-living				Symbiotic	
Aerobes		Anaerobes		Leguminous plants	Nonleguminous plants
Heterotrophs	Phototrophs	Heterotrophs	Phototrophs		
Bacteria: *Azobacter* spp. *Klebsiella* spp. *Beijerinkia* spp. *Bacillus polymyxa* *Mycobacterium flavum* *Azospirillum lipoferum* *Citrobacter freundii* Methylotrophs (various but not all)	Cynobacteria (various but not all)	Bacteria: *Clostridium* spp. *Desulfovibrio* spp. *Desulfoto maculum*	Bacteria: *Chromatium* spp. *Chlorobium* *Rhodospirillum*	Soybeans, peas, clover, locust, etc., in association with members of the genus *Rhizobium* or *Bradyrhizobium*	*Alnus, Myrica, Ceanothus, Comptonia,* in association with actinomycetes of the genus *Frankia*

2.4 Plants and bacteria

Basically, 80-90% of the reactions in soils are mediated by microbes (Coleman and Crossley, 1996). In agro-ecosystems, bacteria are responsible for diverse metabolic functions that affect plant health and soil fertility including nutrient cycling, soil structure, organic matter formation and decomposition, and plant growth promotion. The presence of microorganisms in the soil will depend on the number and volume of available microhabitats and bacterial activity to the amounts of available metabolic substrates found in those microhabitats (Nannipieri, 2003). These soil properties in turn depend not only on the fauna and vegetation but also on the geographical, geological, hydrological, climate, and anthropogenic influences (Liesack *et al*., 1997). Soil contains many different microhabitats thus increasing the bacterial diversity.

2.4.1 Nitrogen fixing bacteria and plants

A direct influence of the plant on the development of bacteria is suggested by Döbereiner (1961). Studies on *Beijerinckia* sp. showed that roots as well as leaves and stems had a positive influence on its populations. This was influenced by exudation of substances into the soil by the roots during rainfall (Döbereiner, 1970). Plant associated nitrogen fixing bacteria have been considered as one of the possible alternatives for inorganic fertilizer for promoting plant growth (Ladha and Reddy, 2000). Soil diazotrophs are the most important microorganisms to play the role in biological fixation.

2.4.2 The importance of biological nitrogen fixation

In natural systems, nitrogen for plant growth comes from the soil, from rainfall or other atmospheric deposition and biological nitrogen fixation. Organic and inorganic forms of nitrogen are needed and will be recycled by natural nitrogen cycle. Among the nitrogen cycle, biological nitrogen fixation takes the role of biological conversion of atmospheric nitrogen to forms available nutrient for plant and microbial growth. However, in many situations, nitrogen deficiency for plant growth still occurs especially in agricultural industry. As deficiency of nitrogen in the soil often limits crop yields, nitrogenous fertilizers are one of the most widely used chemical fertilizers. Generally, less than 50% of the added nitrogen is available to the plants. Since biological nitrogen fixation is one of the primary processes involved in the nitrogen cycle, this process recover the loss of nitrogen from soil-plant ecosystems.

Biological nitrogen fixation is reported to have contributed 65% of the nitrogen used in agriculture (Burris and Roberts, 1993). Much of this is via symbiotic nitrogen fixation, non symbiotic and associative fixation (Sylvia *et al*., 1999). Basically, ammonia as well as nitrate levels are often low and only a few prokaryotes can carry out nitrogen fixation. Hence, those factors also tend to limit plant growth in many situations (Prescott *et al*., 1999). Introduction of new technology and enhancement of biological nitrogen fixation efficiency offers an alternative to the use of expensive ammonium based fertilizer nitrogen, in other words biofertilizers compete with chemical fertilizers.

2.5 Erosion

Erosion refers to the displacement of solids (soil, mud, rock and other particles) by the agents of water, wind or ice, by downward or down-slope movement in response to gravity or by living organisms (Mokhtar *et al.*, 2006). It is different from weathering, where no movement is involved in the decomposition of rock and particles through processes, although the two processes may be concurrent. Erosion reduces levels of soil organic matter, removes topsoil and result in the breakdown of soil structure. The final impact depends on a combination of many factors, including the amount and intensity of precipitation, the texture of the soil, the gradient of the slope, ground cover (from vegetation, rocks, etc.) and land use. Rain is one of the main agents for erosion. Basically, areas with high-intensity precipitation, sandy or silty soils and steep slopes are the most erosive (Mokhtar *et al.*, 2006).

2.5.1 Vegetation cover

Soil erosion is directly driven by the forces of climate (effects of wind and rainfall) and specially occurs when the vegetation and upper soil horizons have their storage functions diminished under the influence of human actions. Vegetation cover is an important parameter used in assessing the relationship between vegetation and soil erosion. However, the intensity of soil erosion actually changes not only with vegetation cover but also with differences in vegetation type and structure (Zhongming *et al.*, 2010). Vegetation controls soil erosion rates significantly. The decrease of water erosion rates with increasing vegetation cover is exponential (Gyssels *et al.*, 2005). Scientists have shown that plant cover can play a major role in reducing the erosive power of rainfall by retaining water. Compared to bare-soils, plant cover decreases runoff by 41% to 81% depending on the plant types and decreases soil erosion by 58% to 98% depending on the plant types. Low

scrubs such as thymus protect more efficiently from soil erosion and runoff than medium-sized scrubs such as lavender. In mountains areas, plant cover enhances the development of microorganisms and therefore increases carbon content in soils (Durán Zuazo. 2006).

There are large numbers of vegetation species available for erosion control. The ability of vegetation in retarding soil, water and nutrient erosion varies for different species. Each vegetation species has its special characteristics and suitability for certain soil type. Some of the vegetation species and its characteristics are shown in Table 2.2 .

Table 2.2 Vegetation species for erosion control (Mokhtar *et al.*, 2006).

Name	Form & Habit	Rooting Characteristics	Planting Condition	Comments
Red Alder *(Alnus rubra)*	Deciduous tree; Seeds prolifically on bare soil	Fibrous, moderately deep	Bareroot seedlings up to 3 ' tall; larger plants in containers	Fast grower in poor mineral soils; typical 40-50 year lifespan; large limbs become brittle; provides food for birds
Pacific Willow *(Salix lasiandra)*	Deciduous multi-stemmed tree; does not spread	Fibrous, Moderately deep and widespread	Rooted plants to10' tall in containers; cuttings 18" - 24"; whips 4'	Fast grower in saturated or shallowly flooded areas; 25 year lifespan -large limbs become brittle, tend to break off; stumps produce long, fast growing whips; easily rooted
Scouler Willow *(Salix scouleriana)*	Deciduous tree or shrub; does not spread	Fibrous, Moderately deep and widespread	Rooted plants to10' tall in containers; cuttings 18" -24"; whips 4';whips not recommended	Of the willows listed here, this species tolerates the driest conditions.
Sitka Willow *(Salix sitchensis)*	Deciduous tree or shrub; does not spread	Fibrous, Moderately deep and widespread	Rooted plants to10' tall in containers; cuttings 18" -24"; whips 4'; whips not recommended	Fast grower in moist to saturated soils; widely used for streambank stabilization
Douglas Fir *(Pseudotsuga menziesii)*	Coniferous tree; does not spread	Tap - Modified Tap, Shallow to deep and widespread	12" - 18" bareroot seedlings; larger plants in containers	Generally not considered a primary species for slope face stabilization; high root strength but typical shallow rooting characteristics in thin coastal soils; can be planted in stands in slope crest greenbelts; good eagle and osprey perch and nest trees; potential for wind throw in thin or disturbed soil
Northern Black Cottonwood *(Populus trichocarpa)*	Deciduous; does not spread	Fibrous, Shallow to deep, and widespread, extensive	Rooted plants to10' tall in containers; cuttings 18" - 24"; whips 4' tall	Fast grower in moist to saturated soils; also widely used for streambank stabilization; potential wind throw
Red-Osier Dogwood *(Cornus stolonifera or Cornus sericea)*	Deciduous shrub; does not spread	Fibrous, shallow	Rooted plants to 6' tall in containers; bareroot & cuttings 18" - 24" tall	Attractive shrub that produces bright red stems
Black Twinberry *(Lonicera involucrata)*	Deciduous shrub; does not spread	Fibrous, shallow	Rooted plants to 6' tall in containers; bareroot 18" - 24" tall	Produces yellow twin flowers and black twin berries; some success reported from cuttings
Ninebark *(Physocarpus capitatus)*	Deciduous shrub; does not spread	Fibrous, shallow	Rooted plants to 6' tall in containers; bareroot 18" - 24" tall	Produces masses of tiny white flowers which change to reddish seed clumps
Cascara *(Rhamnus purshiana)*	Deciduous tree/shrub; does not spread	Tap - Moderately deep	Rooted plants to 6' tall in containers; bareroot 18" - 24" tall	Shiny black berries are favored by Cedar Waxwings
Salmonberry *(Rubus spectabilis)*	Deciduous shrub; spreads by underground runners to form thickets	Fibrous, Shallow	Rooted plants to 4' tall in containers; bareroot 6"-8" tall; cuttings 18"-24"	Spreads quickly once established; berries provide food for a variety of songbirds

2.5.2 Hydrological role of vegetation

Plant is a major component of soil-plant-atmosphere continuum (SPAC) (Coppin *et al*. 1990). Water transports minerals through the soil to the roots where they are absorbed by the plant and the process occur throughout plants almost continuously. There is a constant movement of water from the soil to the roots, from the roots into the various parts of the plant, then into the leaves where it is released into the atmosphere as water vapor through the stomata and this process is called transpiration. Combined with evaporation from the soil and wet plant surfaces the total water loss to the atmosphere is called evapotranspiration. By absorbing part of the ground water, plants thus play a significant role towards the drying of slopes (Huang and Nobel, 1994). This absorbed soil water will subsequently be removed through the transpiration process into the atmosphere. This phenomenon would demand a large amount of water absorption by the root to produce a flow in the SPAC (Huang and Nobel, 1994). Ultimately, this water cycle system would result in drier and more stable slopes. It has been shown that 99% of water lost due to transpiration and only 1% is due to evaporation (Hazlifah, 1995).

2.5.3 Mechanical role of vegetation

Vegetation contributes to mass stability by increasing soil shear strength through root reinforcement (Gray, 1995). Roots naturally grip the soil to restore the physical condition of the plants. Instead of taking part in its original function to supply nutrients to plants and grasses, roots also play an important role to avoid them from being flushed away by soil erosion. The posibility of slope failure is increase when trees are cut down and their roots started to decay. This gradual decay of interconnected root systems was the principal cause of increased slope failure (Abe, 1997). The combined effects of vertical root

anchorage and lateral root traction are significant in prohibiting slope instability (Schroeder, 1985).

2.6 Microflora and slope stability

Organisms in the soil are both numerous and highly diverse. Many soil organisms are small and can only be seen with the aid of magnification. Microflora refers to the smallest organisms which are bacteria, actinomycetes, fungi and algae. Microbes exhibit an enormous diversity of functions in the soil. For example, they decompose organic compounds and release inorganic elements, a process called mineralization, reduce oxidized forms of elements. Also, the reduction of dinitrogen to a biologically utilizable form and degradation of organic wastes and pollutants to carbon dioxide and water are important functions of soil microbes. (Zuberer, 2005).

Microflora are responsible for the formation of soil from barren rocks due to the collective activity of algae, moss and lichens that colonise the bare rocks; produce organic acids which dissolve the primary minerals and release the nutrients contained in them for plant growth. Microflora improves soil structure by improving the soil texture, i.e. by making the soil more loamy. For example, algae and some bacteria that have exopolysaccharide secretion onto their cell surface due to their hygroscopic properties, bind more water molecules to their surface. Presence of these microbes in a sandy soil, converts the soil to more loamy by binding more soil particles onto their surface (by increasing the moisture). Such soils improved their mineral binding capacity and thus their fertility. The presence of soil microflora would also allow a lot of gaseous exchange and thus favours better soil aeration and tend to maintain the balance of pH in the soil by excreting metabolites (acidic and basic) in order to facilitate better absorption of mineral nutrients by

the plants. Other than that, the root and its associated microflora have a major effect upon soil structure and the stability of aggregates. The formation of these aggregates is an important prelude to soil stabilisation (Poh *et al*., 2006).

2.7 Characteristics of slope grass, ***Axonopus compressus***

Axonopus compressus was also known as blanket grass, broadleaf carpet grass, or rumput parit as well (in Malaysia). It was often used as a permanent pasture, ground cover and turf in moist, low fertility soils, particularly in shaded situations. Once established, this grass was an effective soil conservation tool to preserve valuable topsoil. *Axonopus compressus* can grow under shady conditions, making it a valuable cover crop in mature orchards with a fuller canopy cover (Smith and Valenza, 2002) . The characteristics of the grass were shown in Table 2.3. There were numbers of important soil quality benefits such as improved soil structure, better water infiltration rates, and increased soil water-holding capacity provided by this plant.. Other than that, a mine in Tak province, Thailand, has used *Axonopus compressus* (Sw.) P. Beauv to protect erosion of cadmium contaminated soil from floods and to remediate cadmium-contaminated soil (Sao *et al*., 2007). This was due to the cadmium accumulation the grass involved the cadmium precipitation in the stable form of cadmium silicate which was non toxic to the plant.

Table 2.3 Characteristics of *Axonopus compressus* (Sw.) P. Beauv (Smith and Valenza, 2002)

Characteristics	***Axonopus compressus* (Sw.) P. Beauv**
Common names	Carpetgrass
Life cycle	Perennial
Growth habit	Stoloniferous
Photosynthetic	C4 photosynthetic
Stem height	Maximum height of about 20-50 cm
Uses of plants	For controlling erosion, suppressing weeds. Improved soil structure, better water infiltration rates, and increased soil water-holding capacity. Some research indicated that it can fix atmospheric nitrogen and can add this nutrient to the soil.
Reproduces	The creeping stems of carpetgrass are compressed and root at each joint. Spreads by both stolons and seed. It is usually only propagated vegetatively by stolons.
Climate	Tropical and subtropical areas. Requires a minimum annual rainfall of about 30 inches (750-775 mm.)
Habitat	All type of soil, sandy soils with a high water table, full sunlight.
Tolerates	Acidic (pH 4.0-7.0) and low fertility soils, Shade tolerant, requires little fertilizer

CHAPTER 3

MATERIALS AND METHODS

3.1 Description of the sites

The soil samples of this experiment were collected from three slopes of 65^{o} gradient at 124m above sea level (longitude E 101^{o} 39’ 25.9’’, latitude N 03^{o} 07’ 51’’) Faculty of Language, University of Malaya, Malaysia (Figure 3.1). Three soil samples were collected together with the grasses which are naturally attached to the soil. In order to avoid any influence of plant species in this study, only soil samples with grasses from species of *Axonopus compressus* were chosen three different slopes namely slope A (50-70 kPa), slope B (80-100 kPa) and slope C. The shear strenght value were set to be highest for slope A (130-140 kPa) followed by slope B (80-100 kPa) and slope C (50-70 kPa) by using a vane tester.

(a) Slope A with shear strength values ranging from 130 to 140 kPa

(b) Slope B with shear strength values ranging from 80 to 100 kPa

(c) Slope C with shear strength values ranging from 50 to 70 kPa

Figure 3.1 The sampling sites which have three different range of soil shear strength.

3.2 Soil treatment

For each slope, a cylindrical soil cores was sampled by using a soil-coring apparatus (Figure 3.2). The soil samples (together with the grasses attached to the soil) were kept in a plastic cylinder and tightly closed at each end of the cylinder to avoid any contamination and evaporation. A long time exposure of the soil to environment will contribute to a significant loss of soil water content. The samples were transported to the laboratory and the grass roots were detached from soil for further microbial isolation on the same day.

3.3 Measurements

In this study, soil water content (SWC) and field capacity (FC) were calculated to determine the saturations level of slope soil. Shear strength, in which to determine the soil stability was measured by using a field inspection vane tester.

3.3.1 Soil water profile

Soil samples were oven-dried (80^{o}C) to a constant weight and SWC calculated as [(fresh weight-dry weight)/fresh weight]×100% (Appendix 1). Field capacity (FC) of the soil was determined by pouring excess water into a container (10cm×10cm×15 cm) filled with soil so that the soil was supersaturated. The excess water was drained out through small holes at the bottom of the container. Once the water stopped dripping, this saturated soil was weighed (SW) and oven-dried at 80^{o}C to obtain a constant dry weight (DW). FC was calculated by FC = [(SW−DW)/SW]×100% (Appendix 2). Thus, the saturation level was determined as : $\frac{SWC}{FC}$ x 100%

a. A hammer was used to help soil-coring apparatus penetrate the earth skin and a long metal rod was used to pull it out.

b. Soil sample was transferred into a plastic cylinder which was tightly closed at each end.

Figure 3.2 Soil sampling for water profiles and bacterial identification was obtained by using soil-coring apparatus.

3.3.2 Shear strength

Shear strength was measured at 30 cm soil depth by using a field inspection vane tester (Figure 3.3), which can provide values ranging from 0 to 260 kPa(± 10%). Soil shear strength were set to be the highest (130-140 kPa - slope A), moderate (80 - 100 kPa - slope B) and lowest (50-70 kPa - slope C) before the soil was collected for further characterisation.

Figure 3.3 A vane tester (Eijkelkamp Agrisearch Equipment model 14.05, The Netherlands) used to measure soil shear strength.

3.4 Isolation and purification medium

Burk's N-free medium (1 litre) containing: 10g glucose, 0.41g KH_2PO_4, 0.52g K_2HPO_4, 0.05g Na_2SO_4, 0.2g $CaCl_2$, 0.1g $MgSO_4.7H_2O$, 0.005g $FeSO_4.7H_2O$, 0.0025g $Na_2MoO_4.2H_2O$, 1.8g agar for semi-solid and 15g agar for solid medium (Wilson and Knight, 1952) was used throughout the study for isolation and purification. The pH of the medium was adjusted to 7±0.1 before autoclaving at 121^oC for 15 minutes at 15 p.s.i.

3.5 Isolation of nitrogen fixing bacteria

Approximately 1g of grass roots from each of the samples of slope type A, B and C were cut out and washed with distilled water. All the samples were surface sterilized by soaking in 1% NaOCl (2 minutes) and followed by 70% ethanol (1 minute). Following this, the samples were rinsed three times using distilled water. Finally, all samples were grounded in 1 ml Ringer's solution (¼ strength) by using sterilized mortar and pestle. One hundred µl of the mixture were taken out for serial dilution with 900 µl of Ringer's solution as diluents to get 10^{-1} solution. Aliquots were serially diluted ten-fold to 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. One hundred µl of each 10^{-3} to 10^{-6} dilution were inoculated onto the Burk's N-free agar medium in triplicates. All the plates were then incubated at 32^oC for 7 days.

3.6 Purification and preservation of isolates

After a week of incubation several kinds of different colonies appeared on the media surface of the isolation plates. The colonies were picked up by using flamed inoculating loop and streaked on Burk's N-free medium in order to produce a single colony. The plates were incubated for a week or more at 32^oC to get a pure culture. Bacterial strains that grow well were inoculated into Burk's N-free medium slant and

incubated at 32^{o}C for 7 days. The slants were kept as short term storage. The pure cultures were kept in glycerol (20%, v/v) at -20^{o}C freezer for long term storage.

3.7 Enumeration of bacteria

The bacterial populations were estimated for each original sample. Colonies that were formed on the isolation medium were enumerated by using colony counter. Bacterial populations were reported as mean number of colony forming units (cfu) per gram weight of grass roots.

3.8 Colonial characterisation of bacterial strains

Colonial and cultural characters of the bacterial isolates were examined according to methods described in Bergey's Manual of Systematic Bacteriology (Stanley *et al*., 2005). The isolates were characterised for the following traits: colour, elevation, size, shape, margin, surface appearance and density. The Gram reaction was performed as per standard procedures.

3.9 Molecular characterisation of bacterial strains

3.9.1 DNA extraction

Total genomic DNA from 61 strains were extracted using a method modified from the procedure by Sambrook *et al*. (1989). A loopful of bacterial cells was suspended in 150 µl of TE buffer containing glass beads (ca. 50 µg; <106 µm; Sigma G-4649), 2.5 µl of lysozyme (50mg/ml) and proteinase K (20 mg/ml). The suspension was mixed by vortexing, incubated at 37^{o}C for 2 hours and centrifuged at 14 000 rpm for 10 minutes. The supernatant was transferred to a new tube, incubated at 75^{o}C for 15 minutes and centrifuged. The DNA preparation was stored at -20^{o}C until required.

3.9.2 Agarose gel electrophoresis

The quality of DNA preparations was checked by electrophoresis using agarose gel (0.8%, w/v). 2 µl of DNA were mixed with 2µ loading dye and loaded into each well. Electrophoresis was carried out for 30 minutes at 100V (0.5X TAE buffer). Subsequently, the gel was stained with ethidium bromide (0.5 µg/ml) for 10 minutes and the gel was washed in distilled water and ready for DNA detection. Gel images were captured using a UV transilluminator (Cleaver SC, UK). Besides, the quality of PCR amplification product was checked by electrophoresis using agarose gel at 1.0 %(w/v) of concentration.

3.9.3 Amplification of DNA fragments using REP primers

For REP-PCR, the pair of 18-mer inosine-containing primers, REP1R-I (5'-III ICG ICG ICA TCI GGC-3') and REP2-I (5'-ICG ICT TAT CIG GCC TAC-3') was used (Versalovic *et al.* 1991). The reaction mixture (total volume 25 µl) contained 100 ng of genomic DNA, 50 pmol of each primer, 750 µM each of four dNTPs, 3 mM $MgCl_2$ and 2 U Bio *Taq* DNA polymerase (Bioline). PCR amplification was performed in a *Multigene*^{TM} *II* Personal Thermal Cycler (Labnet International, USA) with the following temperature profile: initial denaturation at 95^{o}C for 5 minutes; followed by 35 cycles each of denaturation at 94^{o}C for 1 minute, annealing at 40^{o}C for 1 minute and extension at 68^{o}C for 8 minutes, and a final extension at 68^{o}C for 16 minutes.

3.9.4 Amplification of DNA fragments using ERIC primers

For ERIC-PCR, the set of 22-mer primers, ERIC1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC2 (5' –AAG TAA GTG ACT GGG GTG AGC G-3') was used (Versalovic *et al.,* 1991). The reaction mixture (total volume 25 µl) contained 100 ng of genomic DNA, 50 pmol of each primer, 750 µM each of four dNTPs, 3 mM $MgCl_2$ and 2

U Bio *Taq* DNA polymerase (Bioline). PCR amplification was performed in *Multigene*^{TM} *II* Personal Thermal Cycler (Labnet International, USA) with the following temperature profile: initial denaturation at 95^{o}C for 5 minutes; followed by 30 cycles each of denaturation at 94^{o}C for 1 minute, annealing at 50^{o}C for 1 minute and extension at 68^{o}C for 8 minutes, and a final extension at 68^{o}C for 16 minutes.

3.10 PCR fingerprint analysis

Cluster analysis includes a broad suite of techniques designed to find groups of similar items within a data set. In this study, the REP-PCR profiles were analysed using The GelCompar II (Applied Maths NV, Belgium) cluster analysis program. Figures from 3.9.3 and 3.9.4 were converted to JPEG format as an input for the program.

CHAPTER 4

RESULTS

4.1 Profile of slope soil

4.1.1 Soil water profiles

In general, the ranges of SWC in all types of slopes studied were lower compared to their FC (Table 4.1). The saturation of soil slope type A, B and C were 58.1%, 60.4% and 65.6%, respectively. As a whole, soil in slope type C was the most saturated among the three slopes. Nevertheless, SWC in each type of slopes showed not much different among them at every soil depth studied (Figure 4.1).

4.1.2 Shear strength

Shear strength of the slopes were shown in Table 4.2. The result showed there was a significant difference amongst the slopes studied ($LSD_{p<0.05}$ = 19.55) (Figure 4.2). Slope type A had the highest value followed by slope B and C, respectively.

Table 4.1 Soil water content (%) and field capacity (in parentheses) of the slopes studied

Type of slope	Range	Mean	Median	% Saturation (median)
A	23.0-23.9 (39.6-39.0)	22.9 (39.4)	23.0 (39.6)	58.1
B	21.7-22.0 (37.1-34.3)	22.5 (35.9)	22.0 (36.4)	60.4
C	24.0-24.3 (32.8-36.6)	23.9 (35.4)	24.0 (36.6)	65.6

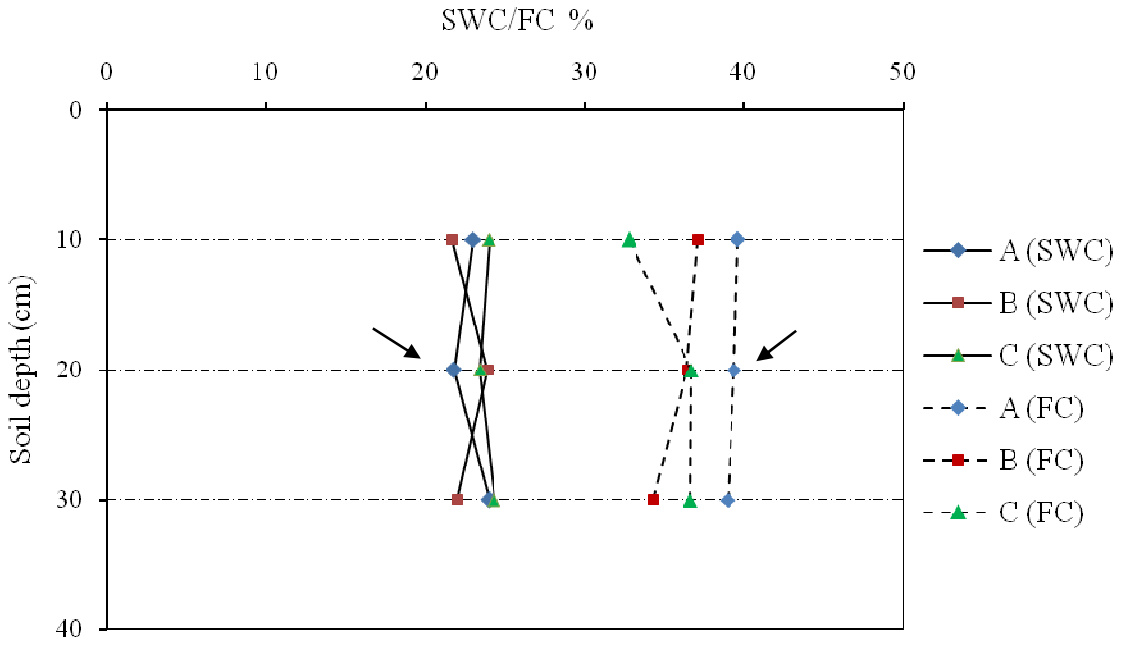


Figure 4.1 Soil water content (—) and field capacity (---) of the studied slopes. Arrows indicate mean of SWC.

Table 4.2 Shear strength of the three soil slopes. Three values (triplicates) of shear strength were taken for each type of slope .

Type of slopes	Shear strength (kPa) (Triplicates)			Mean (kPa)
	1	2	3	
A	66 x 2 = 132	68 x 2 = 136	70 x 2 = 140	136.00± 4.00
B	51 x 2 = 102	46 x 2 = 92	40 x 2 = 80	91.33±11.02
C	30 x 2 = 60	26 x 2 = 52	38 x 2 = 76	62.67±12.22

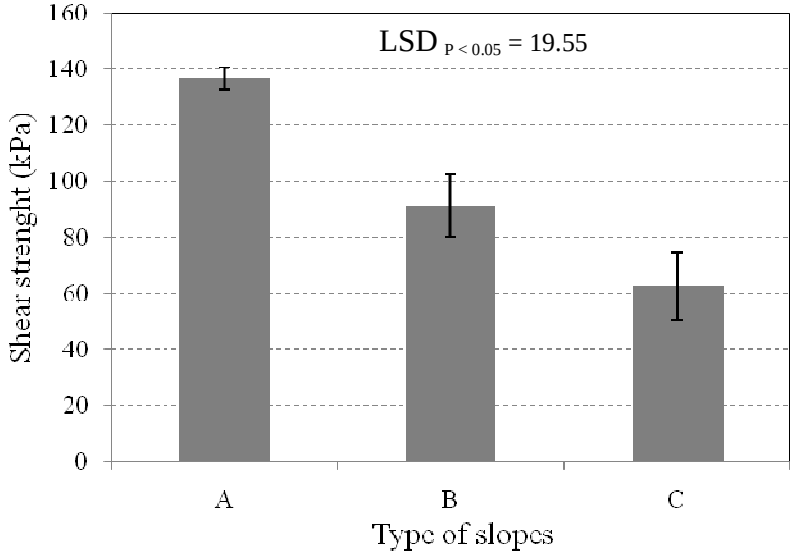


Figure 4.2 Shear strength value (kPa) at 30 cm of soil depth (n=3). Vertical liner represent a significant difference among the slopes studied at $LSD_{P<0.05}$

4.1.3 Correlation between shear strength and soil profiles

The correlation between shear strength and SWC was analysed. The SWC values of all soil samples were consistent, ranging from 22.5% to 23.9% (Table 4.3). Table 4.4 showed the correlation between soil shear strenght and soil saturation level. The soil saturation level were analysed in triplicates. The results indicated that the higher soil shear strenght had lower saturation level. As shown in Figure 4.3, there was no relationship observed between the SWC and shear strength. However, there was a negative relationship between shear strength and soil saturation level ($r^2 = 0.58$, $p<0.05$). (Figure 4.4).

Table 4.3 Soil water content (%) and shear strength (kPa) of the slopes studied

Type of slopes	SWC (Triplicates) 1	2	3	Shear strength (Mean)
A	23.03	21.84	23.91	136.00± 4.00
B	21.69	23.92	22.00	91.33±11.02
C	23.95	23.42	24.29	62.67±12.22

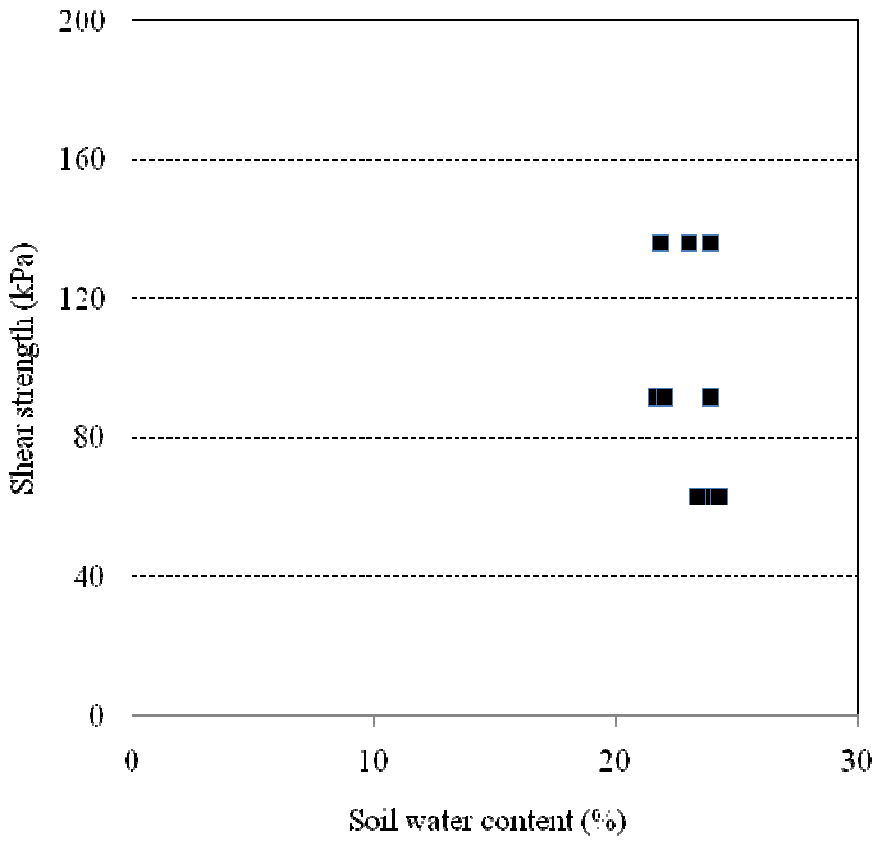


Figure 4.3 Correlation between shear strength and SWC.

Table 4.4 Shear strength (kPa) and saturation level (%) of the slopes studied

Type of slopes	Triplicates	SWC	FC	Saturation level (SWC/FC)(%)	Shear strength (Mean)
A	1	23.03	39.64	58.1	136.00± 4.00
	2	21.84	39.43	55.4	
	3	23.91	39.02	61.3	
B	1	21.69	37.06	58.5	91.33±11.02
	2	23.92	36.45	65.6	
	3	22.00	34.28	64.2	
C	1	23.95	32.79	73.0	62 67±12.22
	2	23.42	36.71	63.8	
	3	24.29	36.59	66.4	

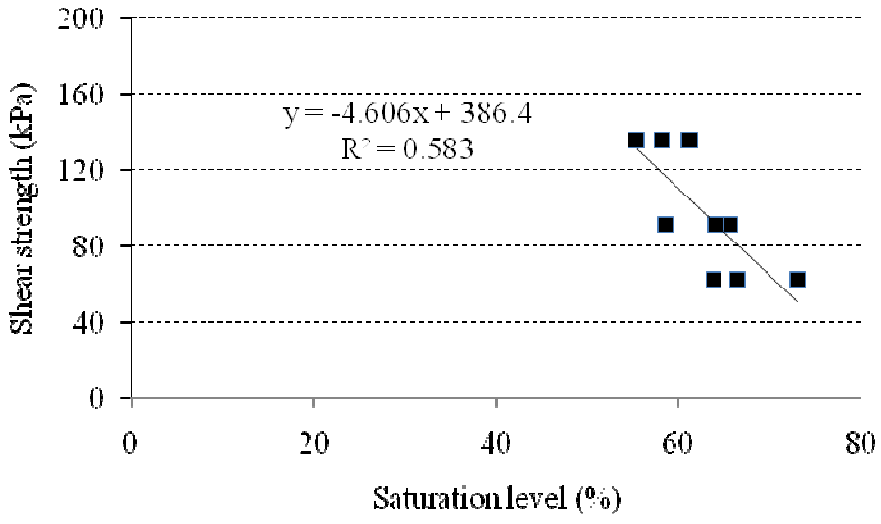


Figure 4.4 Correlation between shear strength and saturation level of the soils.

4.2 Isolation of nitrogen fixing bacteria

After a week of incubation at 32^{o}C, many types of colonies were formed on the Burk's N-free agar plates. The colonies were chosen randomly and inoculated onto new N-free medium in order to get pure cultures. The strains were named according the source of samples (slope type A, B or C) and the dilution factor of the inoculums (Table 4.5). A total numbers of 77 colonies were isolated from slope A (25 colonies), B (22 colonies) and C (30 colonies) (Appendix 3).

4.2.1 Gram staining

Since not all of the strains were able to grow well, only 73 of the strains were ready for further analysis. Gram staining on the strains indicated that 51 out of 73 strains were Gram-negative and the rest (22 strains) were Gram-positive (Table 4.5). Obviously, this study revealed that Gram-negative bacteria were dominant (as 70% was Gram-negative) (Table 4.6). Majority of the strains were rods and cocci.

Table 4.5: Colonies grown on N-free medium. Observation was made after more than 7 days of incubation. All the colonies were chosen randomly from original plate (mixed culture).

Strain[a] label	Dilution factor	Gram result	Strain[a] label	Dilution factor	Gram result	Strain[a] label	Dilution factor	Gram result
1A6	6	+	1B6	6	-	1C5	5	-
2A6	6	-	2B5	5	+	2C5	5	+
3A5	5	-	3B5	5	-	4C5	5	-
4A5	5	-	6B4	4	-	5C5	5	-
5A5	5	-	7B4	4	-	6C5	5	-
7A4	4	-	8B4	4	-	7C5	5	-
8A4	4	-	12B4	4	-	8C5	5	-
9A4	4	-	13B4	4	-	9C4	4	-
11A4	4	+	14B4	4	-	10C4	4	+
12A4	4	-	15B4	4	-	11C4	4	+
13A4	4	-	16B3	3	+	12C4	4	+
14A4	4	+	18B3	3	-	13C4	4	-
16A4	4	+	20B3	3	-	14C4	4	+
17A3	3	+	21B3	3	-	15C4	4	-
18A3	3	+	22B3	3	-	16C4	4	+
19A3	3	-	23B3	3	-	17C4	4	+
20A3	3	-	24B3	3	-	18C4	4	+
21A3	3	-	27B3	3	-	19C3	3	+
22A3	3	+	30B3	3	+	20C3	3	+
23A3	3	+				21C3	3	-
24A3	3	-				22C3	3	-
25A3	3	-				23C3	3	-
26A3	3	-				24C3	3	-
27A3	3	-				25C3	3	-
28A3	3	-				26C3	3	-
						27C3	3	-
						28C3	3	-
						29C3	3	+
						30C3	3	-

[a] Each strain is labeled in reference to type of slope and dilution factor

\+ refer to Gram-positive

\- refer to Gram-negative

Table 4.6 Distribution of culturable Gram-positive and Gram-negative free-living nitrogen fixing bacteria in the slopes as a comparison.

Type of slope	Gram Positive	Gram Negative	Total
A	8 (32.0%)	17 (68.0%)	25
B	3 (15.8%)	16 (84.2%)	19
C	11 (37.9%)	18 (62.1%)	29
Total	22 (30.1%)	51 (69.9%)	73

4.2.2 Colonial and cultural characters

Most of the diazotrophs which were successfully isolated from the grass roots had different colonial character when grown on N-free medium. The main characteristics of all isolates examined were compared to the standard clasification of Bergey's Manual of Systematic Bacteriology (Stanley *et. al*, 2005). The isolates were characterised for the following traits: colour, elevation, size, shape, margin, surface appearance and density on Burk's N-free agar. For Gram-positive strains, the results showed that 64% of them were cream/cream yellow in colour, 68% of the colonies showed raised elevation (Table 4.7). Other than that, 86% of the colonies were small in size and 77% of them were circular. Margin and surface appearance showed more than 50% of the colonies were filamentous and dull/dull rough respectively. Yet, only 27% of the colonies were opaque. The rest were translucent. The observation on 51 Gram-negative diazotrophs showed that 45% of them were cream in colour and 45% of the colonies showed raised elevation (Table 4.8). Other than that, 86% of the colonies were small in size and 67% of them were circular. Margin and surface appearance showed more than 50% of the colonies were entire and glistening respectively. 49% of the colonies were translucent and 41% were transparent.

Table 4.7 The observation of 22 Gram-positive diazotrophs (After a week of incubatioan at 32^{o}C).

Strains	Color	Elevation	*Size	Shape	Margin	Surface appearance	Density
1A6	Cream	Raised	Small	Circular	Filamentous	Dull	Translucent
11A4	Cream-yellow	Raised	Small	Circular	Filamentous	Dull-rough	Opaque
14A4	Ice-white	Convex	Small	Circular	Entire	Glistening	Transparent
16A4	Cream	Doom	Small	Circular	Entire	Glistening	Transparent
17A3	Cream-yellow	Raised	Small	Circular	Filamentous	Dull-rough	Translucent
18A3	Cream-yellow	Raised	Small	Circular	Filamentous	Dull-rough	Translucent
22A3	Cream-yellow	Raised	Small	Circular	Filamentous	Dull-rough	Translucent
23A3	Ice-white	Convex	Medium	Circular	Entire	Glistening	Transparent
2B5	Cream-yellow	Raised	Small	Circular	Filamentous	Dull-rough	Opaque
16B3	Cream	Convex	Small	Punctiform	Irregular	Glistening	Translucent
30B3	Cream	Raised	Small	Punctiform	Irregular	Creamy	Translucent
2C5	Cream	Doom	Medium	Circular	Entire	Granular	Transparent
10C4	Cream	Convex	Small	Punctiform	Undulate	Glistening	Translucent
11C4	Cream	Convex	Small	Circular	Entire	Glistening	Translucent
12C4	Cream-orange	Raised	Small	Circular	Filamentous	Dull-Rough	Opaque
14C4	Cream-yellow	Raised	Small	Circular	Filamentous	Dull-Rough	Translucent
16C4	White	Raised	Small	Circular	Filamentous	Dull-Rough	Opaque
17C4	White	Raised	Small	Circular	Filamentous	Dull-Rough	Opaque
18C4	Ice-white	Raised	Small	Punctiform	Entire	Glistening	Translucent
19C3	White	Raised	Small	Circular	Filamentous	Dull-Rough	Opaque
20C3	Ice-clear	Raised	Medium	Irregular	Undulate	Glistening	Transparent
29C3	White	Raised	Small	Circular	Filamentous	Dull-Rough	Translucent

*Size (diameter in mm) – large (greater than 1 mm), medium (1 mm) and small (less than 1mm)

Table 4.8 The observation of 51 Gram-negative diazotrophs.

Strains	Color	Elevation	Size	Shape	Margin	Surface appearence	Density
2A6	White	Raised	Small	Circular	Filamentaous	Dull-rough	Translucent
3A5	Cream	Doom	Small	Circular	Entire	Glistening	Transparent
4A5	White	Raised	Small	Punctiform	Entire	Glistening	Transparent
5A5	Cream	Convex	Small	Circular	Entire	Glistening	Translucent
7A4	Cream	Convex	Small	Circular	Entire	Glistening	Translucent
8A4	Cream	Raised	Small	Punctiform	Circular	Creamy	Translucent
9A4	Ice-white	Doom	Medium	Circular	Entire	Creamy	Translucent
12A4	White	Convex	Small	Circular	Entire	Granular	Translucent
13A4	White	Raised	Small	Punctiform	Entire	Glistening	Transparent
19A3	White	Raised	Small	Circular	Filamentous	Dull-rough	Opaque
20A3	Ice-clear	Doom	Small	Circular	Entire	Glistening	Transparent
21A3	Ice-clear	Raised	Medium	Circular	Entire	Glistening	Transparent
24A3	White	Raised	Small	Punctiform	Entire	Glistening	Transparent
25A3	Cream	Raised	Small	Punctiform	Irregular	Creamy	Translucent
26A3	White	Raised	Small	Circular	Filamentous	Dull-rough	Translucent
27A3	Cream	Convex	Small	Punctiform	Irregular	Glistening	Translucent
28A3	White	Convex	Small	Circular	Entire	Granular	Opaque

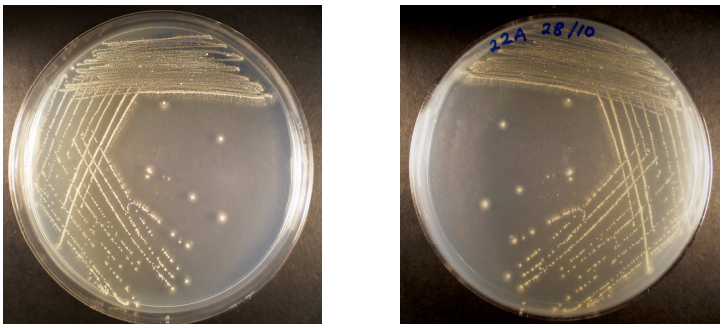
Table 4.8 (*cont*.)

Strains	Color	Elevation	Size	Shape	Margin	Surface appearance	Density
1B6	Cream	Doom	Small	Circular	Entire	Glistening	Translucent
3B5	Ice-clear	Convex	Small	Punctiform	Irregular	Glistening	Transparent
6B4	Cream	Doom	Small	Circular	Entire	Glistening	Translucent
7B4	Ice-clear	Convex	Small	Circular	Entire	Glistening	Transparent
8B4	Cream	Doom	Small	Circular	Entire	Glistening	Translucent
12B4	Pink-white	Raised	Small	Punctiform	Entire	Glistening	Translucent
13B4	Cream-yellow	Raised	Small	Circular	Filamentous	Dull-Rough	Translucent
14B4	Cream	Doom	Small	Circular	Entire	Glistening	Translucent
15B4	Cream	Doom	Medium	Circular	Entire	Creamy	Translucent
18B3	Cream	Doom	Small	Circular	Entire	Creamy	Translucent
20B3	Cream	Doom	Small	Circular	Entire	Glistening	Translucent
21B3	Ice-clear	Raised	Small	Punctiform	Entire	Glistening	Transparent
22B3	Cream-yellow	Raised	Small	Circular	Irregular	Glistening	Translucent
23B3	White	Raised	Small	Circular	Entire	Dull-Rough	Translucent
24B3	Dark brown	Raised	Small	Circular	Filamentous	Dull-Rough	Opaque
27B3	Ice-clear	Convex	Small	Circular	Entire	Glistening	Transparent

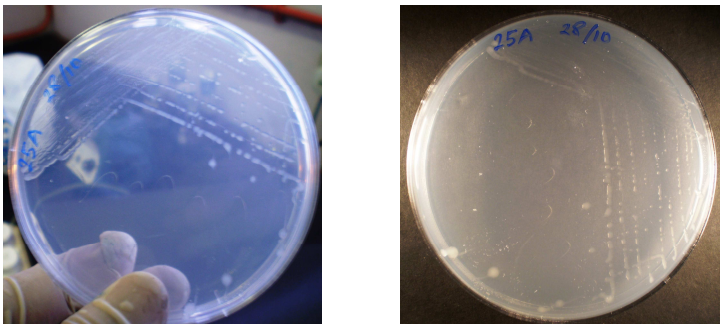
Table 4.8 (*cont*.)

Strains	Color	Elevation	Size	Shape	Margin	Surface appearance	Density
1C5	Cream	Doom	Medium	Circular	Entire	Granular	Transparent
4C5	Cream	Raised	Small	Circular	Filamentous	Dull	Translucent
5C5	Ice-clear	Convex	Small	Punctiform	Undulate	Glistening	Transparent
6C5	White	Raised	Small	Circular	Filamentous	Dull-Rough	Opaque
7C5	Cream-yellow	Doom	Small	Circular	Entire	Dull-Rough	Transparent
8C5	Ice-white	Raised	Small	Punctiform	Entire	Creamy	Translucent
9C4	Cream	Raised	Small	Punctiform	Entire	Creamy	Translucent
13C4	Cream	Raised	Small	Irregular	Filamentous	Dull-Rough	Opaque
15C4	Cream	Doom	Small	Circular	Entire	Glistening	Transparent
21C3	Cream	Convex	Small	Punctiform	Undulate	Glistening	Translucent
22C3	Ice-clear	Convex	Medium	Circular	Entire	Glistening	Transparent
23C3	Cream	Raised	Small	Punctiform	Undulate	Creamy	Translucent
24C3	Ice-clear	Doom	Medium	Circular	Entire	Glistening	Transparent
25C3	White	Raised	Small	Circular	Filamentous	Dull-Rough	Translucent
26C3	White	Convex	Small	Circular	Entire	Glistening	Transparent
27C3	Ice-clear	Convex	Small	Punctiform	Undulate	Glistening	Transparent
28C3	Cream	Raised	Small	Circular	Entire	Glistening	Transparent
30C3	Ice-clear	Convex	Medium	Circular	Filamentous	Glistening	Transparent

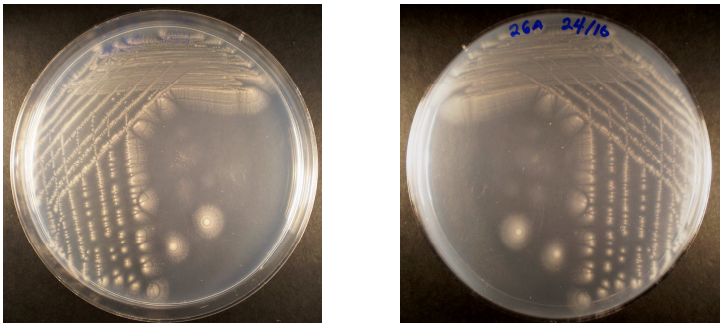
Different types of bacteria produced different colonies (Appendix 3). Some of colonial characteristic of the strain were shown in Figures 4.5, 4.6 and 4.7.



Strain 22A3. Small size colonies with cream-yellow colour, circular shape and dull rough surface appearance.

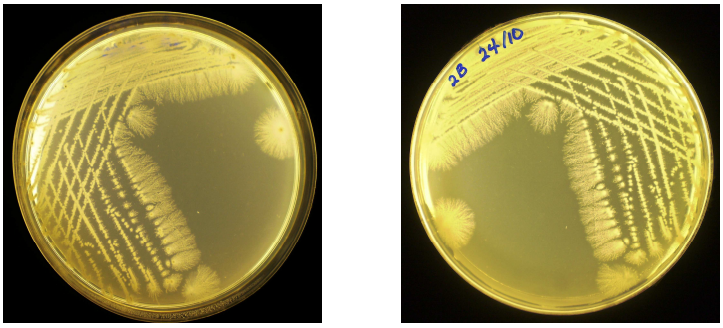


Strain 25A3. Small size colonies with cream colour, punctiform shape and creamy surface appearance. Slow growing strain and translucent.

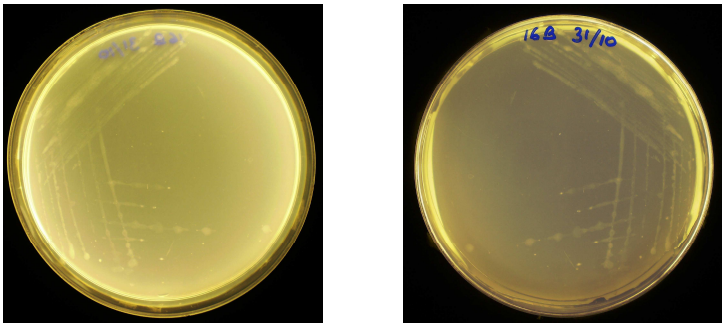


Strain 26A3. Small size colonies with white colour, circular shape and dull-rough surface appearance. Filamentous edge was also observed.

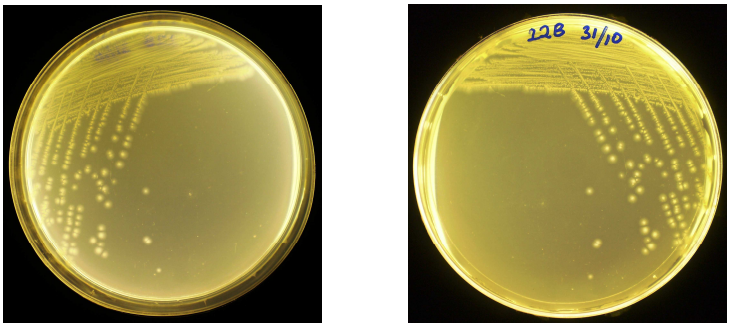
Figure 4.5 Selected strains isolated from slope type A.



Strain 2B5. Small size colonies with cream-yellow colour, circular shape and dull rough surface appearance. Filamentous edge was also observed.

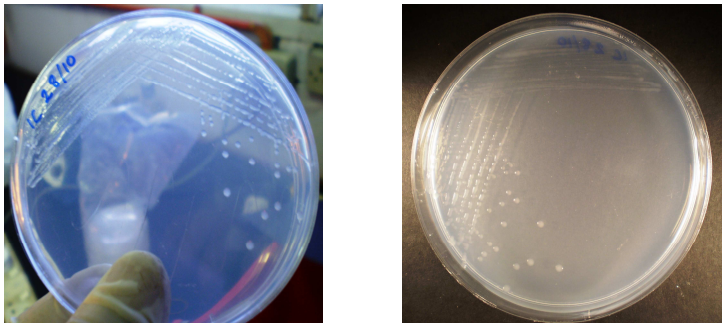


Strain 16B3. Slow growing and small size colonies with cream colour. Punctiform shape and glistening surface appearance.

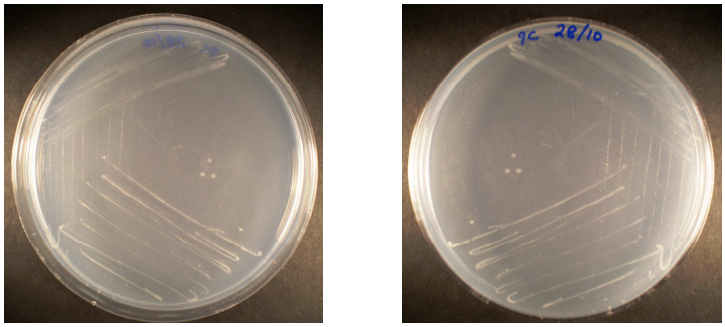


Strain 22B3. Small size colonies with cream-yellow colour, circular shape and dull rough surface appearance.

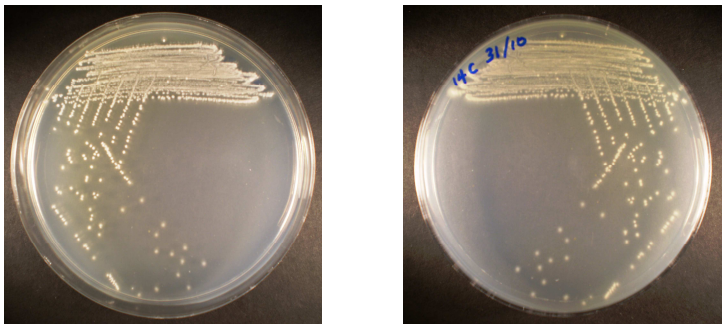
Figure 4.6 Selected strains isolated from slope type B.



Strain 1C5. Medium size colonies with cream colour and circular shape. Ganular surface appearance and transparent characters were also observed.



Strain 9C4. Slow growing and small size colonies with cream colour and punctiform shape. creamy surface appearance appearance was also observed.



Strain 14C4. Small size colonies with creamy colour and circular shape. Dull surface appearance was also observed.

Figure 4.7 Selected strains isolated from slope type C.

4.2.3 Enumeration of bacteria (cfu/g)

The bacterial populations were estimated in each original grass root sample. Colonies that form on the N-free medium were counted. Isolated bacterial growing on the media were reported as mean number of colony forming units (cfus) as shown in Table 4.9. Statistically, only those plates with 30 to 300 colonies were used for calculation. Hence, bacterial population at dilution factor of 10^4 was used for further discussion. Since 100 µl or 0.1 ml of bacterial culture were plated onto the medium, colony forming unit (cfu/g) were 6.6 x 10^6, 8.2 x 10^6 and 1.06 x 10^7 for slope type A, B and C respectively. Population size of diazotroph in slope type C was the highest followed by slope type B and type A.

Table 4.9 Mean number of cfu/g from triplicate plates of isolation medium

Type of slope	Dilution factor			
	10^3	10^4	10^5	10^6
A	283	66	6	3
B	249	82	4	2
C	383	106	12	0

4.2.4 Correlation between mean number of cfu/g and soil profile

The result in Table 4.10 indicated that the number of colonies in the slope type A was the lowest whereas slope type C was the highest. The mean number of cfu and level of saturation was shown in Table 4.11. As shown in Figure 4.8, there was no relationship between the number of cfu/g and SWC. Nevertheless, there was a positive relationship between mean numbers of cfu/g and the saturation level (Figure 4.9) ($r^2 = 0.60$, $p<0.05$).

Table 4.10 Mean number of cfu/g ($x10^5$) and SWC (%) of the slopes studied

Type of slopes	SWC (Triplicates)			Mean number of cfu/g ($x10^5$)
A	23.03	21.84	23.91	66
B	21.69	23.92	22.00	82
C	23.95	23.42	24.29	106

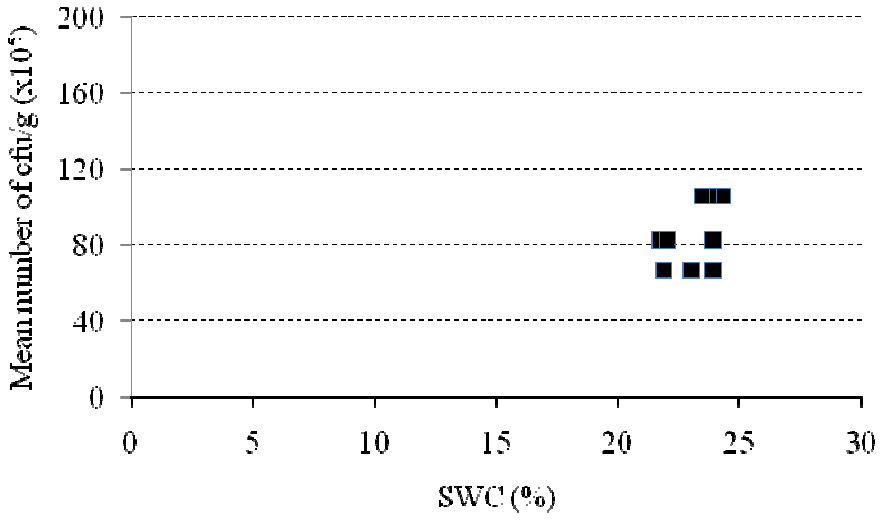


Figure 4.8 Correlation between mean numbers of cfu/g and SWC

Table 4.11 Mean number of cfu/g ($x10^5$) and level of saturation (%) of the slopes studied

Type of slopes	Saturation level (SWC/FC) (%) (Triplicates)			Mean number of cfu/g ($x10^5$)
A	58.1	55.4	61.3	66
B	58.5	65.6	64.2	82
C	73.0	63.8	66.4	106

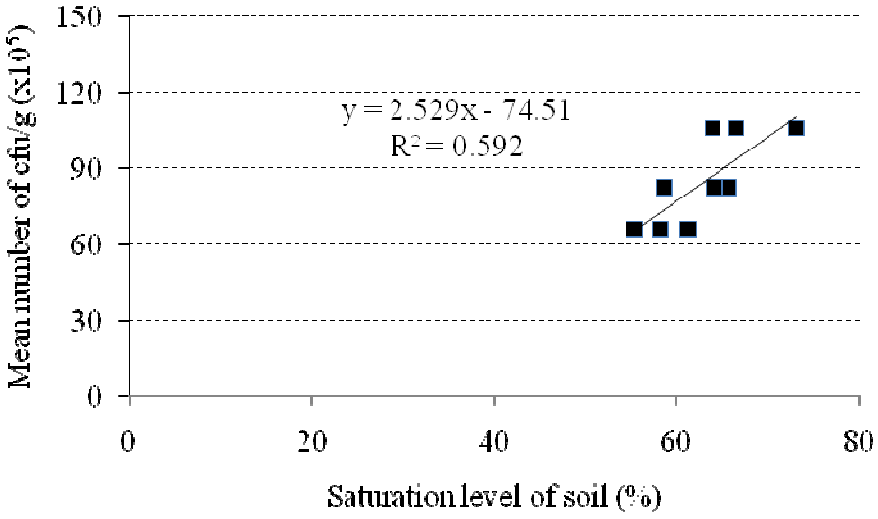


Figure 4.9 Correlation between mean numbers of cfu/g and saturation level of soil.

4.3 Molecular Characterisation

4.3.1 DNA extraction

From a total of 73 strains, only DNA of 23 strains from slope type A, 19 strains from slope type B and 19 strains from slope type C were successfully extracted (Appendix 4). Figure 4.10 showed some of the strains which genomic DNA were successfully extracted by using the procedure in this study. No traces of protein found in the well of the gel were an indication that the DNA samples were of good quality. Single and clear bands were observed except on strain 23B3. This could be resulted from DNA denaturation.

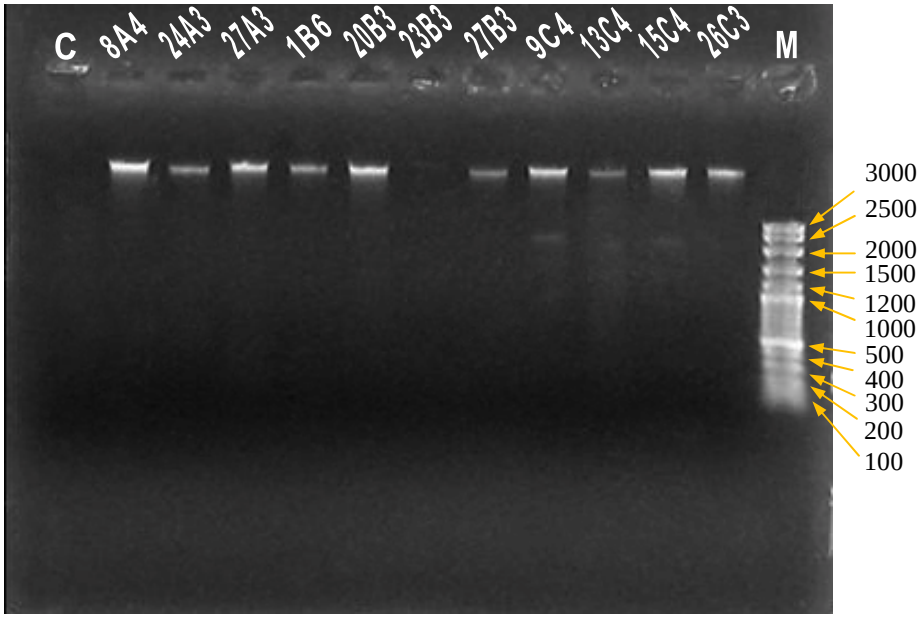


Figure 4.10 Agarose gel electrophoresis (0.8%) of the product from DNA extraction. DNA was extracted from all strains except strain 23B3. Lane C was a control and lane M was a 100bp marker Plus DNA Ladder (vivantis).

4.3.2 DNA fingerprinting profile

Initially, two methods of dereplication; ERIC-PCR and REP-PCR, were used on five selected strains to compare the two approaches. The result is shown in Figure 4.11. In general, all of the samples showed distinguishable pattern of DNA bands in both types of PCR. The patterns produced among each PCR methods also differ to one another. Hence, both PCR methods were suitable approaches to be used for DNA characterisation. Nevertheless, as shown in Figure 4.12, amplification by REP primers produced clearer and higher number bands compared to ERIC primer. Consequently, REP-PCR was chosen for further DNA profiling.

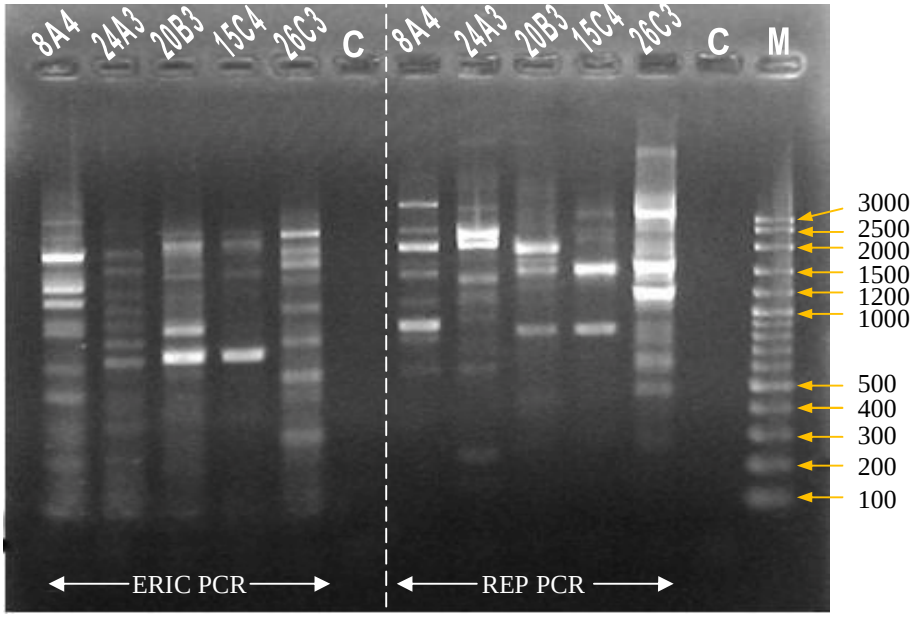


Figure 4.11 Agarose gel electrophoresis (0.8%) of five DNA sample from different strain (were chosen randomly according to their physiological and morphological different). Lane C was a control and lane M was a 100bp Plus DNA Ladder (vivantis).

As shown in Figure 4.12 (a), numbers of bands for REP-PCR profile for slope type A ranging from 11 (strain 12A4) to 15 (strain 5A5). DNA band with size 1 000bp were observed in most of the strains. No bands was observed from strain 7A4. Since all of the samples were selected from samples that showed positive result during DNA extraction, this condition could be due to the denaturation of DNA. Strains 13A4 and 24A3 had a similar REP-PCR profile. Nevertheless, strain 13A3 showed a clearer and sharper band. Presumably they were originated from close taxonomic group.

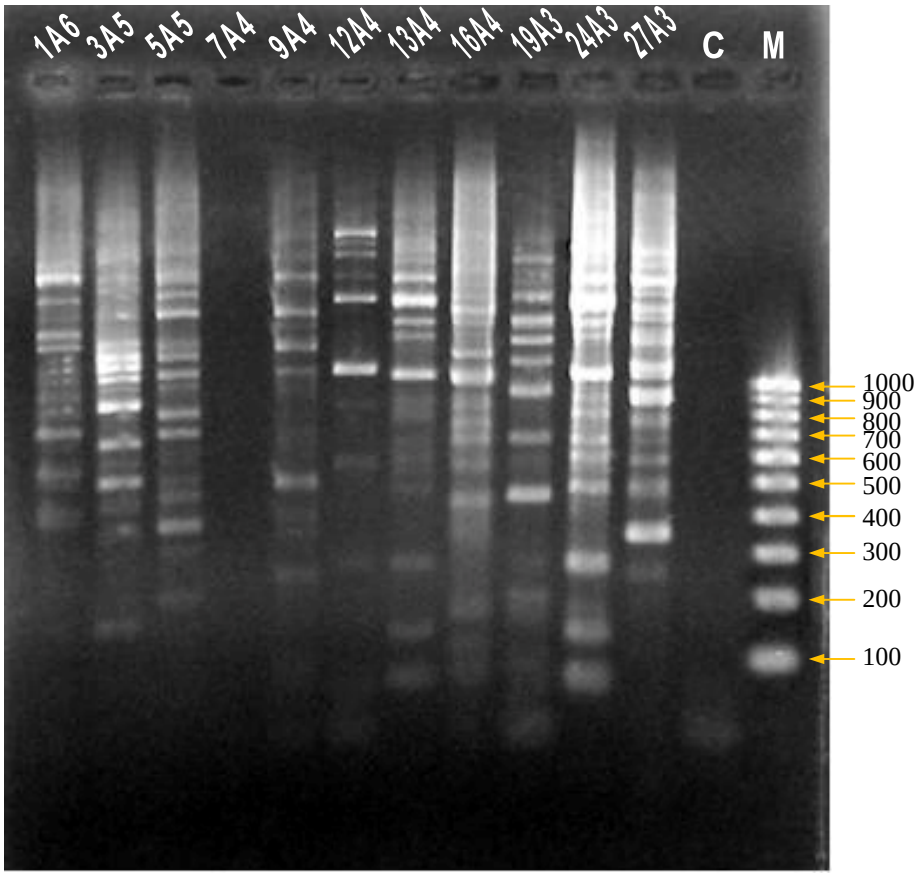


Figure 4.12 (a) REP-PCR profile of strains from slope type A. All samples showed positive result except strain 7A4.

Agarose gel electrophoresis (1%) of PCR amplified product. REP-PCR profile generated with the REP 1R and REP 2 primers. A total 11 DNA sample were chosen according to their physiological and morphological differences. Lane C was a control and lane M was a 100 bp DNA Ladder (iDNA).

The result for slope type B was shown in Figure 4.12 (b). All samples showed clear bands except strain 3B5 which was an indication of low DNA concentration. The size ranging from 300-2500 bp. No band was observed on strain 6B4 and discarded for further analysis. None of the strains showed complete similarity to one another. The number of bands ranging from 7 (strain 27B3) to 14 (strain 15B4).

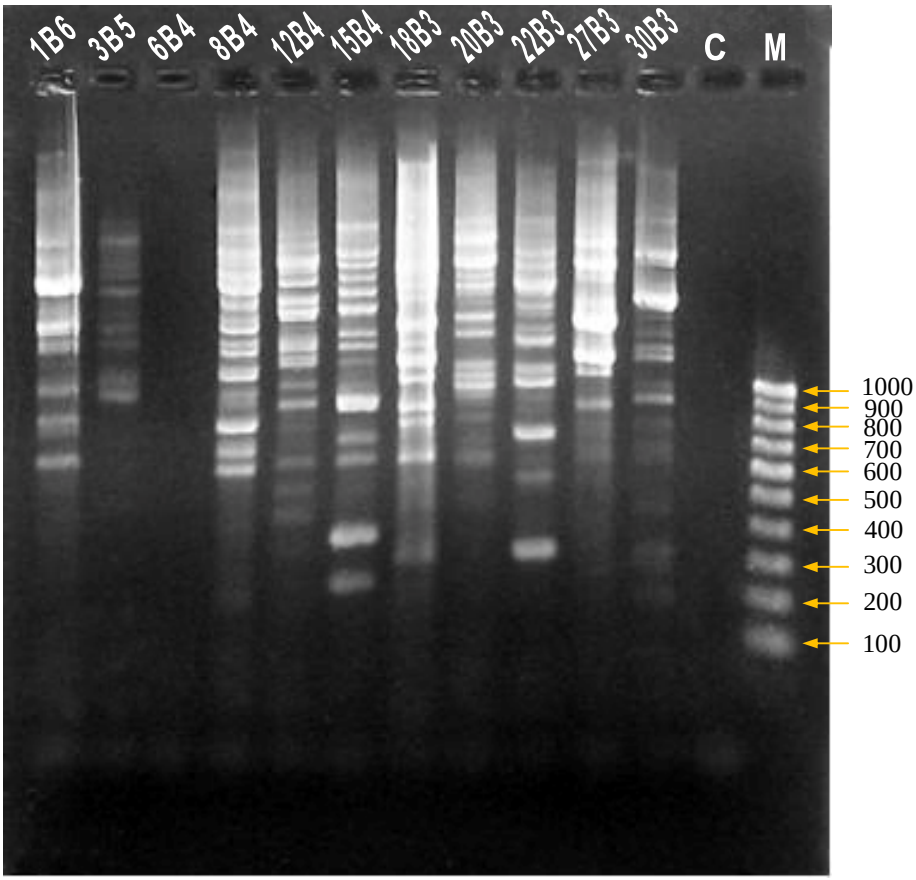


Figure 4.12 (b) REP-PCR profile of strains from slope type B. All samples showed positive result except sample 6B4.

As shown in Figure 4.12 (c), numbers of bands observed from REP-PCR profile of strains from slope type C were lower compared to strains from slope type A and B. The numbers of bands ranging from 5 (strain 4C5) to 11 (strain 2C5). The size ranging from 100-2500 bp. Since strain 15C4 and 28C3 had a similar REP-PCR profile, they were probably from closely related taxonomic group.

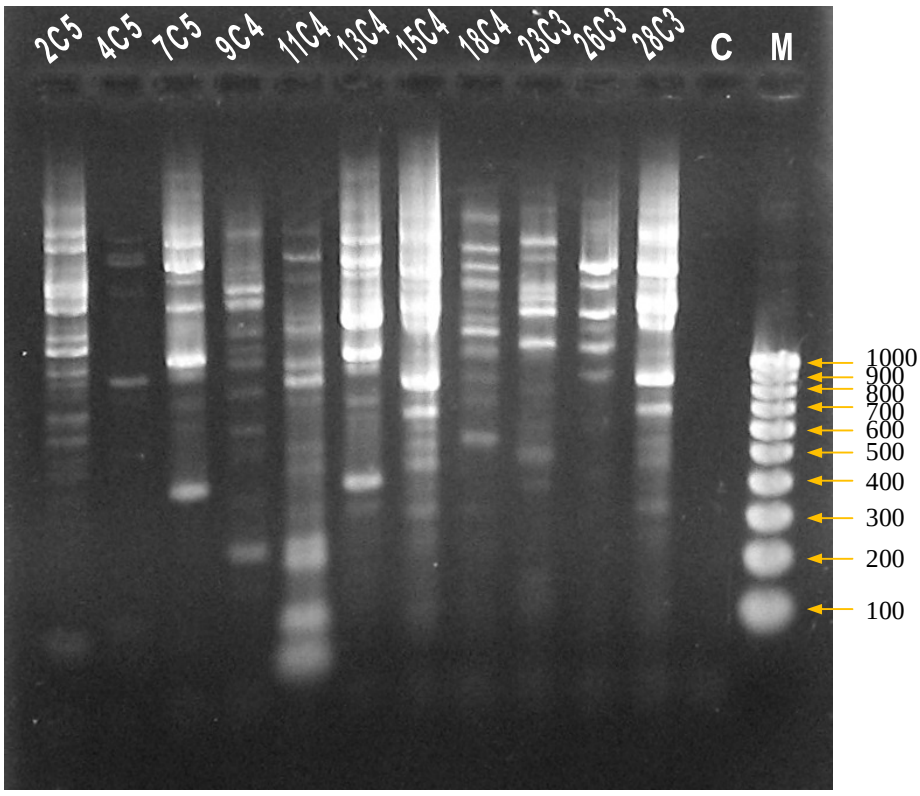


Figure 4.12 (c) REP-PCR profile of strains from slope type C. Strain 15C4 and 28C3 showed a similar DNA profile.

4.3.3 Fingerprint analysis and dendogram.

Cluster analysis of REP-PCR profile for each type of the slope were done individually. The result, a dendogram, was automatically produced after all of the inputs were set on the program. Figure 4.13, 4.14 and 4.15 were the results for each sample from different slopes.

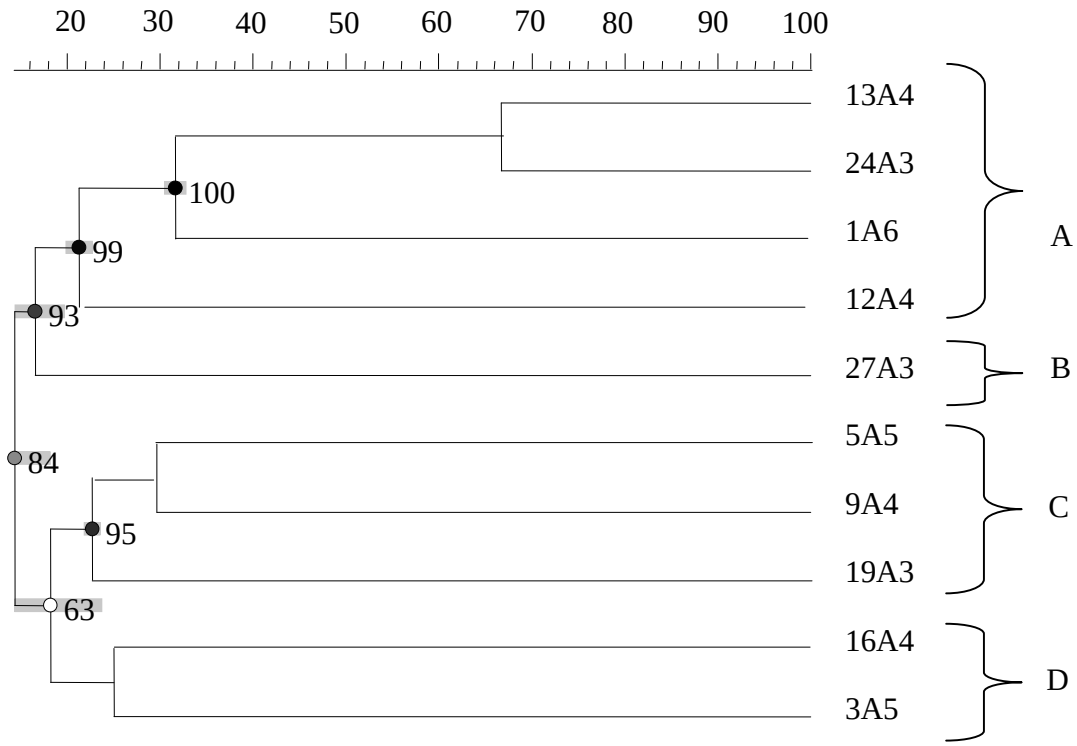


Figure 4.13 Dendrogram obtained from cluster analysis of REP-PCR profile of strains from slope type A. The strains can be grouped into clade A, B, C and D. Strains 13A4 and 24A3 showed a clear relationship at the similarity more than 65%. The cut-off point for clustering was at 20%.

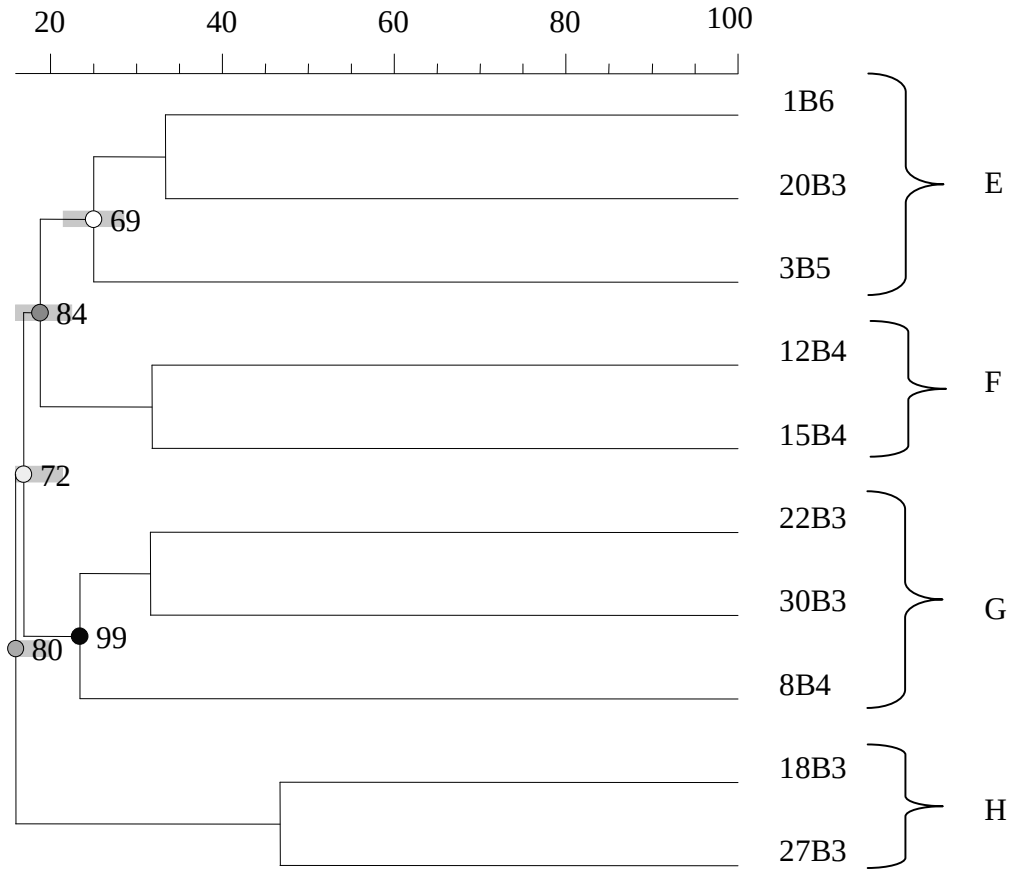


Figure 4.14 Dendrogram obtained from cluster analysis of REP-PCR profile of strains from slope type B. The strains grouped into clade E, F, G and H. The cut-off point for clustering was at 20%. Strain 18B3 and 27B3 had more than 40% of similarity.

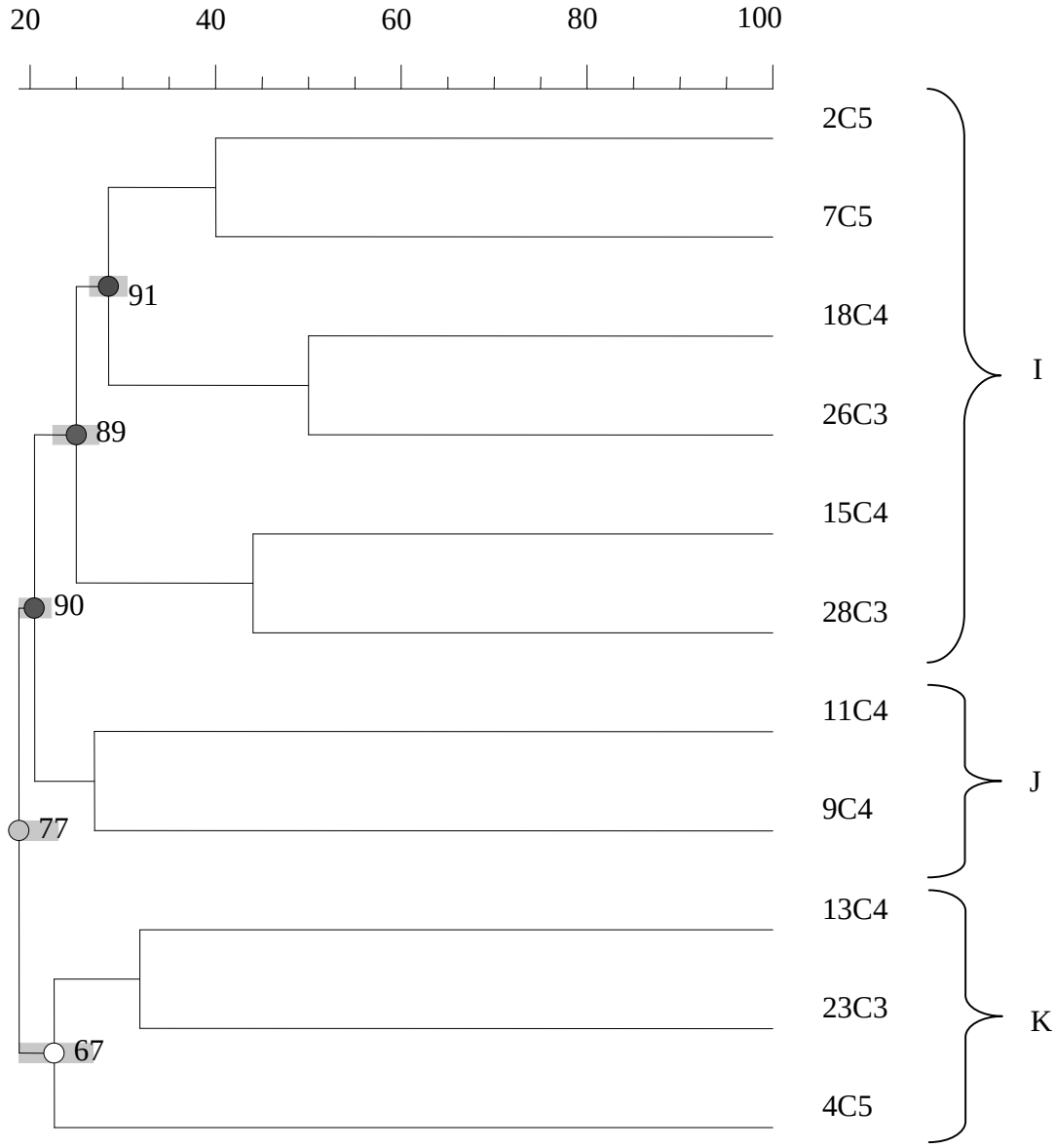


Figure 4.15 Dendrogram obtained from cluster analysis of REP-PCR profile of strains from slope type C. The cut-off points for clustering at 20%, the strains can be grouped into clade I, J and K. Strain 4C5 did not fit the cluster well.

CHAPTER 5

DISCUSSIONS

5.1 Soil profile

In order to avoid any influence of plants species on the experiment, the same species of grasses, *Axonopus compressus,* (which grow naturally) were selected. The existence of plant specific bacteria was also observed by Baldani and Baldani (2005). It has been shown that the soil type was also responsible for bacterial diversity (Seldin *et al*. 1998). Since the soil samples used in this study were collected from the area located near to one another, the soil content were homologous and not much distinguished. Hence, it is assumed that the soil type was not a source of variable and has no influence in this study.

Since water content usually varies with depth throughout the root zone, SWC were measured at several depths within the soil (Appendix 2 (a), (b) and (c)). The result showed that SWC in each slopes was not much different among the soil depth studied. SWC of slope type A, B and C, ranging from 23.0 to 23.9%, 21.7 to 22.0% and 24.0 to 24.3%. respectively (Table 4.1) Whereas the saturation level (median) of slope type A, B and C were 58.1%, 60.4% and 65.6%, respectively (Table 4.1). Figure 4.1 showed that SWC of all the slopes were lower compared to their FC respectively, which was an indication that all of the slopes were categorized as stable. However, in a comparison, slope type A was considered to be the most stable followed by slope type B and type C.

As indicated in Table 4.2, the shear strength of slope type A was the highest and slope type C was the lowest and there was a significant difference amongst the slopes studied (Figure 4.2) ($LSD_{p<0.05}$ = 19.55). There was no relationship between the SWC and shear strength but a negative correlation was observed between shear strength and soil

saturation level (Figure 4.4) (r^2 = 0.58, $p<0.05$). This was parallel with the study done by Normaniza and Barakbah (2006) which concluded that there was a negative relationship between SWC and shear strength up to the value of 50 kPa. However, beyond this point, the shear strength was constant.

The shear strength of a soil decreased in line with the increase of soil saturation. Thus, high shear strength of the slopes soil will reduce the capability of water to reach the space between soil molecules and SWC will reduce as well. Cheng and Feng (2008) reported that the shear strength of soils may decrease noticeably with increasing soil moisture content.

5.2 Culturable bacterial populations

In general, about 70% of the free-living nitrogen fixing bacteria found in this study were Gram-negative (Table 4.6). Study by Parka M. *et al.* (2005) found that three strains Gram-negative with no endospores and two Gram-positive producing endospores of diazotroph. It was also reported that Gram positive spore-forming nitrogen fixing bacteria of the genus *Bacillus* was successfully isolated from saline soils of Egypt. (Zahran *et al.,* 1995).

More than 60% of Gram-positive were cream/cream yellow in colour, raised elevation, circular and filamentous. Other than that, majority the colonies were small in size and translucent. Half the colonies were dull/dull rough surface appearance (Table 4.7). Gram-negative diazotrophs colonies showed that most of them were cream in colour and showed raised elevation. Nearly all of them colonies were circular and small in size. Moreover, half of the colonies were entire and glistening (Table 4.8). All of the

characteristics were close to *Azotobacter* sp. especially strains 4C5, 13C3 and 23C3 (Atlas and Bartha, 1998). Nevertheless, some biochemical test should be done to support the assumption. In this study, the results from DNA fingerprinting were used to study the diversity of the bacterial population.

This study showed that the population of free-living nitrogen fixing bacteria was estimated ranging from 6.6×10^6 to 1.02×10^7 cfu/g dry weight (Table 4.9). Slope type C showed the highest bacterial population size followed by slope type B and A. The study by Wright and Weaver (1980) showed that the population free-living nitrogen fixing in roots of forage grasses ranged from approximately 10^4 to 3×10^7 per g of root. In addition, Xie *et al*. (2003) found that the number of cultivable free-living nitrogen fixing bacteria isolated from the paddy field ranged between 1.41×10^6 cfu/g and 1.24×10^8 cfu/g dry weight of the soil.

This study revealed that no relationship was observed between bacterial population with SWC (Figure 4.8). Nevertheless there was an obvious correlation between bacterial population and soil saturation level (Figure 4.9) ($r^2 = 0.60$, $p<0.05$). As discussed previously, there was a negative correlation between shear strenght and soil saturation level. Hence, the higher soil shear strenght will also had lower number of bacterial population. The result showed that the slope which had the higher soil saturation level (slope type C followed by B and A) was also showed the higher bacterial population. This was an indication that water had significant contribution on the bacterial population. The effects of soil moisture on the survival of three diazotrophic bacteria species (*Azospirillum amazonense*, *Gluconacetobacter diazotrophicus* and *Azospirillum brasilense*) had been tested by André *et al*. (2004). The study showed that soil moisture had little influence on

the survival of *A. brasilense*, which was considered a free-living species. On the other hand, increased soil moisture extended the survival of the endophytes *A. amazonense* and *G. diazotrophicus.* These results indicated that nitrogen-fixing endophytic species were more affected by soil moisture than associative nitrogen-fixing bacterial species. More saturated soil meant that higher volume of water was able to reach the grass roots. This could be the factor which can enhanced the microbial growth since this phenomenon would supply more water needed by microorganism and stabilized the microenvironment as well. Study by Kana and Tjepkema (1978) showed that the rates of N_2 fixation were usually higher in the root zone of flooded soil systems than in well-drained soils, and substantial N_2 fixation rates have been reported in the root zones of several non-nodulating marsh plants, as well as in nodulating legumes of riparian systems (Moreira *et al*., 1992). N_2 fixation in bacteria occurs only on anaerobic condition unless the oxygen is neutralized by chemical like leghemoglobin.

5.3 DNA fingerprinting as measure of diversity

In this study, a total of 61 genomic DNA were extracted (Appendix 4). The quality of DNA preparations were checked by agarose gel (0.8%, w/v) electrophoresis. Since the size of bacterial DNA was between 1000kbp to 9000kbp (Trevors, 1996), the present of any single band in the gel prior to the marker DNA size 3000bp was an indicator that the interested DNA was successfully extracted and will be used in further characterisation (Figure 4.10). Genetic characterisation or strain classification had been accomplished by a number of different approach. In general, the approaches should be able to differentiate all of the organism within a species. Free-living nitrogen fixing bacteria isolated from rhizosphere of seven different plants were successfully identified based on phenotyphic and 16S rDNA sequences analysis (Park *et al*., 2005).

Two approaches, REP-PCR and ERIC-PCR were chosen as a mean to determine the diversity of free-living nitrogen fixing bacteria. The enterobacterial repetitive intergenic consensus (ERIC) sequences and the repetitive extragenic palindromic elements (REP) were two classes of short intergenic repeated sequences families in enteric bacteria which contained highly conserved central inverted repeat that did not share significant homology (Stern *et al*., 1984). These consensus sequences were used to design REP and ERIC specific oligonucleotide primers and to probe the genomes of a variety of eubacteria for the presence of REP- and ERIC like-sequences using the polymerase chain reaction (PCR) (Versalovic *et al*., 1991). Recently, Ogutcu *et al*. (2009) suggested that rep-PCR (ERIC-, REP- and BOX-PCR) fingerprinting methods could be a good choice for the genotypic characterization and phylogenetic analysis of *Rhizobium leguminosarum* subsp. *ciceri* strains isolated from perennial wild chickpeas.

As shown in Figure 4.11, both REP-PCR and ERIC-PCR were pre-tested on five samples (chosen randomly) to check on the suitability of the method to be used in this experiment. As not much difference resulted in both methods, REP-PCR was used in further characterisation due to it accessibility in the lab. Due to time constraint, only 11 strains from each slope were chosen for genetic characterisation. Selections of the strains were based on their morphological characteristic. The entire DNA was successfully amplified except for strain 7A4 and 6B4. This was due to denaturation of the DNA. Hence only 10 samples from A and B, and all samples from C (11 strains) were used for further phylogenetic analysis (Figure 4.12).

As a comparison, the DNA profile of strains from slope type C showed lesser bands than strains from other slopes (Figure 4.12 (c)). The result also indicated that strains from slope type B had bigger size ranging from 300 to 2500 bp compared to strains from the other slope (Figure 4.12 (b)). The result also revealed that there were two pairs of strains that showed similarity on the REP-PCR profile (strain 13A4/24A3 and 15C4/28C3). Hence, the strains presumably originated from a close family in the phylogenetic hierarchy among each other.

The dendogram constructed for strains from slope type A can be grouped into four clades (Figure 4.13). Strains 13A4 and 24A3 had a closer relationship to each other compared to other strains at the similarity more than 65%. This was parellal with the result showed in the REP-PCR of the strains. Strain 27A3 was the most isolated in the cluster. Strains from slope type B was also can be grouped into four clades: Clade E, F, G and H (Figure 4.14). Strains 18B3 and 27B3 in clade H show clear relationship between them (at the similarity more than 40%) but join the large cluster sharing weak phenetic affinity of less than 20%. Presumably the two strains had close relationship between one another but not with the other strains in the cluster. For strains from slope type C, the dendogram can be group into clades I, J and K (Figure 4.15). As shown in clade I, strains 15C4 and 28C3 showed close relationship at the similarity of 50%. This relationship was also observed on the REP-PCR profile in the previous discussion. Hence, the two strains probably originated from the same family.

In Figure 4.16, the dendogram was constructed as a combination of all of the strains isolated from the three slopes showed they can be group into four clades (Clade I, I, III and IV). Most of the Gram-positive bacteria used in REP-PCR were in clade I (4 out of 6

strains) and all of strains in clade II were Gram-negative. There were no bacteria from slope A in clade I. The results also revealed that strain pairs 13A4/24A3, 18B3/4C5 and 18C4/26C3 showed some clear relationship between one another at the similarity more than 50%. The phylogenetic tree clearly showed that strain 12A4 did not cluster with the others and it’s position in the tree is a single member cluster. These results showed that the diversity of nitrogen-free fixing bacteria in this study were quite high.

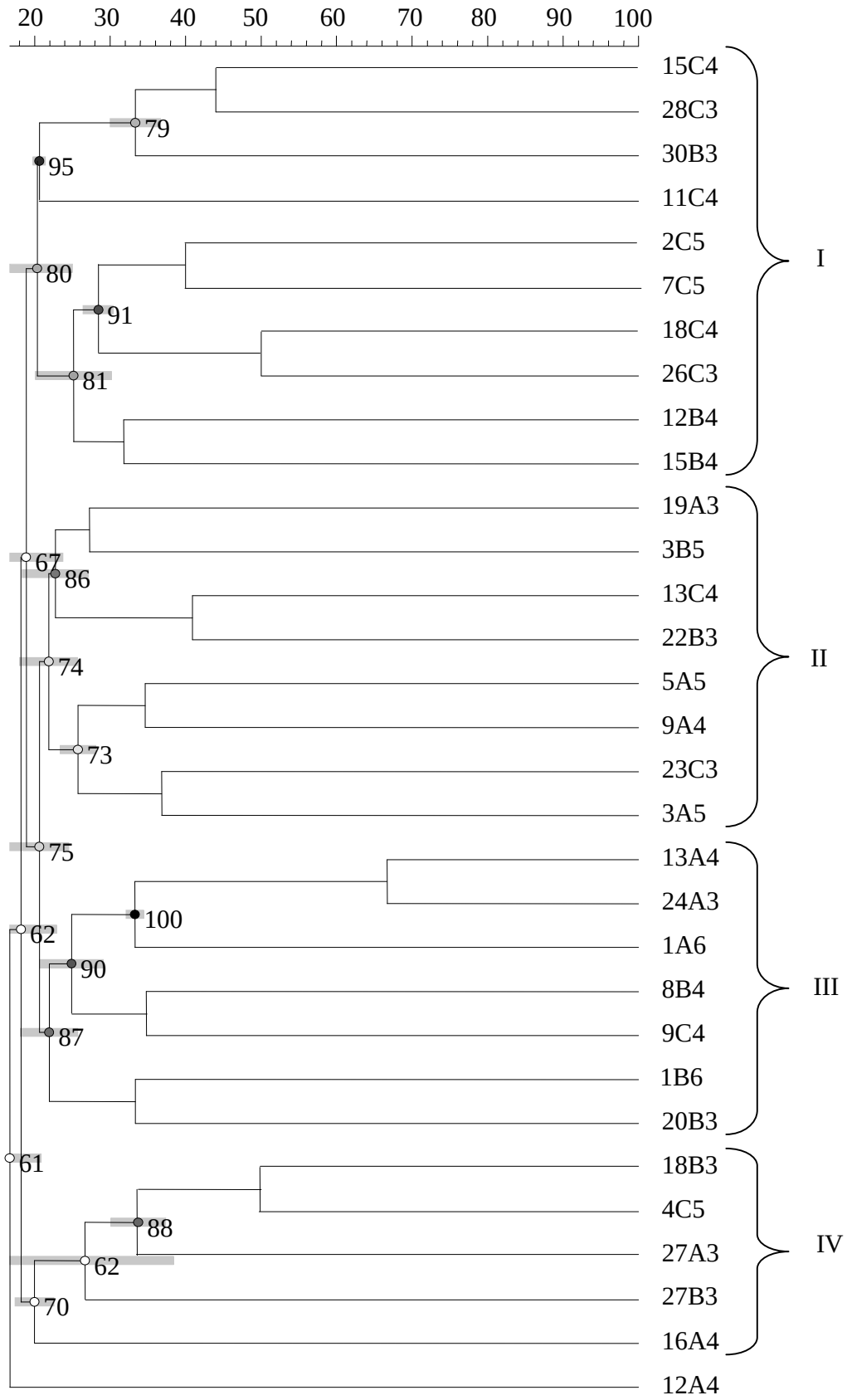


Figure 4.16 Dendrogram obtained from all of 31 strains. At the cut-off point for clustering was 22%, the strains can be grouped into 4 clades (I, II, III and IV). Strains 12A4,16A4 and 11C4 did not fit the cluster.

CHAPTER 6

CONCLUSIONS

In general, all slopes in this study are in a range of stable slope. This is parallel to previous study which showed that the more stable slopes will also have a low SWC. The use of SWC as an indicator of slope stability is proven as a good approach to be practiced in the future study. This study revealed that there is no relationship between shear strength and SWC but a negative correlation is observed between the soil shear strength and soil saturation level. As a whole, this study shows that Gram-negative bacteria is dominant. The population of free-living nitrogen fixing bacteria ranging from 6.6×10^6 to 1.02×10^7 cfu/g dry weight . Slope type C showed the highest bacterial population size followed by slope type B and type A. No relationship between bacterial population and SWC but there is positive correlation between bacterial population and soil saturation level.

A total of 77 colonies isolated from the pure culture is an indicator that the N-free medium and the growth condition used in this study is suitable to be used for further research in culturable free-living nitrogen fixers. Since many of free-living nitrogen fixing bacteria are unculturable (Sarita *et al*., 2008), the use of *nif*H PCR and 16rRNA PCR are good methods to be used for bacterial identification. Studied by Meadow *et al.* (1994) showed that bacteria (*Pseudomonas atlantica*) itself have direct influence on slope stability in which the bacteria bind particles together with their extracellular polymeric material.

Observation on the colonial characteristic showed that strains 4C5, 13C3 and 23C3 resembled members of the genus *Azotobacter*. Nevertheless, more study should be done since this type of characterisation is influenced by many factors and variables. Based on the

result of DNA fingerprinting and cluster analysis, the bacterial diversity in this study is high. Nevertheless, strains pair 13A4-24A3 and 15C4-28C3 shows strong relationship to one another.