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In the name of Allah Most Gracious Most Merciful

STUDIES ON THE ENVIRONMENTAL MICROBIOLOGY AND BIOGEOCHEMISTRY OF DESERT SURFACE SOILS

By

Reda Hassan Amasha

MSc., King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia

MPhil., University of Sheffield, England

Thesis submitted in partial fulfillment for the requirements of the Degree of Doctor of Philosophy

Department of Molecular Biology and Biotechnology

The University of Sheffield, England

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DEDICATION

To the memory of my beloved parents

Certainly, you both are proud of me, and I do hope that we all meet in heaven, with Allah’s willing

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First of all, I wholeheartedly thank Al Mighty Allah, who blessed me with health, courage, enthusiasm and for giving me the power and endurance to complete this Thesis. I deepest thank my supervisor Professor Dr Milton Wainwright for his guidance, support, helpful advice, encouragement all time and assisted me in each step to complete the Thesis.

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ABSTRACT

Microorganisms play a key role in the functioning of the environment, particularly in relation to the biogeochemical cycles. Here, a study was made of the microbial activity of primitive desert surface soils in comparison with that exhibited by a fertile agricultural loam soil. The microbial transformations studied included nitrification, the hydrolysis of urea, the oxidation of elemental sulphur to sulphate and phosphate solubilisation; these processes were collectively used to study the biogeochemical activity of desert surface soils. Bacterial population densities in the desert surface soils, fertile loam soils and volcanic, cave rock samples were also determined. A variety of bacterial isolates from desert surface soils and cave rock samples have been identified using molecular identification techniques like DNA extraction, PCR amplification, determinations of 16S and 18S rRNA gene sequences. The isolation and characterization of extremophilic bacterial strains from a dormant volcano on the island of Reunion is reported, using molecular identification, morphological and physiological studies. As the area of the volcano, from which these bacteria were isolated, has not been recently active, it was considered of interest to determine if these bacteria grow, or merely survive, in a mesophilic environment. Nuclear magnetic resonance spectroscopy (NMR) was used to study the compatible solutes in these isolates when growing under high temperatures, low and high pH stresses and at various concentrations of NaCl. Finally, various environmental samples were tested in order to detect the presence of *Mycoplasma* using an EZ-PCR *Mycoplasma* Test Kit.

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CHAPTER 1

CHAPTER1

LITERATURE REVIEW

1.1. Introduction to biogeochemical cycles

1.1.1 The Nitrogen Cycle

Nitrogen is one of the most common elements found in cells, and makes up about 12% of cell dry weight (Maier *et al*, 2009). Living organisms principally rely on nitrogen to produce a number of complex organic molecules like proteins and nucleic acids (Pidwirny, 2004). Nitrogen can be in many different forms, both inorganic and organic, and can also exist in a variety of different oxidation states (Table 1.1).

Table 1.1: The nine oxidation states of nitrogen. (*Reproduced and updated from Bengtson, 2010*).

Oxidation state	Species	Name
-3	NH_3, NH_4^+	Ammonia, ammonium ion
-2	N_2H_4	hydrazine
-1	NH_2OH	hydroxylamine
0	N_2	Nitrogen gas
+1	N_2O	Nitrous oxide
+2	NO	Nitric oxide
+3	HNO_2, NO_2^-	Nitrous acid, nitrite ion
+4	NO_2	Nitrogen dioxide
+5	HNO_3, NO_3^-	Nitric acid, nitrate ion

The Nitrogen cycle is a complex biogeochemical process, which involves the transformation and translocation of nitrogen in soil, water, and both living and dead organic matter (Francis *et al*., 2007). This process is highly dependent upon the activity of microorganisms within the ecosystem. Fig 1.1 shows that various forms of nitrogen exist, and are converted to intermediates or to end products in the nitrogen cycle (Park, 2010). The atmosphere supplies the main reservoir of nitrogen as (N_2); other main source of nitrogen includes organic matter in soil and in the oceans.

Organic nitrogen is converted into inorganic form during these biogeochemical processes. There are five main processes operating in the N-cycle. Nitrogen fixation is the processes by which atmospheric nitrogen (N_2) is converted into ammonia (NH_3), a process achieved by specific bacteria. Once ammonia is produced by bacteria, it goes through another process called nitrogen uptake (i.e. assimilation) where other organisms or bacteria will make use of ammonia and thereby produce other organic nitrogen compounds. Nitrogen mineralisation is the process by which organic nitrogen is converted to inorganic nitrogen, i.e. ammonium (NH_4^+). In the nitrification processes, the ammonium salt is oxidized to nitrate (NO_3^-). Denitrification is the processes whereby nitrate and nitrite (NO_2^-), are reduced to nitrites and nitrous oxide (N_2O), and finally to a nitrogen gas (Harrison, 2003; Pidwirny, 2006).

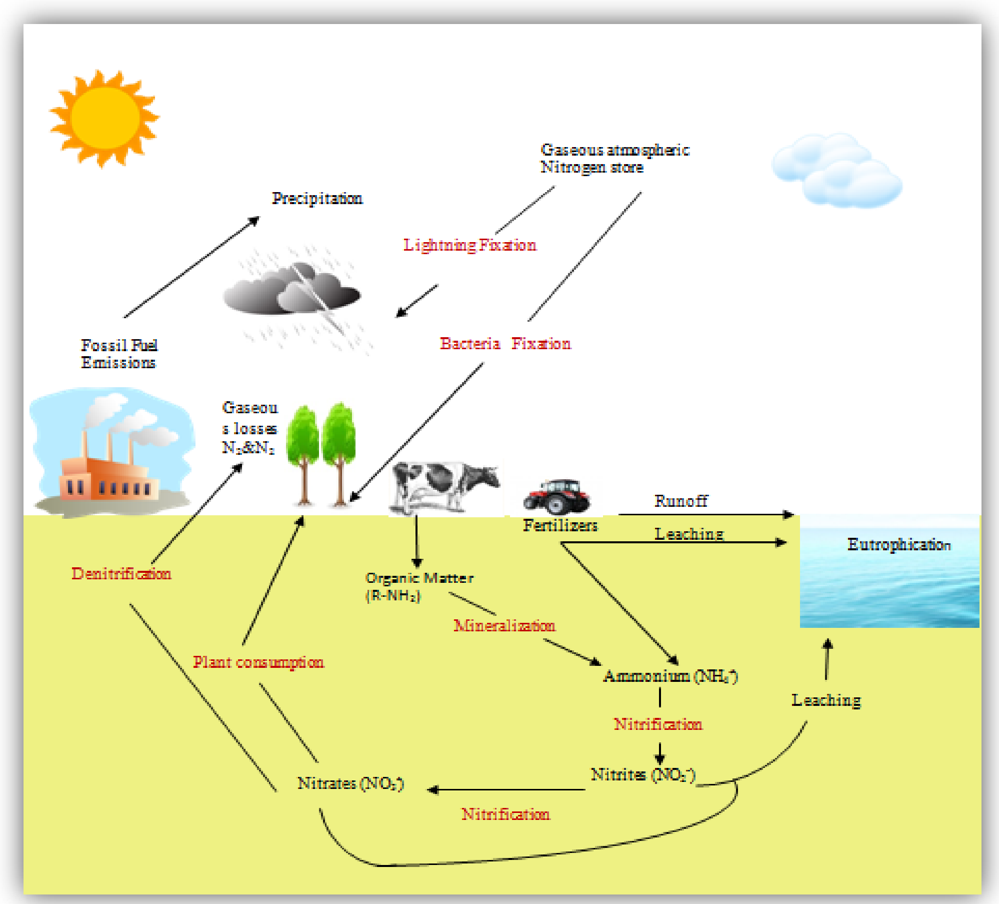

Figure 1.1: The Nitrogen Cycle (*Reproduced and updated from Pidwirny, 2006*)

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1.1.1.1. Nitrogen fixation

Nitrogen fixation is the process by which nitrogen gas (N_2) from the atmosphere is fixed into ammonia (NH_3) by means of over 100 different free-living (both aerobic and anaerobic) bacteria, including actinomycetes and cyanobacteria. Nitrogen fixers include, *Azotobacter*, *Beijerinckia*, *Acetobacer* and *Pseudomonas* (aerobic), *Klebsiella*, *Bacillus* and *Rhodospirillum* (facultative anaerobic), *Clostridium* (anaerobic), cyanobacteria *Anabaena* and *Nostoc* (aerobic), and actinomycetes *Frankia*. Nitrogen fixation is limited to bacteria and it is an energy-intensive process. (Soomro, 2000; Maier *et al.,* 2009). The most important genus of bacteria for nitrogen fixation is *Rhizobium* (Harrison, 2003).

1.1.1.2. Nitrogen mineralisation (Ammonification)

Nitrogen mineralisation is the conversion of organic nitrogen to ammonium, which makes the nitrogen assimilable by various plants or for further oxidation to nitrate (NO_3^-) during the process of nitrification (Harrison, 2003). Ammonification is the final result of the breakdown of organic matter, for example dead plants and animals or waste materials such as faeces. This process is accomplished by a variety of bacteria, fungi and actinomycetes. These organisms convert dead organic materials to ammonia via two stages: firstly, proteins are hydrolysed by enzymes to form simple amino acids, and secondly, microorganisms convert the produced amino acids into ammonia. The resulting ammonia dissolves in water as ammonium ions. Numerous factors; such as temperature, pH, moisture, aeration and the carbon: nitrogen (C: N) ratio in the environment determine the activity of ammonifying microorganisms, and thus mediate the ammonification of organic nitrogen (Hart *et al*., 1994).

1.1.1.3. Immobilisation

Immobilisation is also known as ammonium assimilation. As ammonia is the end product of N_2 fixation process in the soil, the pH value makes a balance between ammonium ions and ammonia in solution. Generally, the ammonium (NH_4^+) form is easily assimilated by microorganisms and converted to amino acids and then proteins. Cell wall components, for example purines, pyrimidines and N-acetylmuramic acid, are used to form nucleic acid. As shown in Fig1.2, there are two pathways which microorganisms use for the immobilisation of ammonium. First, is a reversible reaction, which extracts ammonium from the amino acid glutamate, and then ammonium is built-in into $\propto -$ketoglutarate to form glutamate. The second reaction is determined by ATP and two enzymes: *viz* glutamine synthetase and glutamate synthetase (GOGAT). In the first phase of the reaction, an accumulation of

ammonium into glutamate to produce glutamine takes place, whereas, in the second stage, the ammonium molecule is transferred from glutamine molecule to the $\propto -$ketoglutarate molecule resulting in the formation of two glutamate molecules (Maier *et al.*, 2009).

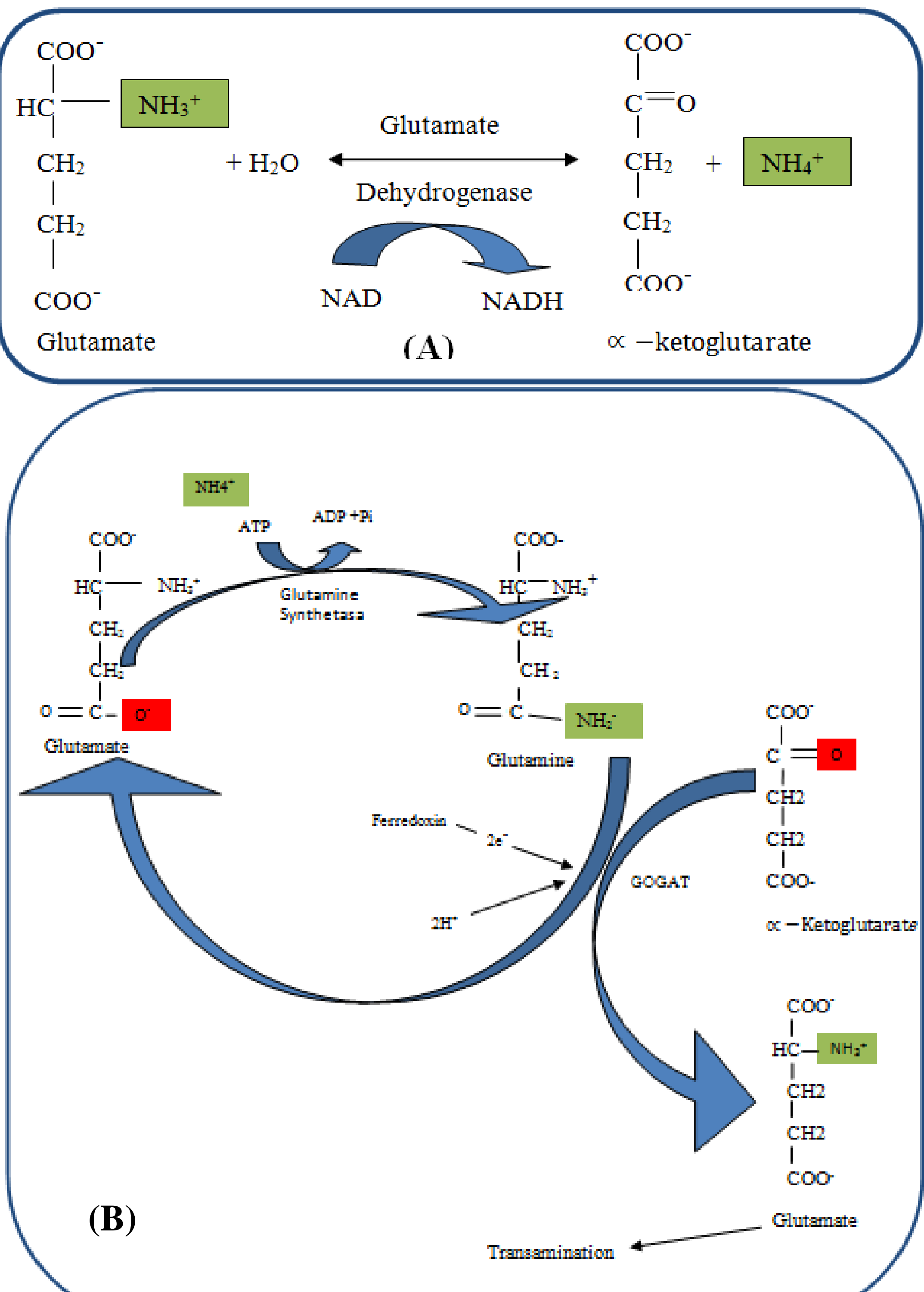


Figure 1.2: Microbial ammonium immobilisation pathways: "(A) The enzyme glutamate dehydrogenase catalyzes a reversible reaction that immobilizes ammonium at high ammonium concentration (B) The enzyme system glutamine synthase-glutamate synthetase that is induced at low ammonium concentrations. This ammonium uptake system requires ATP energy" (*Reproduced and updated from Maier et al., 2009 (Figure 14.12) p. 304*). Used with permissions

1.1.1.4. Nitrification

Nitrification is a microbially mediated oxidation of ammonium to nitrate. It is exclusively an aerobic process which mainly involves chemoautotrophic bacteria, although some heterotrophic fungi and bacteria can also perform the oxidation (Maier *et al*., 2009). Essentially, the nitrification process involves the following two steps: The first involves the formation of nitrite (NO_2^-), when ammonia (NH_3) or ammonium (NH_4^+) ions are oxidised by chemoautotrophic nitrifying bacteria (*Nitrosomonas*):

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H+ + H_2O \qquad \Delta G = -267.5\ kJ.mol^{-1}$$

The second step involves the oxidation of (NO_2^-) to (NO_3^-), a process which also involves nitrifying (*Nitrobacter*) chemoautotrophic Gram-negative bacteria. Plants readily take up nitrates through their roots and assimilate them into organic compounds.

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \qquad \Delta G = -86.96\ kJ.mol^{-1}$$

In the nitrification process, the generated energy is used for carbon dioxide fixation. Nitrification occurs over an optimum pH range of 6.6 – 8.0. The rate of the nitrification become slower at pH values below 6.0 and is completely inhibited at pH values below 4.5 (Philips *et al*., 2002). Nitrification is affected by a variety of environment factors such as temperature, pH, oxygen supply, moisture content, organic and inorganic matter, carbon dioxide availability, and the cation exchange capacity of a soil (Soomro, 2000).

Nitrogen does not usually accumulate in the environment, and there is rarely an excess of ammonia in a natural ecosystems. On the contrary, serious environmental problems may occur because of nitrate build up in many managed agricultural systems and feedlots, as well as in septic tanks and landfills, where a large input of nitrogen fertiliser occurs.

Since nitrate is a negatively charged anion, and since soil is also anionic, nitrate ions are extremely mobile in soil water and hence, can be leached from soil into ground water and surface waters, which can lead to several health concerns related to excessive levels of nitrate and related compounds (Gershuny e*t al*., 1995).

1.1.1.4.1. Health and environmental effects of nitrification

Nitrates and ammonium are known to cause a number of health effects in humans and animals, for example high levels of nitrates in water can cause methemoglobinemia, especially in infants which are less than six months old. The stomach acid of an infant is not as strong as in older children and adults and for this reason, a large number of bacteria exist, which can readily convert nitrate to nitrite in baby's stomachs. The absorbed nitrates in the blood and haemoglobin is then, converted to methemoglobin. Methemoglobinemia causes brain damage and death. Nitrates and nitrites are also carcinogenic and can also cause a decrease in the functioning of the thyroid gland (Delellis, 2004). The accumulation of N contaminants in water systems also has a harmful consequence, termed "eutrophication". This results from an increase in the concentration of nitrogen in water systems and result in the rapid growth of algae and higher plants, which when they rot remove oxygen from the system. Some algae also release poisonous toxins into the water course and cause toxicity-related environmental problems (Park, 2010).

1.1.1.4.2. The biological characteristic of nitrification

Schloesing and Muntz were the first researchers to study the biological nature of nitrification in 1877. However, they could not isolate the nitrifying bacteria, which awaited the work of Winogradsky (1889), who showed that nitrifying bacteria grow autotrophically in media with no organic carbon. In recent years, the understanding

of the biology and ecology of nitrification has developed as follows: Firstly, the rate of nitrification has been shown to not be solely limited by soil pH as was previously thought. Secondly, two types of nitrification are recognized: chemoautotrophic and heterotrophic nitrification, with chemoautotrophic nitrification generally dominating in agricultural soils, whereas heterotrophs tend to play a more important role in acidic soils (Falih, 1995).

1.1.1.4.3. Chemoautotrophic nitrification

Chemoautotrophic bacteria are identified as bacteria that can obtain the energy required for their growth from oxidation of inorganic compounds such as hydrogen sulphide or ammonia. Chemoautotrophic bacteria are classified into two groups:

i) Ammonium oxidising bacteria: bacteria which derive energy for cell synthesis by the oxidation of ammonium. This group of bacteria have genera names usually begin with (Nitroso), and

ii) Nitrite oxidising bacteria: bacteria which derive energy from the oxidation of nitrite. This group of bacteria have genera names begin with (Nitro). *Nitrosomonas* and *Nitrobacter* have been identified the main genera as the most common ammonium oxidisers in soil. Ammonium oxidisers are classified on their DNA (G+C) contents, shape, membrane constituents and phylogenetics as illustrated in Table 1.2 (Madigan *et al*, 2003). Oxidation reactions provide the energy for nitrifiers; ammonia monooxygenase being the enzyme which oxidizes NH_3 to hydroxylamine then to NO_2^- .The nitrite generated in this reaction is oxidized to nitrate by the nitrifying bacteria. The chemical reaction occurs in two stages: Ammonia is oxidized to nitrite by *Nitrosomonas*

$$NH_3 + O_2 + 2\,e^- + 2H^+ \longrightarrow NH_2OH + H_2O$$

$$NH_2OH + H_2O + 1\tfrac{1}{2}\,O_2 \longrightarrow NO_2^- + 2\,H_2O + H^+ \quad \Delta G = -275 \text{ kJ/reaction}$$

Nitrite is oxidized to nitrate by *Nitrobacter*

$NO_2^- + ½O_2 \longrightarrow NO_3^-$ ΔG = -74.1 kJ/reaction (Madigan *et al*, 2003).

Table 1.2: Characteristics of the chemoautotrophic nitrifying bacteria. (*Reproduced and updated from Madigan et al, 2003*).

Genus	Phylogentic group	DNA (mol%GC)	Habitats	Characteristics
Oxidize ammonia				
Nitrosomonas	Beta-Proteobacteria	45-53	Soil, Sewage freshwater, marine	Gram-negative short to long rod, motile or non motile
Nitrosococcus	Gamma - Proteobacteria	49-50	freshwater, marine	Large coccid, motile
Nitrosospira	Beta-Proteobacteria	54	Soil	Spirals, motile
Nitrosolobus	Beta-Proteobacteria	54	Soil	Pleomorphic, lobular, motile
Nitrobacter	_	54	Soil	Slender, curved rods
Oxidize nitrite				
Nitrobacter	Alpha-Proteobacteria	59-62	Freshwater, marine, soil	Short rods, reproduce by budding, motile
Nitrospina	Delta-Proteobacteria	58	Marine	Long, slender rods, non motile
Nitrococcus	Gamma-Proteobacteria	61	Marine	Long cocci, motile
Nitrospira	Nitrospira group	50	Marine, soil	Helical to vibrioid – shaped cells, non motile.

1.1.1.4.4. Heterotrophic nitrification

Heterotrophic nitrifying microorganisms oxidise ammonium or organic nitrogen compounds (e.g. pyruvic acid, oxaloacetic acid and $\alpha - \text{ketoglutaric acid}$) . These microbes do not gain energy directly from nitrification (Syliva *et al*, 1999).

The association of heterotrophis with nitrification was first recognized in 1894 and since, a large diversity of heterotrophic nitrifying microorganisms have been found which are capable of oxidising both organic and inorganic nitrogen (Table 1.3) (Alexander, 1965). The microorganisms involved include both Gram-positive and Gram-negative bacteria, anaerobes and spore or non spore formers as well as some species of fungi and species of actinomycetes. The heterotrophic bacteria include *Agrobacterium, Azotobacter, Bacillus, Clostridium,* and *Pseudomonas.* Fungi include species of *Aspergillus, Penicillium,* and *Cephalosporium.* Actinomycetes involved include *Micromonospora*, and various spices of *Nocardia* and *Streptomyces*.

The relative important of autotrophic and heterotrophic nitrification in the environment is however, not readily determined.

Table 1.3: Nitrogenous substrates and products of some heterotrophic nitrifying microorganisms (*Reproduced and updated from Alexander, 1965*).

Microorganisms	Substrate	Product
Bacteria		
Agrobacterium spp.	pyruvic oxime	nitrite
Azotobacter chroococcum	NH_4	bound NH_2OH
Bacillus spp.	NH_4	nitrite
Clostridium butyricum	N_2	nitrate
Corynebacterium simplex	nitrophenols	nitrite
Mycobacterium rubrum	NH_2	nitrite
Pseudomonas spp.	NH_2OH	nitrite
Pseudomonas methanica	NH_4	nitrite
Actinomycetes		
Micromonospora spp	NH_4	nitrite
Nocardia spp.	NH_4	nitrite
Nocardia coralline	pyruvic oxime	nitrite
Nocardia spp.	p-nitrobenzoate	nitrite
Streptomyces spp.	NH_4	nitrite
Fungi		
Aspergillus flavus	NH_4	bound NH_2OH, nitrite $\beta^{\sim}$-nitropro- ionate, nitrate.
Aspergillus flavus	amino	aspergillus acid
Aspergillus niger	NH_4	NH_2OH
Aspergillus wentii	nitrite	nitrate
Cephalosporium sp.	NH_4	nitrate
Penicillium spp.	nitrite	nitrate
Penicillium spp.	amino	N-formyl hydroxy-aminoacetate
Sterigmatocystis nigra	NH_4	NH_2OH

1.1.1.5 Denitrification

Denitrification is the biological reduction of nitrate (NO_3^-) to nitrogen gas (N_2). This process is carried out by a large range of bacteria, which utilise dissimilatory nitrate (NO_3^-) or nitrite (NO_2^-) reduction to ammonium (NH_4^+) (Table 1.4). The responsible bacteria are termed heterotrophs and perform conversions from nitrate to nitrite, and from nitrite to various gaseous products (e.g. nitric oxide NO, nitrous oxide N_2O, and free nitrogen gas N_2) (Metcalf, 1991).

Denitrification is generally referred to as "anaerobic process", even though aerobic denitrification can also take place. The microbial reduction of nitrate usually takes place by two processes: the first is assimilatory, where the ion is reduced to nitrite and ammonium; this involves nitrate and nitrite reductases. The products of nitrate assimilation are incorporated into cell material. The second reduction is identified as being dissimilatory, where nitrate ions act as an alternative electron accepter to oxygen in electron transport chain. Dissimilatory nitrate reduction leads to the formation of either ammonium or dinitrogen; where dinitrogen is formed, the process is termed as denitrification. Denitrification, by depleting part of the soils reserve of essential nutrients, has detrimental effects on crop production.

Table 1.4: Bacteria responsible for the dissimilatory reduction of nitrate (NO_3^-) or nitrite (NO_2^-) to ammonium (NH_4^+). (*Reproduced and updated from Maier et al., 2009*).

Genus	Typical habitat
Obligate anaerobes	
Clostridium	Soil,sediment
Deslfovibrio	Sediment
Selenomonas	Rumen
Veillonella	Intestinal tract
Wolinella	Rumen
Facultative anaerobes	
Citrobacter	Soil,wastewater
Enterobacter	Soil,wastewater
Erwinia	Soil
Escherichia	Soil,wastewater
Klebsiella	Soil,wastewater
Photobacterium	Seawater
Salmonella	Sewage
Serratia	Intestinal trace
Vibrio	Sediment
Microaerophilles	
Campylobacter	Oral cavity
Aerobes	
Bacillus	Soil,food
Neisseria	Mucous membranes
Pseudomonas	Soil,water

Denitrification is influenced by many factors in the environment. For example, carbon source is an important factor, simply because heterotrophic bacteria need to use carbon as an energy source. Soil pH is another factor which directly affects the rate of this process. The optimum pH range for denitrification is from 7.0 to 8.5. Temperature can also influence the growth rate of bacteria responsible for the process, with higher growth rates occurring at higher temperatures. Denitrification can occur between 5°C and 30°C, and the rate of the process increases with increasing temperature and amount of organic matter present.

1.1.1.6 Urea hydrolysis

Today urea is the most important solid nitrogen fertiliser used in agriculture worldwide. There are many advantages of using urea over other nitrogen fertilisers. For example: urea has high nitrogen content which exceeds that of ammonium nitrate and ammonium sulphate. Urea is also highly soluble in water and its safe application, cheap production, reliable transport and distribution make it the leader in the world N trade (Ferguson *et al.*, 1984).

Microorganisms release a range of enzymes (lysozymes, proteases, nucleases and urease) which can begin the degradation of nitrogen containing compounds, such as proteins, cell wall, nucleic acids and urea. Ureases are extracellular enzymes secreted by soil microbes, plants and animals. As shown in equation below, urea is converted to carbon dioxide and ammonia by urease in soil (Maier *et al.,* 2009). Many bacteria are capable of hydrolysing urea, such as *Bacillus*, *Pseudomonas*, *Micrococcus*, *Achromobacter, Clostridium* and *Coryneformbacterium,* and actinomycetes, as well as some fungi.

$$NH_2-\overset{\overset{\displaystyle O}{\|}}{C}-NH_2 \;+\; H_2O \longrightarrow 2NH_3 + CO_2$$

1.1.2 The Sulphur cycle

Sulphur is ranked as the tenth most abundant element in the earth's crust, and is an essential element for growth. In biology, sulphur accounts for approximately 1% of the dry weight of a cell. Sulphur exists in numerous oxidation states the highest being +6 for sulphate (SO_4^{2-}) and the lowest being -2 for sulphide (S^{2-}). Table 1.5 shows the most important inorganic sulphur forms present in the environment. Sulphur concentration ranges from 20 to 2000 $\mu g\ g^{-1}$ in most agricultural soil, whereas its concentration in various volcanic ash and organic and tidal-marsh soil

exceeds 3000 μg g^{-1}, and falls to 10μg g^{-1} in some desert soils (Paul and Clark, 1996). Sulphur is an essential element for the synthesis of the amino acids, cysteine and methionine, and some vitamins like vitamin B1 thiamine, hormones such as biotin, coenzymes and lipoid acid. In particular, it is very important element in the process of synthesising of proteins, where disulphide bridges, formed with cysteine residues, govern the protein folding. Although the sulphur cycle is less complex than that for nitrogen, it has a global impact, including its relation to acid rain episodes, mine drainage and the corrosion of concrete and metals (Maier *et al*, 2009).

Table 1. 5: The nine oxidation states of inorganic sulphur compounds. (*Reproduced and updated from Paul and Clark, 1996*)

Oxidation state	Formula	Form
+6	SO_4^{-2}	Sulphate
+4	SO_3^{-2}	Sulphite
-2,+6	$S_2O_3^{-2}(S\text{-}SO_3^{-})$	Thiosulphate
-3, +1.7	$S_4O_6^{-2}$	Tetrathionate
-2	S-C-S	Thiocyanide
+2,+6	$^{-}O_3SSSO_3^{-}$	Trithionate
0	S^0	Elemental
-2	HS^-	Disulfide
-2	S^{-2}	Sulphide

Most of the sulphur in the soil is present in the form of organic matter, and therefore, must be oxidised to inorganic sulphates before it becomes available for use by plants. Even though some non-biological oxidation of sulphur can also occur in soils (MacIntire *et al.*, 1921), the majority of soil sulphate released from inorganic sulphur is produced by microbiological oxidation (Sievert *et al.*, 2007). Sulphur undergoes numerous transformations in soil which together form the sulphur cycle. As shown in Fig1.3, these reactions are largely mediated by microorganisms (Brown,

1982). In the sulphur cycle, microorganisms are responsible for the following processes:

i) Mineralisation of organic sulphur to sulphate,

ii) The oxidation of reduced forms of inorganic sulphate,

iii) The anaerobic reduction of sulphate to sulphides, and

iv) The immobilisation of sulphate as organic sulphur.

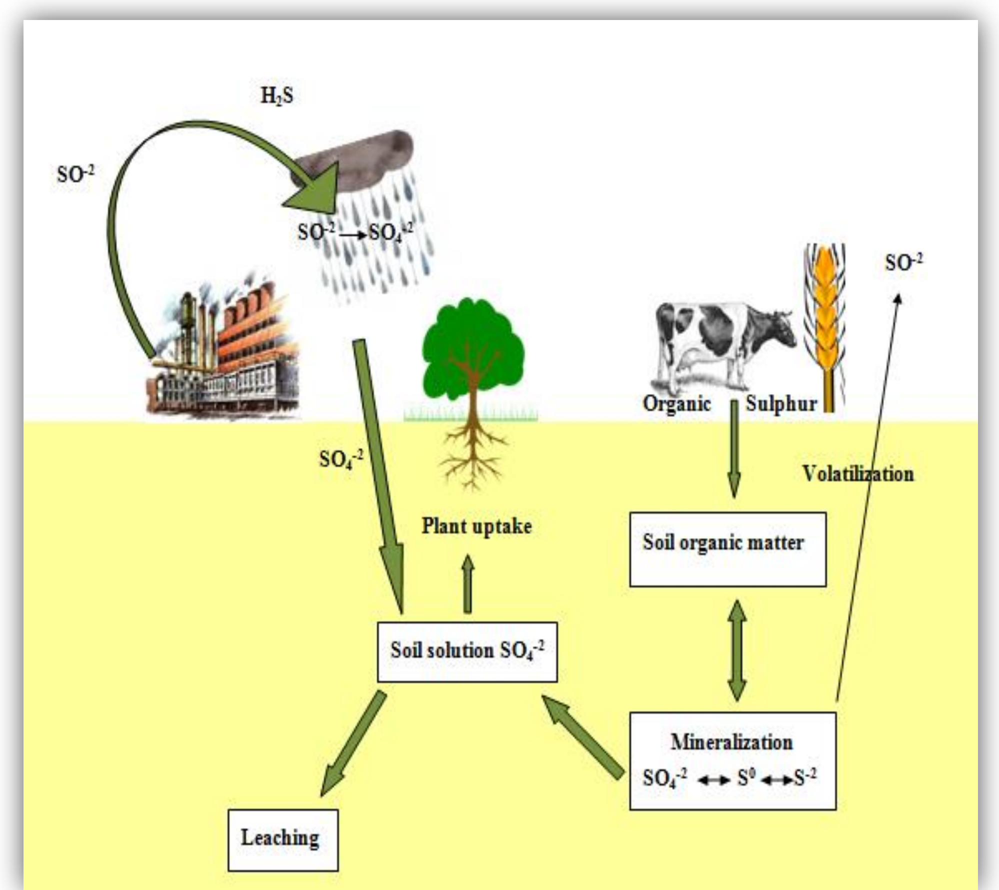


Figure 1.3: The Sulphur Cycle. (*Reproduced and updated from Place et al., 2007*) Used with permissions.

1.1.2.1. Sulphur mineralisation

There are two pathways which convert organic sulphur into inorganic forms: *viz* biological process and chemical process. These processes are also known as sulphur mineralisation and they can occur under both aerobic and anaerobic conditions

(Maier *et al*, 2009). Sulphur mineralisation plays a main role in the metabolism of microbes, and consequently it is also an essential means by which sulphate is mobilised, as well as acting as a source of H^+ ions (Tabatabai, 1985). The mechanisms responsible for sulphur mineralisation are not completely known although two major mechanisms (e.g. biological and biochemical) have been suggested (Grayston, 1987).

(i) Biological sulphur mineralisation: Carbon-bonded sulphur, such as in amino acids, is mineralised by microbes through an oxidative process which leads to energy generation. Numerous inorganic products are utilised by soil microbes for cell synthesis and the remainder is released into the environment. Sulphate is the end product of inorganic sulphur oxidation under aerobic conditions; however, the decomposition of proteinaceous substances eventually results in the formation of hydrogen sulphide under anaerobic conditions (Soomro, 2000).

***(ii) Biochemical sulphur mineralisation*:** This process occurs when non-carbon-bonded organic sulphur ester sulphates are being mineralised during the enzymatic catalysis process of sulphatases produced by a variety of microorganisms external to the cell membrane. Factors that affect process of sulphur mineralisation include changes in oxygen content, varying temperatures especially in the mesophilic range, moisture levels, and presence of lime in acidic soils (Tabatabai and Al-Khafaji, 1980).

1.1.2.2. Sulphur oxidation

Reduced sulphur compounds can be oxidized by chemoautotrophic bacteria in the presence of oxygen under strictly aerobic conditions, and a group of photoautotrophic bacteria can achieve this under strictly anaerobic conditions. In addition, aerobic heterotrophic microbes (including both bacteria and fungi) oxidise

sulphur to thiosulphate or sulphate; interestingly, no energy is derived from this process. Chemoautotrophs are thought to be the dominant sulphur oxidisers in most environments. Heterotrophs can also initiate sulphur oxidation thereby producing a lower pH which promotes the growth and activity of chemoautotrophs. Most chemoautotrophs oxidise sulphide to elemental sulphur, which is then deposited inside the cell as granules.

$$H_2S + ½ O_2 \rightarrow S^0 + H_2O \qquad \Delta G = -218 \text{ kJ/mol}$$

The energy released from the above oxidation reaction is used to fix CO_2 for cell growth. Both oxygen and sulphide are essential in order for this reaction to take place; however, reduced sulphur compounds are generally found in environments that contain little or no oxygen, and consequently, these organisms are micro-aerophilic. These organisms are often found in marsh sediments, where they produce a typical black band resulting from the deposition of reduced sulphur deposits and also “rotten egg” smells-like, due to the presence of hydrogen sulphide. Some chemoautotrophs, notably *Acidithiobaccillus thiooxidans* can oxidise elemental sulphur as follows:

$$S^0 + 1 ½ O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+ \qquad \Delta G = -532 \text{ kJ/mol}$$

Apparently this reaction produces acid. *A. thiooxidans* is extremely acid tolerant and its optimum growth occurring at pH 2, although the species varies widely in their response to acidity (Baker and Banfield, 2003). *Acidithiobaccillus thiooxidans*, in conjunction with *Acidithiobacillus ferrooxidans*, is responsible for the formation of acid mine drainage; a very undesirable consequence; because of its adverse impact on the environment. However, these organisms can also be employed to recover precious metals from the environment.

1.1.2.2.1. Chemoautotrophic sulphur oxidation

The diversity of S-oxidizers allows sulphur oxidation in various soils. Chemoautotrophic (lithotrophs) sulphur bacteria differ in morphology and physiology ranging from specialist obligate (facultative chemolithotrophs which can grow mixotrophically) to specialist heterotrophy. Table 1.6 shows that some of chemolithotrophs may not directly benefit from the oxidation of reduced sulphur compounds (Madigan *et al*., 2003).

Thiobacilli are the best studied species of sulphur chemolithotrophs oxidizers. This bacterium is Gram-negative, rod shaped, motile, nonspore forming. It derives its energy from the oxidation of sulphides (SH^-), elemental sulphur (S^0), thiosulphate ($S_2O_3^{-2}$), tetrathionate($S_4O_6^{-2}$) and thiocyanate ($S\text{-}C\text{-}N^-$); whereas CO_2 or bicarbonate supplies the required carbon for chemolithotrophs growth. Numerous species of *Thiobacillus* are acidophilic and they can be subdivided into two groups: those growing on neutral pH and those which live at acidic pH. (Atlas *et al*., 1997).

Table 1.6: Physiological characteristics of sulphur-oxidising chemolithotrophic bacteria. (*Reproduced and updated from Madigan et al, 2003*)

Genus	**Phylogentic group**	**DNA GC%**	**Range of pH for growth**	**Inorganic electron donor**
Thiobacillus species growing poorly if at all in organic media				
T.thioparus	Beta	61-66	6-8	$H_2S, SH^-, S^0, S_2O_3^{-2}$
T.denitrificsns		63-68	6-8	H_2S, $S^0, S_2O_3^{-2}$
T.neapolitanus		52-56	6-8	$S^0, S_2O_3^{-2}$
T.thiooxidans		51-53	2-4	S^0
T.ferrooxidans		55-65	2-4	S^0, metal SH^-, Fe^{+2}
Thiobacillus species growing well in organic media				
T.novellus	Beta	66-68		$S_2O_3^{-2}$
T.intermedius		64	6-8	$S_2O_3^{-2}$
Filamentous sulphur chemolithotrophs				
Beggiatoa	Gamma	37-51	6-8	H_2S, $S_2O_3^{-2}$
Thiothrix		52	6-8	H_2S
Thioploca		-		H_2S, S^0
Other genera				
Achromatium	Gamma	-	-	H_2S
Thiomicrospira	Gamma	36-44	6-8	H_2S, $S_2O_3^{-2}$
Thiosphaera	Alpha	66	6-8	H_2S, $S_2O_3^{-2}, H_2$
Thermothix	Beta	-	6.5-7.5	H_2S, $S_2O_3^{-2}, SO_3^-$
Thiovulum	Epsion	-	6-8	H_2S, S^0

1.1.2.2.2. Heterotrophic sulphur oxidation

Heterotrophic sulphur oxidation bacteria were firstly studied by Guittoneau in 1927, followed by research conducted by Starkey in 1934. Killham (1994) also reported that heterotrophic bacteria are capable of oxidising thiosulphate ($S_2O_3^{-2}$) to sulphate (SO_4^{-2}) through tetrathionate ($S_4O_6^{-2}$), and no energy is derived in this process.

A wide variety of heterotrophic sulphur oxidation microorganisms can oxidise a wide range of forms of reduced sulphur *in vitro*. Furthermore, several studies have confirmed that numerous bacterial species such as *Arthobacteria, Achromobacter, Bacillus, Beggiatoa, Flavobacterium, Micrococcus, Mycobacterium, Pseudomonas* and *Sphaerotilus* are capable of oxidising sulphur (Trudinger, 1967; Schook and Berk, 1978).

Paul and Clark (1996) reported that some actinomycetes and numerous fungal species can oxidise inorganic S compounds, for example: *Absidia, Alternaria, Fusarium* and *Trichoderma.* The ability of fungi to oxidise sulphur was studied by Armstrong in (1921) and Abbot in (1923), and it was further confirmed when Wainwright and Killham (1980) conducted an extended study; fungi are considered as strict heterotrophs and unlikely to gain energy from sulphur oxidation.

1.1.2.2.3. Photoautotrophic sulphur oxidation

The photoautotrophic sulphur bacteria occur in anaerobic environment such as mud, saline lakes, and stangnant water. These bacteria are limited to only green and purple sulphur bacteria (Table1.7). These microorganisms fix carbon using light energy; however, instead of oxidising H_2O to O_2 they use an analogous oxidation of sulfide to sulphur. These microorganisms play an important role of eliminating

sulfide from the surrounding environment, effectively preventing its movement into the atmosphere and its precipitation as metal sulfide (Maier *et al*, 2009).

Table 1.7: Sulphur oxidising bacteria. (*Reproduced and updated from Maier et al., 2009*).

Grop	Sulphur conversion	Habitat requrirements	Habitat	Genera
Obligate or facultative chemoautotrophs	$H_2S \rightarrow S^0$ $S^0 \rightarrow SO_4^{-2}$ $S_2O_3^{-2} \rightarrow SO_4^{-2}$	H_2S O_2 interface	Mud,hot spring,mining surface,acid mine,drainge ,soil	*Acidithiobacillus* *Sulfobacillus* *Thiomiicrospira* *Achromatium* *Beggiatoa* *Thermothrix*
Anaerobic phototrophs	$H_2S \rightarrow S^0$ $S^0 \rightarrow SO_4^{-2}$	Anaerobic H_2S light	Shallow water, anaerobic Sediments,meta or Hypolimnion, Anaerobic water	*Chlorobium* *Chromatium* *Ectothiorhodospira* *Thiopedia* *Rhodopseudomonas*

1.1.2.2.4. Factors affecting sulphur oxidation in the environment

Several factors affect sulphur oxidation in soil, including temperature, pH, moisture. The addition of sulphur fertilisers to soil can affect soil microbial factors such as the population of sulphur oxidising bacteria in soil (Janzen *et al.*,1982).

(i) ***Temperature:*** the optimum temperature range is between 27°C to 40°C; whereas, some thermopilic bacteria and fungi could also grow at 55°C (Wainwright, 1984).

(ii) ***pH:*** sulphur oxidation can take place over the pH range (pH 2 to pH 9). The sulphur oxidation process directly increases with increasing pH. (Vitolins and Swaby, 1969).

(iii)Moisture and aeration: the moisture content for most rapid sulphur oxidation processes is near field capacity, even though other factors such as soil texture can

effect this level. With the exception of *T. denitrificans* and some other photosynthetic bacteria, sulphur oxidation processes require environments with adequate oxygen levels (Mahfouz, 2005).

(iv) ***Soil microbial influence:*** Sulphur oxidation is highly affected by the size and composition of the soil microbial populations (Soomro, 2000).

1.1.3 The Phosphorus Cycle

In terms of its importance, the phosphorus cycle is second after the nitrogen cycle. Phosphorus is an essential component for all living organisms as it is a critical element involved in formation of adenosine triphosphate (ATP). ATP is involved in most biochemical processes in plants and enables them to extract nutrients from soil. Phosphorus plays a significant role in cell development and (DNA) deoxyribonucleic acid formation (Hyland *et al*., 2005). Principally, phosphorus is available in its oxidised state as orthophosphate mainly as complexes with calcium , iron, almonium and silicate minerals. Phosphorus rich minerals contain fluorapatite, hydroxyapatite, oxyapatite, wavellite and monetite (Paul and Clark, 1996). Phosphate residues in soil are large and often immobile, and can become unavaliable for plant uptake because of its adsorption, precipitation, or exchange of recalcitrant organic forms (Richardson, 1994). Excessive amounts of P in fresh water streams and lakes can cause damaging algal blooms: When algae die, their decomposition results in oxygen depletion which can lead to the death of aquatic plants and animals; this process is called “eutrophication” (Hyland *et al*., 2005). The cycling of phosphorus occurs between inorganic and organic forms; these processes often actively involve actions of microoganisms.

1.1.3.1. Role of microorganisms in the phosphorus cycle

Microorganisms have been acknowledged to transform phosphorus into available sources in a number of ways (Fig 1. 4) as follows:

(i) By altering the solubility of inorganic compounds of phosphorus,

(ii) By mineralising of organic phosphate compounds into inorganic phosphates,

(iii) By converting of inorganic, available anion into cell components (i.e. an immobilisation process), and

(iv) By oxidising or reducing phosphorus compounds; these are however, very limited processes.

Of these mineralisation and immobilisation are the most important reactions/processes in phosphorus cycle, soil phosphates are made available through the secretion of organic acids, (lactic, acetic, formic, fumaric, succinic acids) .Thus, phosphate-dissolving/solubilising soil microorganisms (include *Pseudomonas, Bacillus, Micrococcus, Mycobacterium, Flavobacterium, Penicillium, Aspergillus,* and *Fusarium*) play an important role in reducing phosphorus deficiency of crop plants. Soil phosphorus solubilisation varies according to pH; neutral or alkaline soils prove the most advantageous, as well as, temperature and moisture affected phosphorus solubilisation by microorganisms. The soil biomass phosphorus content could increase with addition of calcium phosphates. Soil microorganisms can derive a significant proportion of their requirement through the mineralisation of phosphorus (Thien and Myers, 1992).

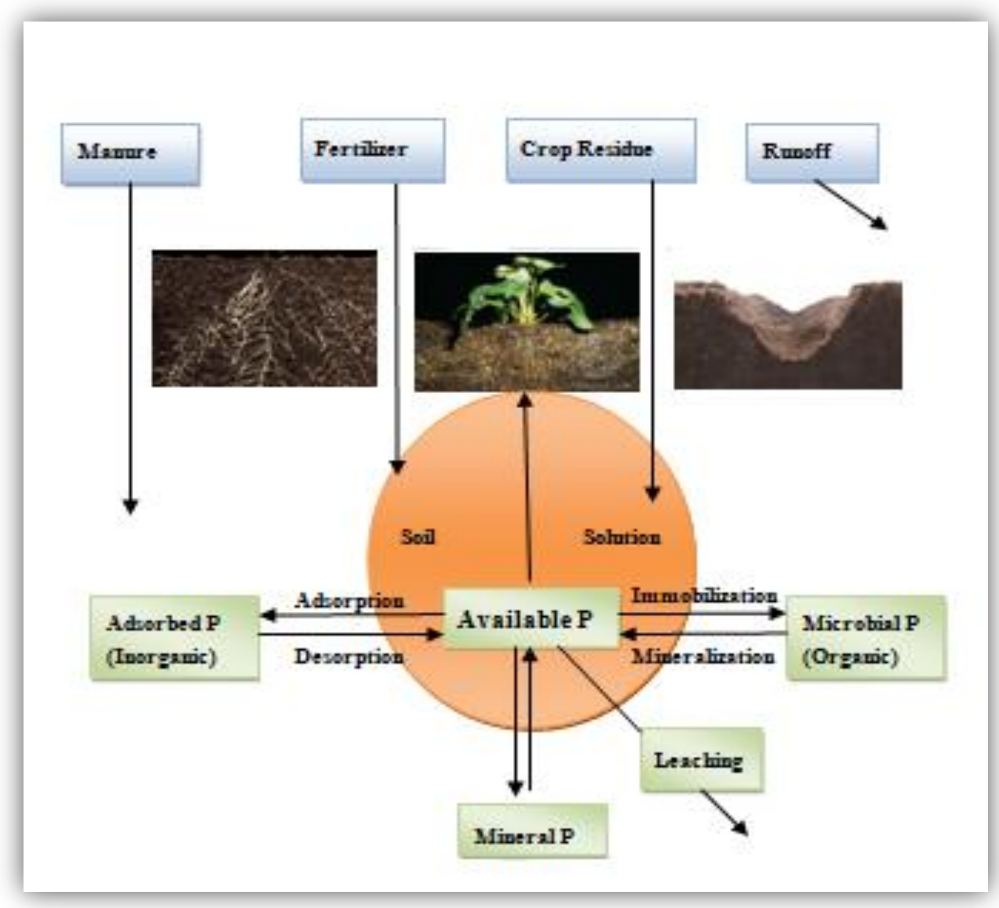


Figure 1.4: The Phosphorus Cycle. (*Reproduced and updated from Hyland et al., 2005*) Used with permissions.

1.1.3.2. Microbial solubilisation of inorganic phosphorus

In soil, phosphorus can be divided into two categories: soluble and insoluble. Insoluble phosphorus is unavailable to plants and microorganisms, which generally comprises of approximately 95-99% of the total P in soil. Insoluble form of inorganic phosphate mainly compounds with iron, calcium and aluminium in different soil types. Numerous microorganisms can solubilise insoluble inorganic compounds of phosphorus. Many species of bacteria as well as some species of actinomycetes and fungi have the ability to solubilise phosphate (Hattori, 1973; Paul and Clark, 1996); P-solubilizing fungi include *Aspergillus, Fusarium, Penicillium* and *Sclerotium* (Alexander, 1977). These fungi grow in medium with calcium phosphate apatite or with similar insoluble resources as a sole phosphate source (Al-Turk, 1990).

Insoluble P is solubilised by reaction with organic acids; this reaction transfers insoluble phosphorus into a dibasic and monobasic phosphates. Certain species of bacteria can liberate hydrogen sulphide, which finally reacts with ferric phosphate to produce ferrous sulphide and soluble phosphate (Hattori, 1973).

1.1.3.3. Mineralisation and immobilisation of phosphorus

Mineralisation is the microbial conversion of organic phosphate to dihydrogen ($H_2PO_4^-$) or monohydrogen (HPO_4^{-2}) phosphate ion forms. In plant, the available phosphate form is identified as orthophosphates (Hyland *et al*., 2005).

The process of mineralisation is mediated by enzymes mainly phosphatase and phytases. These enzymes are hypothesised to hydrolyse the carbon oxygen phosphorous (C-O-P) ester bounds through mineralisation of soil organic material (Sharpley, 1999). Soil microorganisms produce these enzymes in soil; and therefore, soil microorganisms play an important role in the cycling of phosphorus in soils (Dinkelaker and Marschner, 1992; He, 1998). Some species of fungi (e.g. *Aspergillus* and *Penicillium* Spp) and other bacterial species (e.g. *Actinomycetes, Pseudomonas,* and *Bacillus* Spp) can produce the enzymes involved in the mineralisation of organic P (Richardson and Simpson, 2011). There are several factors that affect the process of mineralisation of organic phosphate, which include temperature (the best temperature range is between 18°C to 40°C), soil pH (the optimum accessibility of orthophosphate occurs at a soil pH 6.5), moisture, aeration, cultivation, presence of growing plants, and fertiliser phosphate additions (Hyland *et al*., 2005; Mahfouz, 2005). Phosphorus is important element for microbial growth, and the absorption of phosphorus into microbial protoplasm leads to the accumulation of non-utilisable forms of the element and making it unavailable for plant growth (Stewart and Tiessen, 1987). In fact, plants themselves present a wide range of root morphological

and physiological changes in relation to phosphorus deficiency (Vance *et al.*, 2003; Richardson *et al.*, 2009). Microorganisms are essential components in the cycling process of soil phosphorus; and the localised improvement of microbial activity in the rhizosphere has important implication for the phosphorus nutrition of plants (Sharpley, 2000; Richardson and Simpson, 2011).

1.1.3.4. Ability of microbes to solubilise insoluble phosphates

Various microorganisms play a significant role in dissolving insoluble inorganic phosphates in the soil and converting them into forms which can be utilized by plants (Table 1.8). A wide variety of heterotrophic microorganisms are capable of solubilising P from different sources. P is assimilated by these microbes which also solubilise a large proportion of the insoluble inorganic P releasing it for use by other organisms. Various chemolithotrophic microbes e.g. *Nitrosomonas* and *Thiobacillus* mobilise inorganic P through producing nitrous and sulphuric acids, respectively (Tiessen and Stewart 1985).

Table 1.8: Microorganisms and sources of phosphate which have been reported to be involved in phosphate solubilisation (*Reproduced and updated from Subba, 1982; Rodriguez and Fraga, 1999 and Chen et al., 2006).*

Microorganisms	Phosphate sources
Bacteria: *Bacillus sp* *Pseudomonas sp* *Erwinia sp* *Escherichia sp* *Serratia spp* *Xanthomonase spp* *Flavobacterium spp* *Alcaligenes spp* *Achrombacter sp* *Aerobacter aerogenoes* *Micrococcus sp* *Nitrosomonas spp* *Thiobacillus thiooxidans* *Rizobium sp* *Burkholderia sp*	**Mineral:** Tricalcium phosphate Calcium phosphate Iron phosphate Hydroxayapatite Fluorapatite Rock phosphate
Fungi: *Asprigillus sp* *Penicillum sp* *Fusarium sp* *Pythium sp* *cladosporium sp* *Rhizoctonia sp* *Candida sp* *Phoma sp* *Schwanniomces sp* *Pseudogymnoascus sp* *Rodotorula sp* *Humicola sp* *Paecilomyces sp* *Mycorrhiza*	**Organic:** Calcium phytate Calcium glycerophosphate Phytin Lecithin Phenol 1 phosphate
Actinomycetes: *Streptomyces sp*	

1.2. Desert varnish

Desert varnish has been found on rocks surfaces in arid and semi-arid environments, and the term “rock varnish” is extended to describe similar surface coverings found in alpine regions, glacial deposits, streams and intertidal waters. An understanding of “Rock varnish” is thought to be crucial to understanding the origin of life on earth and perhaps on other planets.

Desert varnish or “rock varnish” is a dark, thin veneer (film), which forms on rock surfaces and the surface of desert soils which have remained undisturbed for thousands of years. Long periods of drought alternating with infrequent periods of heavy rain, extreme UV radiation and temperatures which often exceed 60°C are typical of the environment. Normally (5 – 50μm) thick, such coatings have been found in a variety of deserts (Paul, 2007; Kuhlman *et al*., 2006; Perry *et al*., 2004), including the Sonoran and Mojave Deserts of S. W. United States and Northern Mexico, the Gibson Desert and Great Victoria Desert of Western Australia, the Negev Desert in the Middle East and the Gobi Desert of China (Taylor-George *et al*., 1983).

Research work conducted on “Rock varnish” in streams began as early as 1850 and 1875 by Humboldt and Darwin, respectively (Staley *et al*., 1982). Studies on rock varnish in America began a little later (around the period 1875–1909) and involved researchers such as Loew, Merrill and Surr (Staley *et al*., 1992). However, their work often excluded details about the chemical makeup or origins of the investigated coating samples until 1921, when Francis presented the first credible and acceptable argument for the microbial origins of “rock varnish” (Francis, 1921). In his study, Francis observed black coatings of iron and manganese oxide on rock surfaces on both land and in streams from Australia, and suggested that they are

formed by lichens and algae. Three years later, it was shown that small lichens coatings (0.2 mm average diameter) occur on rock surfaces (Staley *et al*., 1992), and later, Laudermilk (1931) proposed a model for varnish formation involving the dissolution of minerals in rocks by the action of lichen acids. Moreover, it was suggested that minerals would also be absorbed from rocks by lichens leading to the precipitation of iron and manganese hydroxides. This build-up of iron and manganese oxides eventually kills the lichens, and produce acids which help to spread the minerals on the rock surface thereby produce "rock varnish" (Stotzky and Bollag, 1992).

The most common biological forms seen (by the use of either light microscope or scanning electron microscopes) in "rock varnish" are micro colonial fungi (MCF) (Taylor-George *et al*., 1983). Bacteria are rarely seen, even with SEM, perhaps because of:

i) The relatively small field of view at the high magnifications required,
ii) The difficulty of distinguishing bacteria from other particles present, or
iii) Because critical point drying was not used during sample preparation (Dorn *et al*., 1981).

1.2.1. Chemical composition of desert varnish

Desert varnish is mainly composed of oxygen, silicon and aluminum, with small amounts of iron, sodium, manganese, sulphur, calcium, nitrogen, magnesium, potassium, phosphorus, titanium, carbon and barium; rock varnish can also contain amino acids and lipids (Perry *et al*., 2005). The presence of serine was also reported in samples taken from Death Valley, California; this amino acid may also be present in/on the clay matrix or in the amorphous silicate matrix of desert varnish. DNA may also exist in desert varnish silica, or complex Si–O–C bonds, or as complexes

with iron or manganese (Perry *et al*., 2004). Manganese (II) is also often present, this being the soluble form of the element which is available to microorganisms and is stable between pH 6 – 9. Manganese (III) and manganese (IV) form insoluble oxides and oxyhydroxides; microbial manganese (II) oxidation likely accounts for the occurrence of the mineral phase of rock varnish (Ehrlich, 1996). Manganese and iron oxidation takes place at the exterior of the bacterial cell surface and iron hydroxides are often deposited on the remains of biogenic structures. Ferric hydroxide has also been identified as extracellular deposits on iron-oxidising organisms (van Veen *et al*., 1978).

1.2.2. Organic constituents of desert varnish

Various amino acids have been observed in rock varnish samples collected from the Whipple Mountains, California, (Perry *et al*., 2004); suggesting that an intimate association between the bacteria and the varnish material. A large variety of bacteria have also been cultured from rock varnish, including *Bacillus, Geodermatophilus, Arthrobacter, Micrococcus, Curtobacterium, Cellamonas,* (Hungate *et al*., 1987), *Pedomicrobium* and *Metallogenium*-type strain (Dorn, 1981). Numerous *Actinomycetes*, including *Geodermatophilus* have also been isolated (Eppard *et al*., 1996). Three strains of bacteria isolated from varnish from Death Valley, California, were identified as being resistant to UV exposure (for up to 5 minutes) (Khulman *et al*., 2006); one strain showing a 98% 16SrRNA sequence similarity with multiple *Arthrobacter* strains; the second showed similarities with strains of *Curtobacterium flaccumfaciens,* whilst the third strain matched with a *Geodermatophilus obscura*, cluster 1 (Eppard *et al*., 1996, Khulman *et al*., 2005).

1.2.3. Evidence for a biological origin of desert varnish

Investigations on microbial activity in Rock varnish from the Sonoran Desert found no detectable carbon dioxide fixation (measurements used ^{14}C–bicarbonate). This suggests that there was only little or no autotrophic activity by either phototrophic or chemolithotrophic bacteria. Interestingly, the investigated rocks also showed no evidence of lichen activity either, which goes against the theory that lichens are involved in the formation of Desert Varnish. The investigations found: 1) active respiration of radio-labeled sodium acetate, showing heterotrophic activity 2) actinomycete counts ranged from 400-600 cm^{-2} of varnish, and the counts for micro-colonial fungi ranged from 600-1500 cm^{-2} of varnish (Hungate *et al*., 1987). These results agreed with the findings obtained from the Negev Desert, which gave 250-2900 heterotrophic bacteria per cm^2. Counts from rock varnish were always consistently higher than from the surrounding area, suggesting that the organisms were indigenous to the varnish and not carried from the surrounding area by the wind (Taylor-George *et al*., 1983).

It has been repeatedly argued that since "rock varnish" contains oxides of iron and manganese, together with microorganisms; then, microorganisms ought to be somehow involved in depositing the oxides in the varnish, a hypothesis which is widely highly accepted, but needs verification.

Palmer *et al*. (1914) found that bacterial colonies from Sonoran and Mojave Desert were gram-positive (e.g. *Micrococcus, Planococcus, Arthrobacter* and *Bacillus*). A study from the Negev desert has reported similar findings with the isolation of species *Dermatophilus* being reported (Plamer *et al*., 1986). These bacteria were not thought to be capable of gaining energy from manganese oxidation, nor able to fix carbon dioxide, and must therefore be heterotrophs. This finding is in full agreement with the reported results from studies conducted on the Sonoran

Desert, where no primary productivity could be found using radio-labeled carbon dioxide as a carbon source (Taylor-George *et al*., 1983). It is assumed, therefore, that these microorganisms obtain their organic nutrients from windblown dust which also delivers iron, manganese and clay.

Fungi have also been found in "Rock varnish*",* appearing as black colonies of approximately 100 µm in diameter and like bacteria, they rely upon wind-blown organic carbon (Staley *et al*., 1982).

Manganese oxidising bacteria and manganese depositing micro-colonial fungi have been reported to be capable of producing an artificial varnish under laboratory conditions. However, no experiments appear to have exactly reproduced the natural conditions under which "rock varnish" has formed. Since microorganisms grow very slowly in desert environments and rock varnish also develops very slowly it may not be possible to conduct such a conclusive experiment in the laboratory. Even if such an experiment were possible it would not explain a) the formation of the iron deposits, b) the involvement or not of microorganisms, or c) show the ways in which these organisms interact with clays to form varnish (Stotzky and Bollag, 1992).

1.2.4. Does desert varnish exist on Mars?

Shiny rock surfaces found on Mars may constitute a kind of rock varnish and infra red images taken for these rocks confirmed that such varnish is not part of the rock, but it is an extra coating (Di-Gregorio, 2010). All the required parameters for rock varnish production exist on Mars, including harsh UV radiation, low atmospheric pressure (0.6-0.8 KPa), very cold and strongly oxidising conditions; Mars is also likely to be free of the factors which erode rock varnish such as rain or lichens (Parnell *et al*., 2007). Current Mars rovers are fitted with an alpha proton X-ray spectrometer, which fires alpha particles and X-rays at rock surfaces to detect which

chemical elements are present; despite this, evidence for the presence of manganese oxide has proven elusive (Horneck and Baumstark-Khan, 2001; Di-Gregorio, 2010).

1.3. Molecular biology techniques

The rapid development of molecular techniques throughout the past decade has revolutionised the field of microbiology. The discovery of phylogenetically informative DNA sequences such as the 16S rRNA gene completely transformed the theory of microorganisms' relatedness, and provided a universal system for the identification and classification of microbes. It is also now possible to detect, identify microorganisms independent of their cultivability and by these means to determine the diversity and spatial organization of complex microbial communities (Amann *et al*., 1995; Hill *et al*., 2000; Kirk *et al*., 2004 and Rogers, 2008). Microbial diversity studies basically rely on results derived from tests carried out on samples of soil ranging from (1-5g) and it is very difficult to predict spatial distribution on a macro scale from such a small sample size, which may be of unknown heterogeneity (Wintzingerode *et al*., 1997).

1.3.1. Deoxyribonucleic acid (DNA)

DNA is double stranded with two polymeric molecules joined by hydrogen bonding between bases. The strands are arranged in a double helix with major and minor grooves. DNA containing nucleotides consist of a phosphate group, pentose sugar (deoxyribose) and nitrogen containing base pyrimidines cytosine (C), thymine (T) and purine guanine (G), adenine (A) (Fig 1.5) (Pary, 2008; Baker *et al*., 2006). Guanine binds only to cytosine and adenine binds only to thymine to form equal sized base pairs (Maier *et al*., 2009).

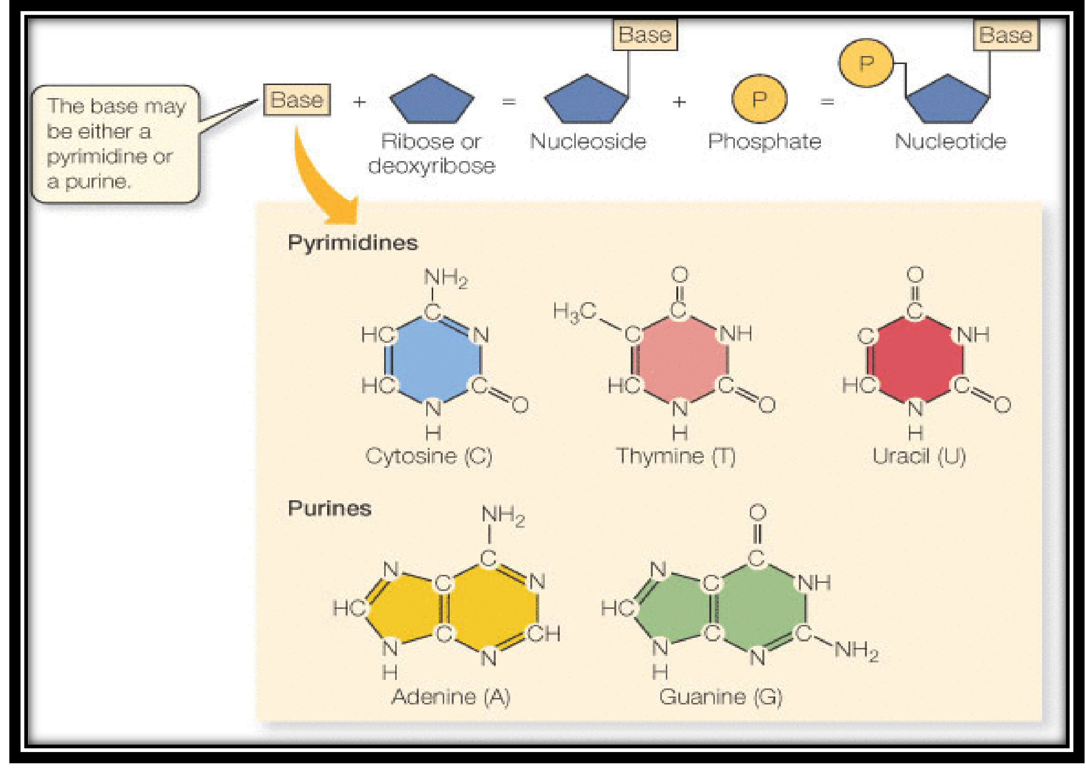


Figure 1.5: Structures of pyrimidine and purine bases in DNA (*Reproduced and updated from Pray, 2008*) Used with permissions.

One strand of DNA is oriented 5` to 3`, while the complementary strand is oriented 3` to 5`. The two strands in resulting double-stranded molecule arranged in an antiparallel fashion (Madigan *et al.*, 2012), which means that the 5` end of one strand is paired with 3` end of its opposite strand (Pary, 2008). The nitrogen bases of each strand forms the inner pare of the double helix while the sugar-phosphate backbone is the outer part (Al'Abri, 2011).

1.3.2. Polymerase chain reaction (PCR) technique

Today, PCR is widely used in many branches of genetic research. PCR was discovered in the mid 1980s by Kerry Mullis who received a Nobel Prize for chemistry in 1993 for this invention (Kubista *et al.*, 2006). The PCR is a molecular technique which is used *in vitro* to amplify the amount of target DNA from trace amounts in a complex mixture of templates. The technique allows for the

multiplication of more than million copies of the target DNA within a few hours (e.g. 10^6 to 10^9 double amplification within 3-4 hours) (Hadidi and Candresse, 2003).

PCR can be advantageous over conventional methods; it is, for example, extremely sensitive, fast and easy to obtain results in only a few hours compared to traditional techniques. In addition, PCR is culture independent technique allowing access to the genomic information from non-culturable microorganisms. Furthermore, presently, PCR determination is applicable in almost all areas of disease identification and to the discovery of pathogens (Hadidi and Candresse, 2003; Maier *et al*., 2009).

PCR is performed in three main stages (e.g. denaturation, annealing and extension) in small thin-walled microcentrifuge tubes (Fig 1.6) (Anderson, 2011). The composition of the PCR solution in the tube consists of components of reaction as shown in Table 1.9 (Baker *et al*., 2006). PCR uses repeated cycles of heating and cooling to produce many copies of a specific region of DNA. The cycle of PCR has three steps: The first step involves the denaturation of the double strand DNA into two single strands of template DNA, achieved by raising the temperature to 94°C. After around 20 to 40 seconds, the block is rapidly cooled to 50 to 70°C for 30 seconds which allows the oligonuclotide primers to anneal to the single template; this is the second step. And finally, the third step involves incubation at a slightly higher temperature (e.g. 72-74°C); in which the enzyme Taq polymerase binds to the primer sequences and adds nucleotides to extend the second strand, also Taq adds bases to the 3`end of both primers extending the DNA sequence in the 5` to 3` directions (Hadidi and Candresse, 2003; Maier *et al*., 2009 and Baker *et al.,* 2006). The previous processes require that the reaction mixture be annealed (i.e. cycled between very high and very low temperatures). When this technique was first invented, the

polymerase was obtained from microbes which could not withstand the high temperatures involved and had to be replenished after every cycle. In order to solve this problem, *Thermus aquaticus* acquired from a stock of samples deposited by Brock some 20 years earlier and a heat stable DNA polymerase was then isolated from it, (Taq polymerase); its tolerance to heat then led to the total automation of the PCR process. In later years, Taq polymerase has sometimes been replaced by Pfu polymerase, extracted from *Pyrococcus furiossus*, which works best at 100°C (Madigan *et al*., 2012). In the PCR reaction, the contamination or non specific priming can lead to false positive results. PCR is also subject to inhibition due to humic substances and metals that may present in environmental samples.

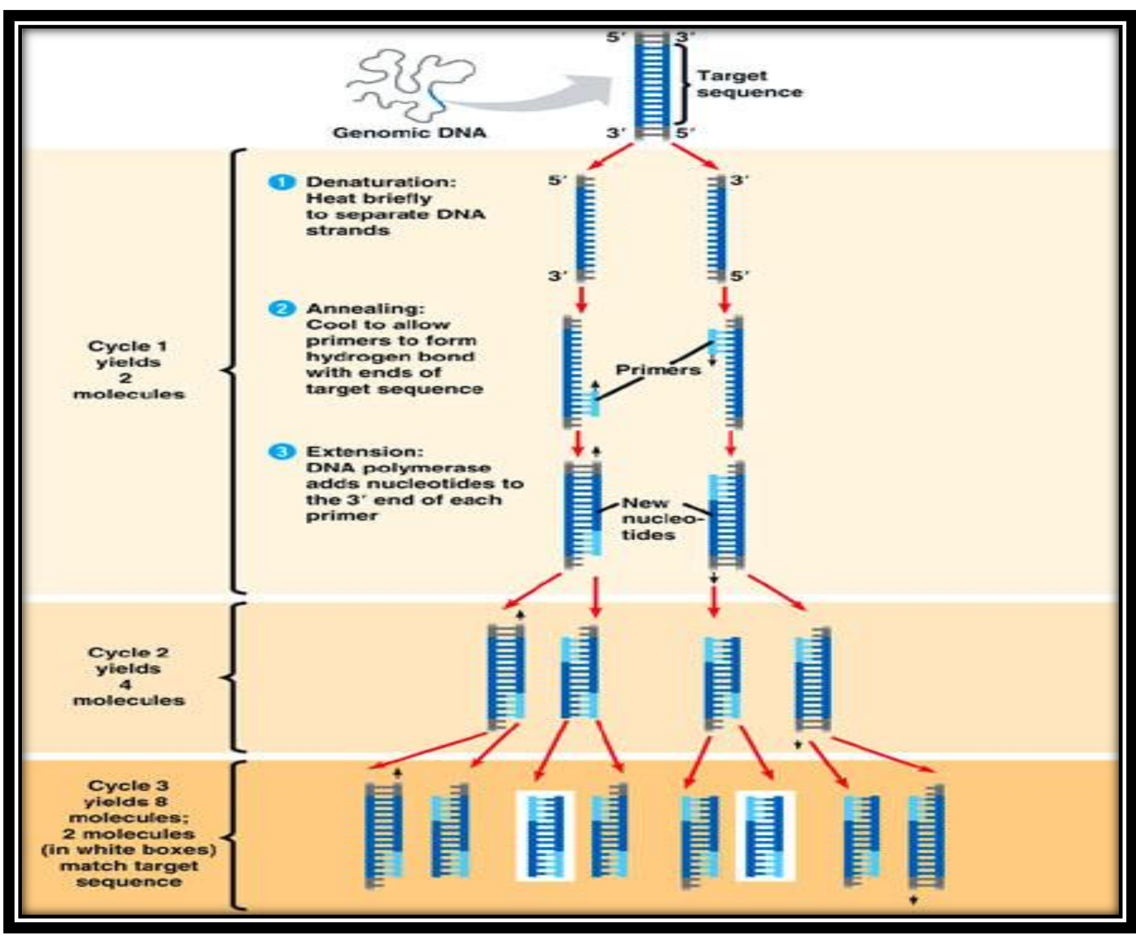


Figure 1.6: The Polymerase Chain Reaction (PCR) cycle. (*Reproduced and updated from Anderson, 2011*) used with permissions.

Table 1.9: Components of the PCR reaction (*Reproduced and updated from Baker et al., 2006).*

PCR Solution Component	Approximate Concentration	Function
Buffer	25-100 mM	Maintains pH at optimum for Taq activity
Magnesium ions	1-5 mM	Essential for Taq activity, binds to template. oligonucleotides and dNTPs
Oligonucleotides Primers (forward and reverse)	In excess	Prime the polymerization. Sequence governs specificity of the PCR
dNTPs (Deoxnucleoside triphosphates)	In excess	Substrate for polymerase
Taq polymerase	1 unit per 100 µL	Enzymatic addition of dNTPs complementary to template
Template	As little as one copy	DNA to be amplified
Water	n/a	Added to bring other component to the correct concentration

1.3.3. The 16S rRNA gene

The 16S rRNA gene has been widely used to study prokaryote diversity and allows for the identification and prediction of phylogenetic relationships (Pace, 1999); rRNA refers to the ribosomal RNA. A ribosome consists of two subunits RNAs and ribosomal proteins. The large subunit (50S) contains 5S and 23S rRNA and 31 proteins, while the small subunit (30S) contains 16S rRNA and 21 proteins (Madigan *et al*., 2012). The 5S rRNA (about 120 nucleotides) has also been widely studied but is too small for reliable phylogenic classifications; 16S (approximately1500 nucleotides) and 23S (roughly 2900 nucleotides); rRNA is however, large enough to be useful (Olsen *et al*., 1986; Madigan *et al*., 2009) and is

the least variable gene in all cells. Sections of rDNA sequence from even distantly related organisms are remarkable similar, hence sequences from distantly related organisms can be precisely aligned, making any differences easy to measure. For this reason, genes that contain the rRNA have been used to determine the taxonomy, phylogenicity and rates of divergence amongst bacteria; 16S rRNA sequences can also be studied to reveal relatedness. This work was begun by Carl Woese at the University Of Illinois, during 1970's; he also proposed the three domain system of microorganism classification, namely–Archaea, Bacteria and Eucarya (Woese *et al*., 1990). Not until almost 20 years later however, did his classification gain complete acceptance (Newman *et al*., 2007). 16S rRNA sequences have hyper-variable regions where sequences have diverged over time and these are often flanked by strongly conserved regions. Primers target are conserved regions and amplify variable regions. The DNA sequence of 16S rRNA gene has been determined for a large number of species, forming a readily accessible and extensive library. Carl Woese recognized the broader potential of rRNA sequencing as a measure of phylogenic relatedness. He initially used a method that compared about ¼ of the nucleotides in 16SrRNA (i.e. the best that could be done with the technology available at the time). By using more advanced techniques, it has now become routine to determine the whole sequence of 16S rRNA molecules, resulting in the greatest accumulation of data available for inferring relationships amongst organisms (Boye *et al*., 1999). 18S rDNA and (ITS) internal transcribed spacer regions have been extensively used to study fungal classes. Nevertheless, the available databases are not as extensive as those for prokaryotes (Prosser, 2002). DNA is extracted from the sample and purified the target DNA (16S) is amplified using universal or specific primers; the resultant products are separated based on necessity (Kirk *et al*., 2004).

1.3.4. Phylogenetic analyses

Phylogenetic analyses have become important for researching evolutionary trees in molecular biology, clinical research, evolutionary studies and plant pathology. Phylogenetics is the study of evolutionary relatedness between groups of organisms which is revealed during molecular sequencing data and morphological data matrices. In addition, phylogenetic analyses are useful for organizing information of biological diversity for structuring, classification and for evolutionary studies (Daum, 2008). 16S rRNA gene sequencing is the most general use of databases for the identification of microorganisms in environmental microbiology. A number of computer software sequencing analysis program packages are available to assist in sequences searches [e.g. (NCBI) National Center for Biotechnology Information and (BLAST) Basic Local Alignment Sequence Tool]; these search programs are used in phylogenetic analysis to identify genetic relationships and heritage (Holmes, 2003; Maier *et al*., 2009).

1.4. Extremophilic microorganisms

Extremophiles are microorganisms which are grouped according to the conditions under which they flourish (see Table 1.10), for example: thermophiles, psychrophiles, halophiles, acidophiles and alkaliphiles, all of which have been widely studied in the last 50, or so, years (Hendry, 2006, Rothschild and Mancinelli, 2001, Rothschild, 2002, Oarga, 2009).

Extremozymes, which help extremophiles to flourish in harsh environments, are of particular interest; For example proteases and lipases, from psychrophiles, are active at low temperature; amylases are used for acidic starch degradation and are produced as secretion from acidophiles; proteases for detergent applications can be produced by alkaliphiles, while finally, halophiles produce enzymes which are active

in non-aqueous solvents (Madigan and Marrs, 1997; van den Burg, 2003 and Hendry, 2006).

Table 1.10: Classification and examples of extremophiles (*Reproduced and modified from Rothschild and Mancinelli, 2001; van den Burg, 2003; Irwin and Baird, 2004*).

Environmental parameter	**Type**	**Characterisation**	**Examples**
Temperature	Hyperthermophile Thermophile Mesophile Psychrophile	Grows at > 80°C 60 - 80°C 15 - 60°C < 15°C	*Pyrolobus fumarii* *Synechococcus lividis* *Homo sapiens* *Psychrobacter, some insects*
pH	Alkaliphile Acidophile	pH > 9 pH < 2	*Natronobacterium* *Bacillus firmus OF4* *Spirulina sp* (all pH 10) *Cyanidium caldarium* *Ferroplasma sp* (both pH 0)
Salinity	Halophile	Salt-loving (2-5 M NaCl)	*Halobacteriacae* *Dunaliella salina*
Radiation	Radioresistant		*Deinococcus radiodurans*
Pressure	Barophile Piezophile	Pressure-loving Pressure-loving	Unknown microbe,130MPa
Oxygen Tension	Anaerobe Microaerophile Aerobe	Cannot tolerate O_2 Tolerates some O_2 Requires O_2	*Methanococcus jannaschii* *Clostridium* *H. sapiens*
Chemical Extremes	Gasses Metals	Metalotolerant	*C.caldarium* *Ferroplasma acidarmanus* (Cu, As, Cd, Zn); *Ralstonia* sp. CH_{34} (Zn, Co, Cd, Hg)

Advanced research on extremophiles has also helped in redrawing the evolutionary tree (Fig 1.7). In the past, it was widely accepted that living creatures could be classified as either bacteria, simple cells lacking a nucleus, and eukarya, whose cells contain nucleus and are more complex (e.g. plants, animals, fungi and protists). Further studies on extremophiles eventually led to the inclusion of a third branch of the tree–the archaea. Similar to bacteria in many ways, archaea in many ways also resemble eukarya; however, they exhibit many unique genes, which suggest that they be included as a separate and third branch of the evolutionary tree (Madigan and Marrs, 1997; Madigan *et al*., 2012). Extremophiles, which flourish at temperatures exceeding 60°C, were first discovered in the modern era in the late 1960's by Thomas Brock and his colleagues. Firstly, *Thermus aquaticus* was identified and named and subsequently led to important and the widespread use of polymerase chain reactions (PCR). Brock's team also found Archaean organisms flourishing in acidic environments, in temperatures as high as 80°C, suggesting that microbes can exist in boiling water (Madigan and Marrs, 1997).

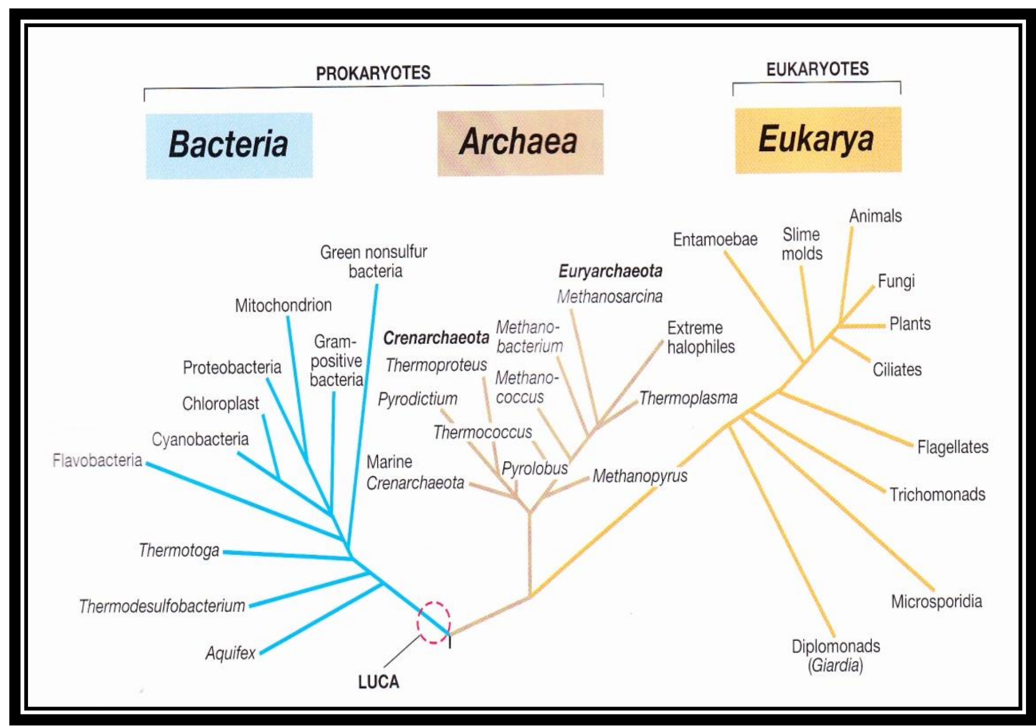


Figure 1.7: Universal phylogenetic tree as determined from comparative rRNA gene (16S or 18S RNA) sequence analysis (*This figure was reproduced and updated from Figure 16.16 in Madigan et al., 2012 and Oren, 2008*). Used with permissions.

1.4.1. Extremes of temperature

Based on extreme temperatures, microorganisms can be divided into three main groups *viz*: psychrophiles (optimal growth temperature ≤ 15°C, maximum 20°C, minimum ≥ 0°C), thermophiles (Growth 60-80°C) and hyperthermophiles (Growth > 80°C) (Magan, 2007; Prieur, 2007).

Basically, thermophiles have stable proteins and cell membranes, whereas psychrophiles have flexible cellular proteins and membranes or contain antifreeze proteins (Irwin and Baird, 2004). Microorganisms posses a number of mechanisms which allow them to survive at temperatures that would usually denaturate proteins, and cell membranes (de Miguel Bouza *et al*., 2006). Most of hyper-thermophiles belong to the archaea. They are capable of growing at temperatures above 90°C and

up to 113°C; cell membranes in archaea are diverse in structure from those of bacteria (Maier *et al*., 2009; Irwin and Baird, 2004).

Numerous thermophilic bacteria are phototrophic bacteria such as cyanobacteria, purple and green bacteria (Seckbach *et al*., 2007) and eubacteria e.g. *Bacillus, Clostridium, Thiobacillus, Desulfatomaculum, Thermos,* lactic acid bacteria, actinomycetes, spirochetes (Rothschild, 2002). Hyper-thermophiles include: for instance *Acidianus infernos, Pyrolobus fumarii, Methanococcus jannaschii, Thermococcus littoralis, Pyrococcus furiosus* (Ghosh *et al*., 2003).

Various species of thermophilic fungi belong to Hyphomycetes, including *Thermoascus thermoplilum, Malbranchea cinnamomea* and Ascomycetes like *Thermoasscus aurantiacus, Thielavia terrestris,* and Zygomycetes for example *Rizomucor miehei, Rizomucor pusillus.* These hyper-thermophilic fungi have been isolated from composts, soil, wood chips and nesting materials of birds. Additionally, a few thermophilic algae e.g. *Achanthes exigua, Mougeotia sp* and protozoa including *Cerosulcifer hamathensis* and *Cyclidium citrullus* can grow at high temperatures (Satyanarayana *et al*., 2005).

1.4.2. Extremes of salinity

Halophiles are extremophile microorganisms that flourish in environments with very high concentrations of salt. They are adapted to life at high salt concentration and are found in all of the three domains of life: Archaea, Bacteria and Eucarya (Ma, *et al*., 2010 and Madigan, 2005). Based on their NaCl requirements, halophilic microorganisms have been classified into three groups: i) halotolerant: which grow in the range of 2 to 5% NaCl[0.34 - 0.85M] NaCl, and the optimum growth range is between 0 and 0.3M NaCl; ii) moderately halophilic: which can grow in a range of 5 to 20 % NaCl [0.85-3.4M] NaCl, and the optimum growth range is between 0.2M

and 2.0M NaCl; and iii) extremely halophilic: that can grow in the range of 20 to 30 % NaCl [3.4-5.5M] NaCl with an optimum growth lies between 3.4-5.1 M NaCl (Gilmour, 1990; Ollivier, *et al*., 1994; Madern and Zaccai, 2004; Echigo, 2005).

Halophilic microorganisms are able to grow in high salt environments and have been found in almost all hypersaline environments. Extremely halophiles organisms can survive at salt concentrations as high as 10 times greater than the saline salt content of normal ocean water. Hypersaline environments are common all over the world, such as the Great Salt Lake in Utah (USA) (concentration of Na 105.3 g/l), the Dead Sea in Jordan which contains salt concentration of about 217 g/l, the Owens Lake (California), and the African Soda Lakes (e.g. Hamara Lake in Wadi El Natroun, Egypt); extremely hypersaline environments are however, uncommon (Margesin and Schinner, 2001; Madigan *et al*., 2012).

Extremely salty environments (of about 30% w/v NaCl) can support extreme halophilic microorganisms (Irwin, 2010). Grant *et al*., (1998) carried out a review on the diversity of halophilic archaea and bacteria and concluded that phototrophic bacteria occur below the cyanobacterial layers in anaerobic, but lit, zones in hypersaline microbial mats (Satyanarayana *et al*., 2005).

Gram-negative aerobic bacteria such as *Marinococcus, Salinococcus, Sporosarcina* and *Bacillus* have been isolated from various hypersaline environments. Globally, natural hypersaline waters contain halobacteria belonging to the archaea genus for example *Haloarcula, Haloterrigena, Halococcus, Haloferax, Halobacterium, Halobaculum,* and *Halorubrum.* Several hypersaline locations show evidence of methanogens, like *Methanohalophilus mahii, Methanohalophilus halophilus* and *Methanohalophilus evestigatum* (Kirkwood *et al*., 2008)

Fungal halophiles are less salt tolerant than that most halophilic bacteria and archaea. The halophilic and halotolerant fungi and yeast include the black yeasts *Hortaea werneckii,* which grows in hypersaline environments containing up to 5M NaCl, *Wallemia ichthyophaga,* requires at least 1.5 M NaCl and *Aureobasidium pullulans* and grow in hypersaline environments which contain up to 3 M NaCl (Gunde *et al*., 2009).

At moderately high salinities, green algae of the genus *Dunaliella* (*D. salina, parva* and *viridis*) have been reported. Moreover, a variety of species of diatoms such as *Amphora coffeaeformis* and *Navicula*, as well as protozoa such as *Porodon utahensis* and *Fabrea salina* have also been isolated from hypersaline environments (Cavicchioli *et al*., 2011). In hypersaline lakes, cyanobacteria are dominant including: *Aphanothece halophytica, Dactylococcopsis salinna*, *Microcoleus chthonoplastes, Phormidium ambiguum, Oscillatoria neglecta, limnetica* and *salina* (Cavicchioli and Thomas, 2004).

High osmolarity in hypersaline conditions can be deleterious to cells, while water is lost to the external medium until osmotic equilibrium is achieved. Generally, halophiles accumulate high organic solute concentrations inside the cytoplasm to prevent loss of cellular water under these conditions (Baati *et al*., 2010; Galinski, 1993). When the isoosmotic balance of the medium is achieved, cell volume is maintained.

1.4.3. Extremes of pH

Acidophiles are defined as the organisms which live at low pH (Irwin, 2010) and include certain types of bacteria, archaea and eukaryotes. Acidophilic microorganisms have been classified into two groups: Acidophilic tolerant (the optimal growth between 0-2 pH) and moderate acidophiles (the optimal growth

between pH 3 to pH 5) (Baker-Austin and Dopson, 2007). Various acidophilic microorganisms have been isolated from a range of acidic environments such as acid mine drainage, in South Africa, pH (0 - 1) (Akcil and Koldas, 2006) and the Rio Tinto River, Spain pH (1.5 – 3.1) (Gonzalez-Toril *et al.*, 2003). Unicellular red algae *Cyanidium caldarium* and the green algae *Dunaliella acidophila*, can grow at pH1, as can some fungi (e.g. *Acontium cylatium, Cephalosporium sp* and *Trichosporon cerebriae*), which grow at pH 0 (Rothschild, 2002). The archaea, such as *Picropilus oshimae* and *Picropilus torridus*, have been isolated at pH 0.7 and 60°C; and *Sulfolobus acidocaldarius* has been isolated at pH 3 and at 80°C (Johanson and Hallberg, 2003). Acidophilic bacteria such as *Leptospirillum ssp, Acidithiobacillus ferrooxidans, Acidiphilium spp, Ferrimicrobium acidiphilum* and *Thermoplasma acidophilum,* have been isolated at pH 0.5-4.0 *and* 55-60°C (Gonzalez-Toril *et al.*, 2003).

Although high acid concentrations can normally break cell membranes and destroy cells, numerous specific mechanisms have been adopted by acidophiles to maintain their internal cellular pH at a neutral level (usually 7.2). These mechanisms are demonstrated into two pathways: "passive" regulation (no energy is required to be exerted by the cell), and "active" regulation (which requires energy) (Booth, 1985).

Alkaliphiles have been classified as extremophiles which can live in high pH environments (e.g. pH > 9, usually between pH 10-12). However, they cannot grow below 9 or at neutral pH 6.5 (Horikoshi, 1999; Ulukanli and Digrak, 2001).

Alkaliphilic microorganisms occur in two groups: (a) haloalkaliphiles: which require high salinity of 33% w/v NaCl and an alkaline pH (e.g. pH > 9); (b) alkaliphiles: which necessitate alkaline pH of 9 or above (Engle *et al.*, 1995; Me *et*

al., 2004). A wide range of alkaliphilic microorganisms have been isolated from various alkaline environments, such as Soda Lakes in Egypt, the Rift Valley of East Africa and Western U.S.A. These locations are characterised by possessing high concentrations of sodium carbonate, magnesium and salinity (5-30 % w/v NaCl) (Le Borgne *et al*., 2008; Rese *et al*., 2004). Alkaliphilic microorganisms include Bacteria, Archaea and Eukaryotes (Mangan, 2007). Many species of alkalitolerant bacteria have now been isolated from alkaline environment such as *Bacillus pasteurii*, *Bacillus alcalophilus*, *Bacillus firmus* and *Chimaerecella alkaliphila; Bacillus firmus* is considered the most alkalitolerant bacterium (Kroll, 1990). Alkaliphilic microorganisms can be isolated from acid and normal neutral environment for example garden soil as well as from extremely alkaline saline environments (Horikoshi, 1996,1999), some species of alkaliphilic *Bacillus, Aeromonas, Micrococcus, Pseudomonas, Actinopolyspora, Paracoccus*; and *Corynebacterium* have been isolated from neutral soils (Satyanarayana *et al*., 2005), while strains of haloalkaliphilic cyanobacteria for example *Arthrospira platensis, Cyanospira rippka, Anabaenopsis sp, Spirulina sp,* and *Thiospirrilum* have been isolated from soda lakes (Kroll, 1990; Jones *et al*., 1998). Environments which are both highly saline and alkaline such as Lake Magadi in the Rift valley of East Africa, Wadi Natrum in Egypt and Owens Lake in California are red in colour because of the presence of haloalkaliphilic archaea, such as *Natronococcus occultus, Natronobacterium greroryi* and *Natronobacterium pharaonis* (Kroll, 1990; Satyanarayana *et al*., 2005).

1.4.4. Extremes of radiation

Radiation is divided into two groups: (a) radiation in the form of particles such as neutrons, protons, electrons, alpha particles and (b) radiation as electromagnetic waves such as X-rays, ultraviolet radiation, gamma rays, microwaves, radiowaves

and infrared (Rothschild and Mancinelli, 2001; Oarga, 2009). Radioresistance relates to organisms which can survive at high radiation levels and are tolerant of aridity and heavy metals (Irwin, 2010). The solar spectrum of ultraviolet radiation (UV) comprises three regions UV (A) the least harmful range from 320 to 400nm, followed by UV (B) which range from 290 to 320nm, and finally UV(C) which ranges from 200 to 290nm. UV(C) is a short wavelength ionising radiation, which can damage microorganisms such as bacteria, fungi, protozoa and viruses (Freifelder, 1987, Gascon *et al*., 1995, Rothschild, 2002).

Deinococcus radiodurans provides the best example of radioresistance, and can resist up to 20,000 Gy of gamma radiation and UV radiation (doses up to 1,000 joules per square meter) (Battista, 1997). Another example of radioresistant microorganisms is *Streptococcus mitis*, which is considered to be a multi-extremophile (Irwin and Baird, 2004). *Rubrobacter sp* and *Dunaliella bardawil* (a green alga) can also live at high levels of radiations (Ferreia *et al*., 1999) and the cyanobacterium *Synechococcus sp,* can survive exposure to 200 to 400 nm radiation (Seckbach and Oren, 2007). Finally, microbes have developed mechanisms to repair cell damage resulting from radiation effects (Oarga, 2009; Snider *et al*., 2009).

1.4.5. Extremophilic microorganisms in non-extreme environments

Various extremophilic microorganisms have been isolated from non-extreme environments, for example thermophilic microorganisms from hot environments, (e.g. *Geobacillus*) have been found in cold environments (Banat, 2004). Other examples include alkaliphilic microorganisms which have been isolated from neutral and acidic soil environments (Horikoshi, 1999); archaea which are generally linked with extreme environments have also recently been isolated from non-extreme environments (Ventosa, 2006). The culturing and isolation of a microbe from a

certain environment does not therefore always mean that microorganism is able to grow and reproduce there but may merely be able to survive in such conditions (Peters and Conrad 1995; Echigo *et al*., 2005; Al'Abri, 2011).

1.5. Adaptation of bacteria to different stresses

Most microorganisms can adapt to what are regarded by humans as stress, such as low or high temperatures, low or high pH, salinity and high UV stress environments (Beales, 2004; Rahman *et al*., 2004). At high temperatures, thermophilic microorganisms, can adapt by increasing protein stability e.g. by increasing the number of disulfide bridges, exchanges between aromatic peptides and hydrogen bonding between peptides (Elnasser *et al*., 2007; Maier *et al*., 2009). Thermophilic microorganisms also produce solutes (e.g. di-inositol phosphate, diglycerol phosphate and mannosylglycerate) which help stabilize proteins against thermal degradation (de Miguel Bouzas *et al*., 2006; Madigan *et al*., 2012). Acidophilic microorganisms have two mechanisms to enable them to grow and survive in acid conditions (passive and active regulation) in "passive" pH regulation; the mechanism primarily relies on re-enforcing the cell membrane against the extreme acidic environment. The secretion of buffer molecules which help to raise the pH within cells is important approach that microbes can passively regulate their pH (Raven and Smith, 1973; Ahmed and Booth, 1983). Some microorganisms utilize "active" pH regulation mechanisms, which allow them to pump hydrogen ions out of their cells and through their cell walls at a constantly high rate (Baronofsky *et al*., 1984; Gonzalez-Toril *et al*., 2003). By using one of the above mechanisms, microorganisms can maintain their internal pH at optimal values (e.g. 6.5 to 7.0). Alkaliphilic microorganisms can survive and grow at high pH when the adjustment of internal pH is achieved by both passive and active regulation mechanisms

(Habitatlar, 2002). Passive regulation system is achieved in two ways viz: cytoplasmic pools of polyamines and low membrane permeability, while the active regulation mode is driven by sodium ion channels. Some cells have been found to contain cytoplasmic pools of polyamines within passive pH regulation. In particular, some cells, which are rich in amino acids with positively charged side groups (lysine, arginine, and histidine) are able to buffer their cytoplasm in alkaline environments. Another mode of passive regulation is provided by low membrane permeability which means that fewer protons move in and out of the cell.

Halophilic microorganisms have different ways of adapting to high salinity. They can accumulate inorganic ions such as Na^+ and K^+ (Robertoson *et al*., 1990) and organic osmolytes (compatible solutes) which accumulate in the cells of the halophiles are usually sugars, polyols and amino acids such as glycine betaine, ectoine, sucrose, trehalose and glycerol; these compounds do not disrupt metabolic proces and have no net charge at physiological pH (Kempf and Bremer, 1998; Gomes and Steiner, 2004; Kurz, 2008). Major exceptions are provided by halobacteria and some other extreme halophiles which accumulate KCl equivalent to the external concentration of NaCl (Sleator and Hill, 2002; Al'Abri, 2011). Radioresistance organisms protect themselves, either actively or passively from the adverse surroundings. Active protection is accomplished by moving away from lethal environment by engaging mechanisms to repair damaged cell components, and passively by accumulate certain protective compounds in their cells which protect them against the harmful radiation (Oarga, 2009). Ultraviolet and the ionising radiation inhibit microbial photosynthesis and damage nucleic acids. Direct effects result from damage to DNA and indirectly by the breakdown of DNA strands or

otherwise cause DNA mutation by producing reactive oxygen radicals (Rothschild, 2002; Yang *et al*., 2008).

1.5.1. Compatible solutes

Microorganisms synthesise compatible solutes to adapt to extreme environments. Compatible solutes are polar, highly soluble molecules and uncharged in physiological pH (Imhoff and Rodringuez-Valera, 1984; Galinski, 1993; Madigan *et al*., 2003), and are found in bacteria, archaea and eucarya (Kurz, 2008; Madigan *et al*., 2012). A range of different compatible solutes have been identified in microorganisms (Table 1.11). Compatible solutes play a main role in protecting microorganisms against different stresses, including high salinity, high temperature and dehydration (Kempf and Bremer, 1998).

Additionally, compatible solutes and extremozymes are widely used in industry (e.g. DNA polymerase) and are used in PCR, medical biotechnology and bioremediation (van den Burg, 2003; Irwin and Baird, 2004; Maire *et al*., 2009).

Table 1.11: Compatible solutes of microorganisms (*Reproduced and updated from Madigan et al, 2012*)

Organism	Main cytoplasmic solutes	Minimum water activity(a_w)	An example of structure
Nonphototropic Bacteria/ Freshwater cyanobacteria	Amino acids (mainly glutamate or proline[a])/sucrose, trehalose[b]	0.98-0.90	Sucrose
Marine cyanobacteria		0.92	
Marine algae	Mannitol, various glycosides, dimethylsulfoniopropinate	0.92	dimethylsulfoniopropinate
Salt lack cyanobacteria	Glycine betaine	0.92-0.75	Glycine betaine
Halophilic anoxygenic phototrophic purple Bacteria	Glycine betaine,ectoine, trehalose[b]	0.90-0.75	ectoine
Extremely halophilic Archaea and some Bacteria	KCl	0.75	
Dunaliella (halophilic green alge	Glycerol	0.75	glycerol
Xerophilic and osmophilic yeasts	Glycerol	0.83-0.62	
Xerophilic filamentous fungi	Glycerol	0.72-0.61	

1.5.2. Synthesis of compatible solutes

Based on the review by Roberts (2005), compatible solutes are divided in to zwitterionic solutes, uncharged solutes, anionic solutes (carboxylates) and anionic solutes (phosphate and sulphate) (Table 5.2). Organic compatible solutes are synthesised by the microorganisms (i.e. the "low salt-in cytoplasm" type) inorganic ions, mainly K^+ and Cl^-, are accumulated in the cytoplasm to a level which resemble the external salt concentration, (high salt in cytoplasm) In addition to *de novo* synthesis, organic compatible solutes can be accumulated from the environment (Oren, 2002). Mesophilic microorganisms synthesise glutamate, proline, glycine, betaine and ectoine as main compatible solutes, whilst hyperthermophilic archaea genes *Thermococcus* can synthesise aspartate (Empadinhas and da Costa, 2010). A green sulphur bacteria *Chlorobium limicola* (a moderately halophile) accumulate glycine betaine from its surroundings and synthesises trehalose which is then used as an osmolyte (Satyanarayana *et al*., 2005). Halophilic and halotolerant fungi use polyols (such as glycerol, erthritol, arabitol and mannitol), as osmotic solutes and retain a low salt concentration in their cytoplasm. The ascomycetous yeast *Debaryomyces hansenii* uses glycerol and polyol (arabitol) as main compatible solutes for high salt resistance (Almalki, 2012). Moderately halophilic green algae use polyols as compatibale solutes, such as glycerol, for their cell osmoregulation, while halotolerant yeasts and green algae accumulate polyols; many halophilic and halotolerant bacteria accumulate glycine betaine and ectoine (Al'Abri, 2011). The cyanobacteria use glycine betaine as the main compatible solute for their cells salt regulation (Schaechter, 2009). The main organic osmolytes which haloalkaliphilic sulphur oxidising bacteria accumulate are ectoine and glycine betaine (Sorokin and Kuenen, 2005). Gram-positive bacteria such as *Staphylococcus* use the amino acid proline as a compatible solute (Madigan *et al,* 2012). Another example is provided

by *Micrococcus luteus* which uses betaine and glutamate as its main compatible solutes at high salt concentrations (Almalki, 2012). Additionally, *Brevibacterium stationis* synthesizes betaine at high salinity (Abubaker, 2010), whereas, Gram–negative bacteria such as *Escherichia coli* synthesise glutamate, glycine and proline at high NaCl concentration (Cayley *et al*., 1992).

Table 1.12: A variety of compatible solutes regarded as zwitterionic, uncharged, anionic (carboxylates) and anionic (phosphate or sulphate) (*Reproduced and updated from Roberts, 2005*)

Zwitterionic organic osmolytes			
hydroxyectoine	Nγ-acetyldiaminobutyrate	Nε-acetyl-β-lysine	β-glutamine
Uncharged organic osmolytes			
α-mannosylglyceramide	trehalose	N-α-carbamoyl-L-glutamine 1-amide	N-acetylglutaminylglutamine amide
Anionic organic osmolytes (carboxylates)			
L-α-glutamate	β-glutamate	α–glucosylglycerate	α-mannosylglycerate
Anionic organic osmolytes (phosphate or sulphate)			
α-diglycerol phosphate	mannosyl-DIP	di-*myo*-inositol-1,1'-phosphate	sulfotrehalose

1.6. Aims of the Project

A variety of samples were collected from different desert soils, desert varnish, and volcanic ash soils. The biogeochemistry and microbiology are studied in order to determine the microbial activity in these soils and the results were compared with microbial activity in a fertile agricultural loam soil. Bacterial population densities in the desert surface soils and fertile loam soils were also determined. Four microbial processes were selected for study, namely: nitrification, urea hydrolysis, the oxidation of elemental sulphur to sulphate, and finally phosphate solubilisation.

The second aim was to determine the microbial biodiversity of various primitive desert surface soils and a range of rock samples, using molecular identification techniques including both 16S and 18S rRNA gene sequencing of microbial isolates. Classical microbiology and molecular techniques were employed to identify and characterise extremophilic bacteria, isolated from non-extreme environments. The area of the volcano from which this bacterium was isolated, has not been recently active.

The third aim was to determine the accumulation of compatible solutes by *Geobacillus thermoleovorans,* subjected to high temperatures stress, *Enterobacter mori,* subjected to various salinity and pH values and *Pseudomonas putida,* when exposed to a range of salinity concentrations using nuclear magnetic resonance spectroscopy (NMR) to determine how these bacteria are adapted to grow, or do they just survive, in a mesophilic environment.

Finally, various desert surface soils, rock samples and other environmental samples were tested with the aim of detecting the presence of *Mycoplasma* using PCR to detect culturable- independent *Mycoplasma* DNA in these environmental samples.

CHAPTER 2

CHAPTER 2

STUDIES ON MICROBIAL ACTIVITY IN DESERT SURFACE ENVIRONMENTS

2.1. Introduction

Studies on biogeochemical cycles have illustrated the geochemical reactions between living microorganisms and the minerals in soil surfaces, oceans and freshwaters (Schlesinger, 1997; Maier *et al*., 2009; Madigan *et al*., 2012). Biological activity in the soil environment drives a number of the key ecosystem processes that control the global system, notably the cycling of the major important elements such as sulphur, nitrogen, carbon and phosphorus (Fitter *et al*., 2005); microorganisms play the main role in the cycling of elements in soils also in the environment (Atlas and Bartha, 1997).

The aim of the work reported in this Chapter was to determine the biochemistry and microbiology of a variety of primitive desert surface soils and if the thin biotic cover which they maintain influence microbial activities; the results being compared with activity in a fertile agricultural loam soil. Bacterial population densities in the desert surface soils and fertile loam soils were also determined. Four microbial processes were selected for study namely: nitrification, urea hydrolysis, the oxidation of elemental sulphur to sulphate, and finally phosphate solubilisation. The sites studied as shown in the following figures:

A) Desert surface soils with lichen cover (Fig 2.1a) and without lichen cover (Fig 2.1b) were obtained from Tabernas in Spain, located in the north of Almerìa (37.05° N, 2.38° W).

B) Volcanic ash with plant cover (Fig 2.1c) and without plant cover (Fig 2.1d) from the French Indian Ocean Island of Reunion. Located off the southern coast of Africa east of Madagascar (21° 06′ S, 55° 36′ E).

C) Desert varnish (Fig 2.1e) and without varnish layers (Fig 2.1f) from Ashikhara in Oman, located 200 km south of Muscat (21° 51′ 14" N, 59° 34′ 50" E).

D) Agricultural loam soil from the peak district national park near Sheffield, located in northern Derbyshire (53° 21′ N, 1° 50′ W).

2.2. Materials and Methods

Surface desert samples were used (0-5cm). All samples were stored in polythene bags (20cm square) at room temperature until used, chemical analyses were then performed. Special methods were used in the collection of rocks samples:

1. Samples were collected as aseptically as possible.
2. Samples were taken downwind to minimize the risk of possible contamination.
3. Rocks should were collected at arm's length with sterile gloves and placed within sterile Whirl-pak bags or similar.
4. Loose dirt on the underside of rocks was carefully removed in the field.
5. Whirl-pak bags were further wrapped to prevent damage or contamination.
6. Desert varnish was harvested from the sample using a laminar flow cabinet.
7. Desert varnish layer was ground from the rock sample using a Dremel grinding tool with a flame-sterilized course bit, the product being placed in a sterile container.

This approach aimed to minimize the possibility of atmospheric contamination and to ensure, as far as is possible, that the bacteria isolated originated from within the varnish (Khulman *et al*., 2005).

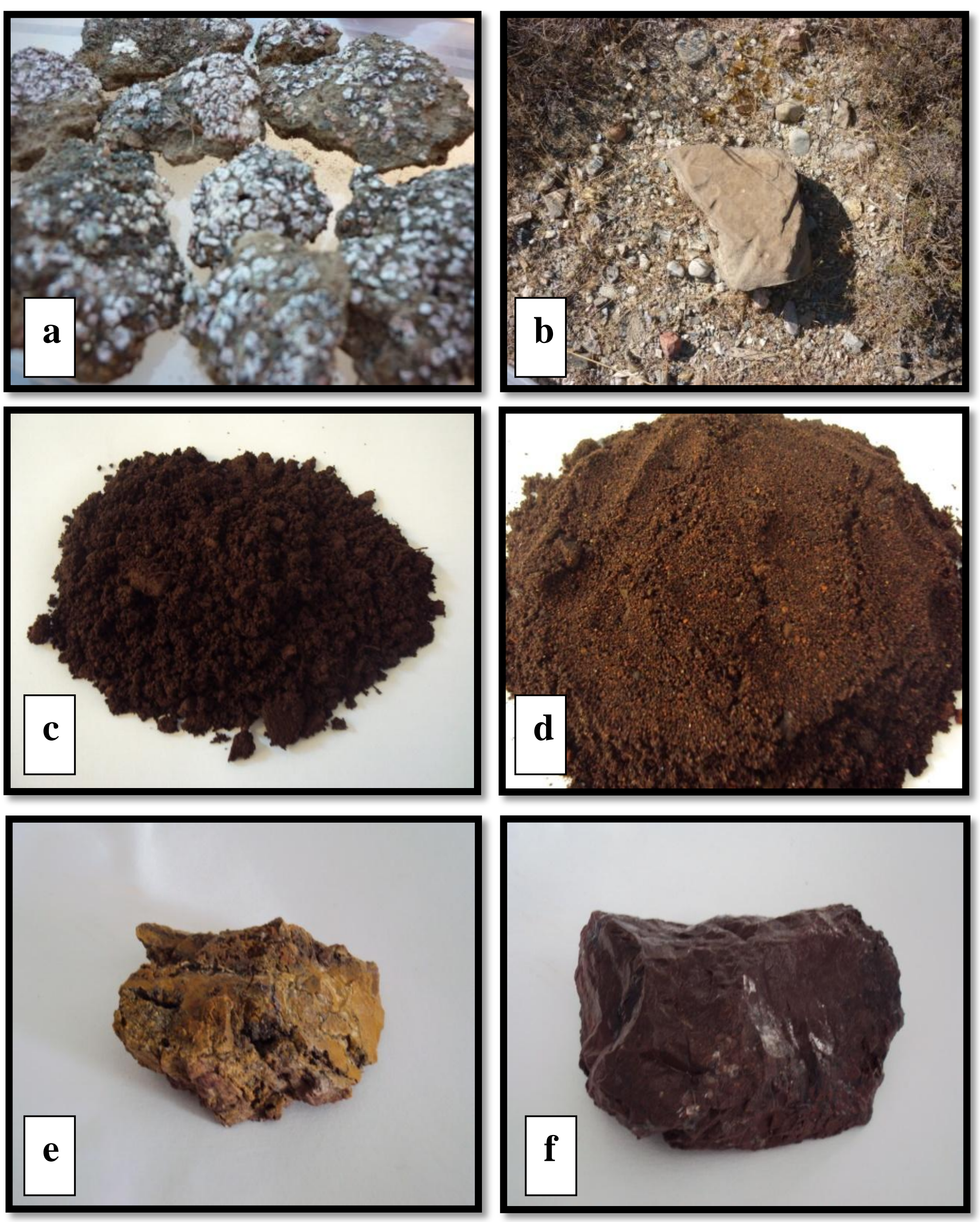


Figure 2.1: Desert surface soils (a) with lichen cover (b) without lichen cover.

Volcanic ash soils (c) with plant cover (d) without plant cover.

Desert varnish (e) with varnish layers (f) without varnish layers.

2.2.1. Preparation of desert varnish powder

Desert varnish or rock varnish samples collected from Ashikhara, Oman were powdered at the preparation laboratory, University of Sheffield Department of Geography by using the mill (Fig 2.2); the powdered samples were stored in polythene bags until used for chemical analyses.

Figure 2.2: Mill used to prepare powdered samples at the University of Sheffield Department of Geography.

2.2.2. Measurement of pH of samples

Soil samples pH were determined by suspending samples in ultrapure water 1:1 (10 grams soil in 10 ml ddH_2O) and shaking them for 30 min on a reciprocal shaker (150 revolutions min^{-1}); pH was determined by using a pH meter fitted with a glass electrode (3310, Jenway Ltd, UK).

2.2.3. Determination of soil microbial numbers (CFU)

Microbial diversity was estimated by selecting, plating and a directly counting the number of viable microorganisms. There are many advantages to plate counting. The procedure is fast, inexpensive and can produce useful information about a population. Plate counts however, favour bacteria and fungi, which grow fast and can produce enormous numbers of spores. The factors which influence the accuracy of this method include temperature, pH and light, all of which can limit the extent of the microbial community (Kirk *et al.*, 2004).

Soil (10g) from each sample was suspended in 90ml of sterile Ringers solution (1/4 strength) shaken for 15min on an orbital shaker (250 rpm) then diluted serially with same solution then 0.1ml was spread on the surface of Plate Count Agar which was prepared by suspending 17.5g, containing (tryptone 5.0g, yeast extract 2.5g, glucose 1.0 g, agar 9.0g and dH_2O 1L) and boiled to dissolve the ingredients completely then the pH was adjusted to pH 7.2 and autoclaved after that, using 3 plates for each sample. The plates were incubated at (25°C ±0.5°C for 1-3 days to enumerate the bacteria (Meintanis *et al.,* 2006).

The total bacterial count was studied at seven day intervals for agricultural loam soil which was collected from the Sheffield district, desert surface soils with lichen cover and without lichen cover from Tabernas, Spain. Volcanic ash with and without plant cover from the French Indian Ocean Island of Reunion was also used. Desert

varnish and control (without varnish layers) collected from Ashikhara, Oman was also used.

2.2.4 Determination of the oxidation of ammonium to nitrate in desert surface soils

All soil samples (50g) were placed in polythene bags and amended with $(NH_4)_2SO_4$ solution (5ml, 100µg NH_4^+-N ml^{-1}) and mixed thoroughly. A control was set-up lacking added $(NH_4)_2SO_4$ solution. The modified soils were incubated in polythene bags closed with a small hole to allow for gas exchange. The bags were set up in triplicate and incubated for 28 days at 25°C± 0.5°C At zero time and at 7 day intervals samples were extracted.

After incubation (1g) soils were placed into screw capped glass bottle with (10 ml) deionised water to extract nitrate, after shaking for 15min at 100 rpm on an orbital shaker, the samples were filtrated through Whatman No.1 filter paper.

Nitrate Determination

Nitrate was determined by using the method of Sims and Grant (1971). Chromotropic acid (CTA) reagent (7ml) was mixed with 3ml of filtrate the mixture was cooled in cool water and incubated at 40°C in water bath for 45 minutes; the yellow colour CTA-NO_3 formed was measured at 410 nm and the concentration of nitrate was determined by reference to a standard curve (0-100 NO_3-N ml^{-1}) prepared from standard solution of $NaNO_3$ (see Appendix A)

Reagents: Chromotropic acid

1- Stock solution

It was prepared by dissolving 1.84 g of Chromotropic acid $C_{10}H_6O_8S_2Na_2$ in 1 litre of sulphuric acid H_2SO_4 .The solution was stored at 4°C for several months.

2- Working solution

A working solution was prepared by diluting 100 ml of stock solution in 990 ml of concentrated H_2SO_4 then added 10 ml concentrated HCl using fume hood and the solution was stored at 4°C for several weeks only.

2.2.5. Determination of the hydrolysis of urea to ammonium in desert surface soils

Each soil (50g) was placed in polythene bags and amended with (0.5g urea) (10mg CH_4N_2O-N g $^{-1}$) and mixed thoroughly. A control was set-up lacking added CH_4N_2O urea. The modified soils were incubated in polythene bags then the bags were closed with a small hole to allow for gas exchange. The bags were set up in triplicate and incubated for 28 days at 25°C. At zero time and at 7 days intervals samples were extracted. Ammonium was extracted from the soil with a solution of KCl (150 g KCl / 1000 water) in the ratio: (1g) soil: (10ml) KCl. The soil was shaken for 30min at 100 rpm on an orbital shaker then filtered through filter paper Whatman No.1.

Determination of ammonium

Filtrate (2ml) was added to (1ml) of EDTA (6% w/v), (7ml) of distilled water, (5ml) of phenolate reagent and (3ml) of sodium hypochlorite solution (10% v/v). The reaction mixture was mixed thoroughly and incubated at 25°C±0.5°C for 20min in the dark. The volume was made up to 50 ml and mixed and the concentration of the indophenol-blue ammonium complex was measured at 630 nm (Wainwright and Pugh, 1973) the concentration of ammonium intensity was then determined by reference to standard curve (0-50µg NH_4-N ml^{-1}) prepared from a standard solution of ammonium sulphate $(NH_4)_2SO_4$ (see Appendix A).

Reagents:

1) Ethylenediaminetetraacetic acid (EDTA):

EDTA (60 g) was dissolved in (900 ml) of distilled water then diluted to1L.

2) Phenol solution:

Phenol (62.5 g) was dissolved in ethanol (25 ml) and adding acetone (18.5ml) to made up to 100 ml. The phenol solution should store in the dark at 4°C.

3) Phenolate reagent:

20 ml of phenol solution was mixed with (20 ml) hydroxide sodium (27% NaOH w/v) and diluting to 100 ml. The reagent was prepared new daily.

2.2.6. Determination the oxidation of sulphur in desert surface soils

Each soil (50g) was placed in polythene bags and amended with (0.5g elemental S) and mixed carefully. A control was set-up lacking added elemental S. The modified soils were incubated in polythene bags, closed with a small hole to allow for gas exchange. The bags were set up in triplicate and incubated for 28 days at $25°C \pm 0.5°C$. At zero time and at 7 days intervals samples were extracted. Soil samples (1g) were shaken with (10ml) of LiCl (0.1 M) for 15 min at 100 rpm using an orbital shaker and then the samples were filtrated through Whatman No.1 filter paper.

Determination of sulphate

The turbidimetric sulphate method was used to determine the oxidation of sulphur (Hesse, 1971). To (5ml) filtrate in 50ml volumetric flask (1g) Barium chloride and (2ml) of gum acacia (0.25% w/v) were added and mixed. The volume was made up 25ml with distilled water. The resultant white suspension was measured at 470nm by using spectrophotometer. The sulphate concentration was determined by reference to

a standard curve (0-100 µg SO_4^{-2}-S ml^{-1}) prepared from a standard solution of Na_2SO_4 (see Appendix A).

2.2.7. Determination of phosphate solubilisation in desert surface soils

Each soil (50g) was placed in polythene bags and amended with (0.5g calcium phosphate $CaPO_4$) and mixed carefully. A control was set-up lacking added calcium phosphate. The modified soils were incubated in polythene bags, closed with a small hole to allow for gas exchange. The bags were set up in triplicate and incubated for 28 days at 25°C. At zero time and at 7 days intervals samples were extracted.

(10g) soils were shaken for 30min with (100ml) of (0.5M $NaHCO_3$) at 100 rpm using an orbital shaker then the samples were filtered through Whatman No.1 filter paper.

Determination of phosphate

Phosphate was determined by using the method described in Falih (1995). A working solution o (7ml) was mixed with 3ml of filtrate then incubated at 37°C for 1 hour; the blue colour formed was measured at 820nm by using a spectrophotometer and the concentration of phosphate was determined by reference to a standard curve of (0-100µg PO_4-P ml^{-1}) prepared from a standard solution of Na_2HPO_4 (see Appendix A).

Reagents:

1- Stock Solution

A) Ascorbic acid 10g was dissolved in100ml of distilled water.

B) Ammonium molybdate 0.42g was dissolved in 100ml of 1N H_2SO_4 (28ml of H_2SO_4 in 1 litre distilled water).

2- Working Solution

1 volume of ascorbic acid (10%) was mixed with 6 volume of ammonium molybdate (0.42%).

Statistics

All observations were presented as Mean ± SE (Standard error). The data was analyzed by SigmaPlot© (Version11.0) $P < 0.05$ was considered as significant.

Paired two or three samples t-test was performed to check whether means were significantly different.

2.3. Results and Discussion

2.3.1. Measurement of pH

Soil pH from 6.6 to 8.0 is generally quoted as being the optimum pH for nitrification meanwhile, the optimum pH for phosphate solubilisations is pH 6.5 and sulphur oxidation can take place in the pH range between pH 2 - pH 9 (USDA, 1996; Maier *et al*., 2009). Iron, manganese and phosphorus are readily available at high pH soil, whereas a low pH soils favours calcium and magnesium availability (USDA, 1996).

The pH of the desert soil with lichen was pH 7.5, desert soil without lichen was pH 8.0, volcanic ash with plant cover was pH 7.5, and volcanic ash without plant cover was pH 8.0, desert varnish ranged between pH 9.0 to pH 8.6 and the agriculture soil was pH 7.6.

2.3.2. Counting of colony forming units (C.F.U)

The total bacterial count of bacteria was determined in soil samples. The highest bacterial count was found in the agricultural soil (Fig 2.3a). As expected, the bacterial count of the desert soil was lower than in non-desert soils and increased in regions associated with lichen and plant cover. The C.F.U. in the agricultural soil was six times higher than in desert soil without lichen cover and four times larger than in desert soils with lichen cover (Fig 2.3a). It can be clearly seen that the highest bacterial were seen in the agricultural soil while much lower numbers were found in

volcanic ash soil (Fig 2.3b). The highest bacterial counts in the agricultural soil being seven times higher than in the volcanic ash without plant cover and six times higher than in volcanic ash with a plant cover; the presence of a plant cover obviously therefore increases the bacterial count of these primitive soils. The number of bacteria present in these soils also decreased during the incubation period. In Fig 2.3c; it is noticeable that there was a gradual decrease in the bacterial count in both the desert varnish and the control. The highest number of colony forming units was, not surprisingly, found in the agricultural soil, while the lowest numbers were found in the desert varnish samples. There was a slight different between desert varnish and control without varnish.

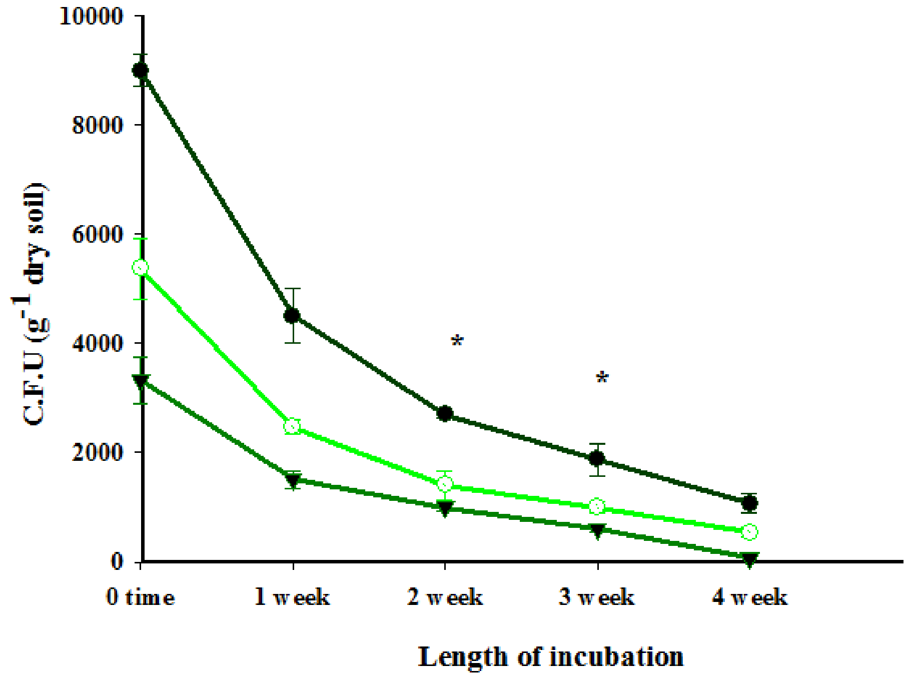


Figure 2.3 a; The total bacterial cell counts on plate count agar of different soils; agricultural soil ( ), desert soil with lichen cover ( ) and desert soil without lichen cover ( ).

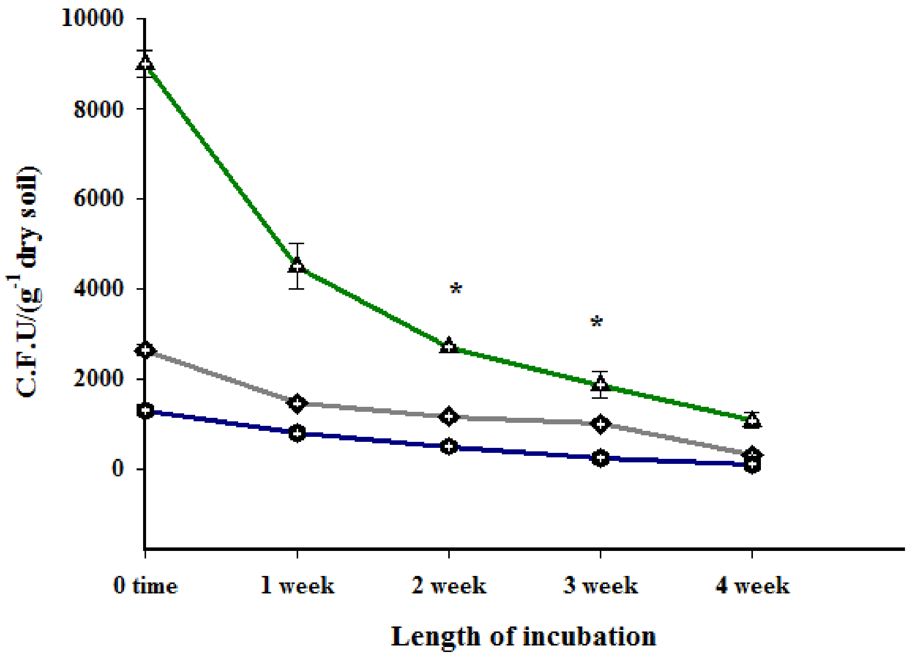


Figure 2.3 b; The total bacterial cell counts on plate count agar of different soils; agricultural soil ( ), volcanic ash with plant cover ( ) and volcanic ash without plant cover ( ).

Means of triplicates (±) standard error (SE).

*significant difference from control value, $P < 0.05$.

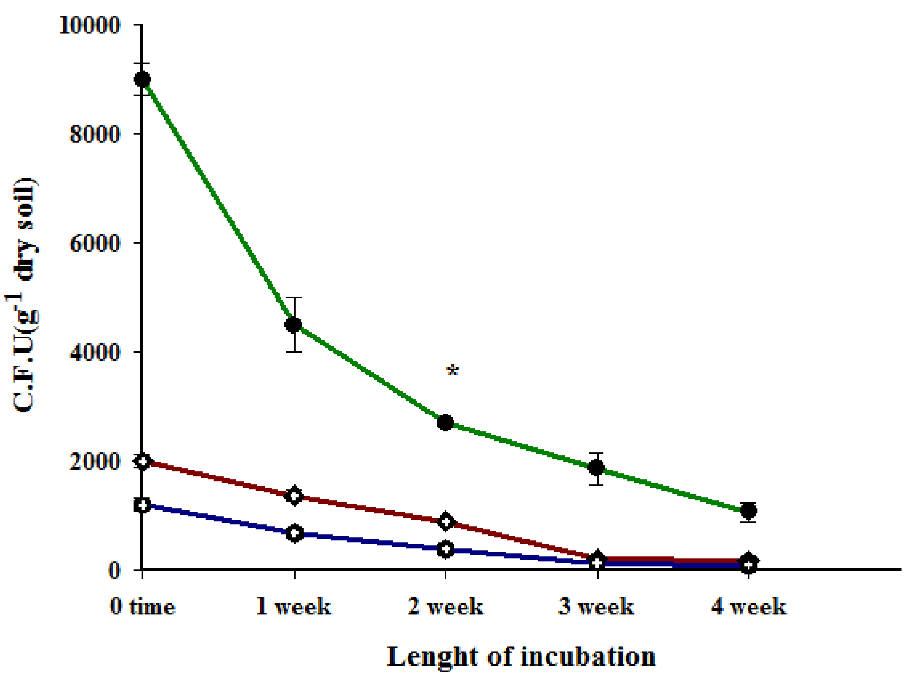


Figure 2.3 c; The total bacterial cell counts on plate count agar of different soils;

Agricultural soil ( ), desert varnish ( ) and control

( ).

Means of triplicates (±) standard error (SE).

*significant difference from control value, P < 0.05.

2.3.3. Net nitrate production from oxidation of ammonium

The oxidation of ammonium to nitrate results from the activity of autotrophic, heterotrophic and chemoautotrophic bacteria, as well and to a lesser extent from heterotrophic fungi. Fig 2.4a, b, c and d show the nitrate production from the oxidation of ammonium over the four week incubation period. Fig 2.4a shows the agricultural soil treatment and control; the maximum nitrate production being found at week 3 in both treatment and control. This shows that the oxidation of ammonium to nitrate is associated with vegetation cover. Fig 2.4b shows desert soil with lichen cover treatment and control. The highest nitrification was established at week 2 then there was a large decrease at week 3 and 4. Fig 2.4c shows the results for the desert soil without lichen cover treatment and control. It can be clearly seen that there was no difference between the treatment and the control over the length of incubation. Fig 2.4d shows a comparison between the agricultural soil (treatment) and desert soil with and without lichen cover (treatment). It shows that the amount of nitrate was found in the agricultural soil increased to 7 day and the highest nitrate production occurred at week 3. However, the highest nitrification in desert soil with lichen cover at week 2, whilst there was no change in the concentration found in desert soil without lichen cover during the whole period of incubation. The expected outcome was seen, namely that the agricultural soil would exhibit the highest rates of nitrate production then the desert soil having a lichen cover, followed by the desert soil without lichen cover.

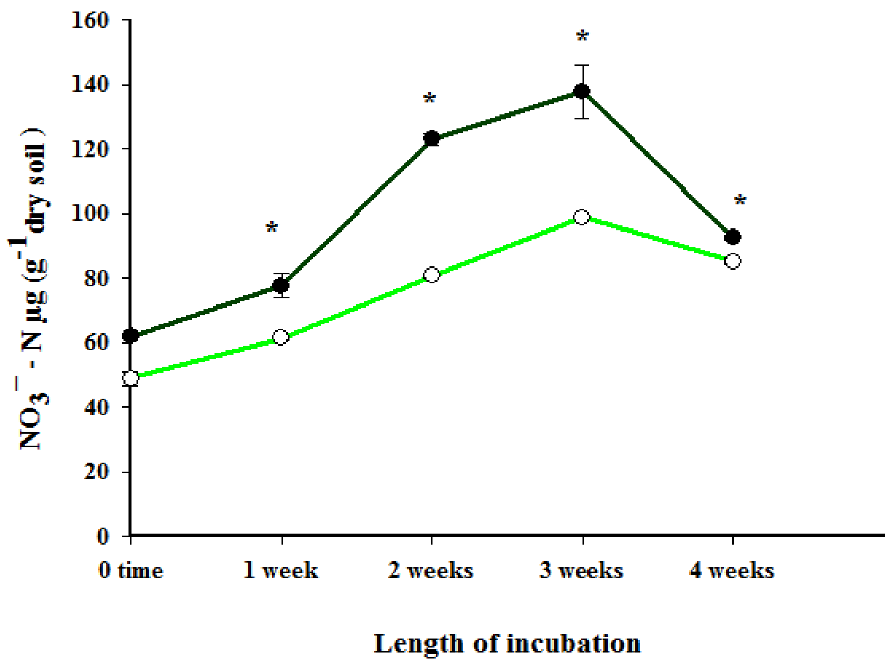


Figure 2.4 a; Nitrate production from the oxidation of ammonium in agricultural soil (—●—) (treatment) and (—○—) control.

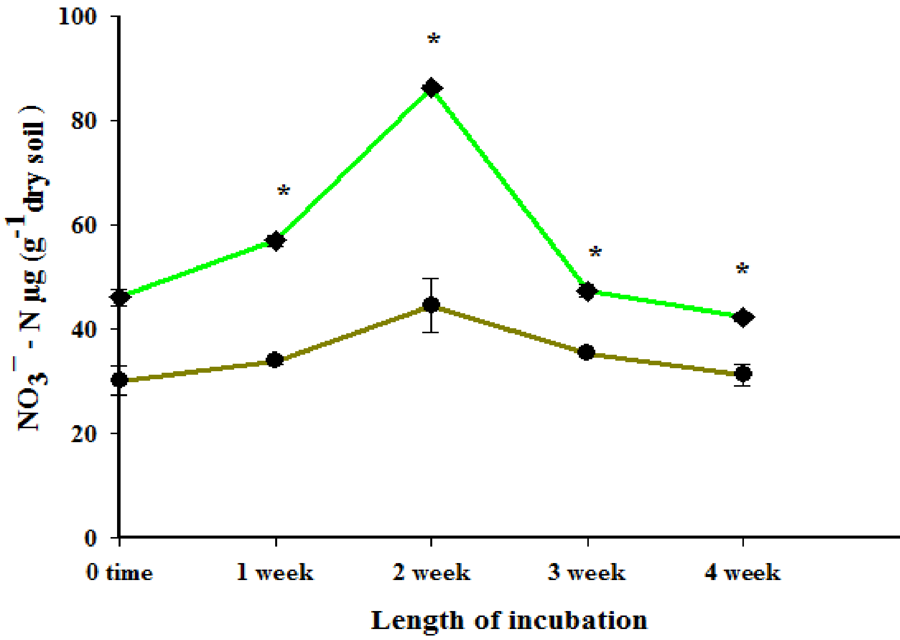


Figure 2.4 b; Nitrate production from the oxidation of ammonium in desert soil with lichen cover (—◆—) (treatment) and (—●—) control.

Means of triplicates (±) standard error (SE).

*significant difference from control value, $P < 0.05$.

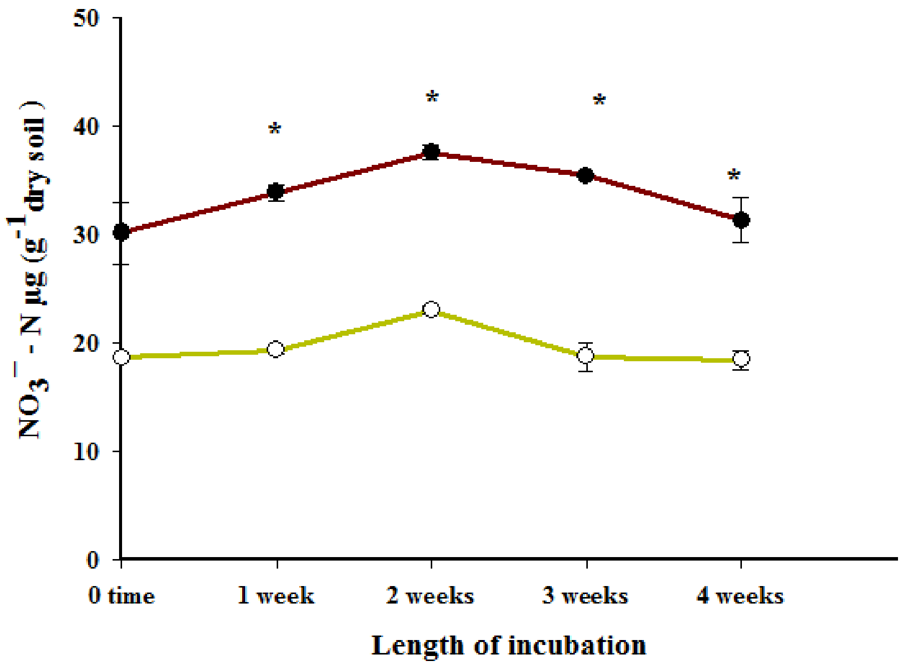


Figure 2.4 c; Nitrate production from the oxidation of ammonium in desert soil without lichen cover ( ) (treatment) and ( ) control.

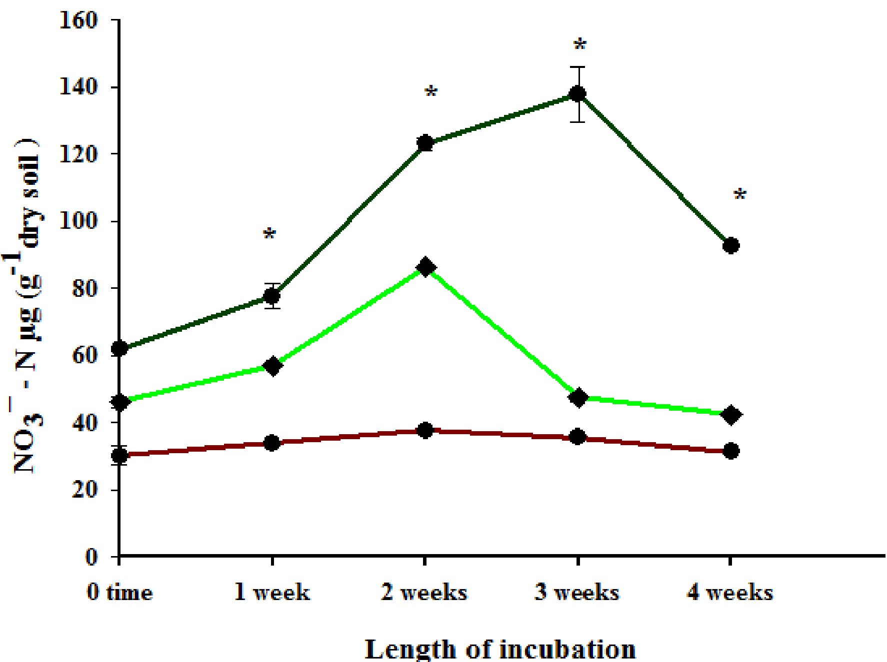


Figure 2.4 d; Nitrate production from the oxidation of ammonium in different soil; agricultural soil ( ) (treatment) desert soil with lichen cover ( ) (treatment) and desert soil without lichen cover ( ) (treatment). Means of triplicates (±) standard error (SE). *significant difference from control value, $P < 0.05$.

The results given in Fig 2.5a and 2.5b show that the oxidation of ammonium to nitrate in volcanic ash with and without plant cover (treatment) and the control over a four week incubation period. Fig 2.5a refers to the volcanic ash with plant (treatment) and the control. There was an increase in the amount of nitrate production from the oxidation of ammonium throughout the whole incubation period in the treatment soil, whereas there was a slight increase of nitrate concentration in the control. Fig 2.5b shows that nitrate production in volcanic ash without plant cover (treatment) and control; it is noticeable that the concentration of nitrate is low in both treatment soil and control. Fig 2.5c illustrates the compare between agricultural soil (treatment) and volcanic ash with and without plant cover (treatment). The results show that there is a significant difference in the rate of nitrate production in the soils examined, with highest rates being found in the agricultural soil. Microbial nitrification occurred at week 3 in the agricultural soil .Moreover, there is a slightly different of the rates of production between volcanic ash with plant and without plant cover. In addition, there is no change in nitrate production in volcanic ash with and without plant cover in throughout the length of incubation. In contrast, it is expected that nitrification is higher in the amended agricultural soil and the amount of nitrate formed is similar in both volcanic ash with and without plant cover. Fig 2.6a and 2.6b show the oxidation of ammonium in desert varnish soil and control. In addition, compare it with the amended agricultural soil. In Fig 2.6a the amount of nitrate production increased gradually and the highest rate found at week 4 in desert varnish. Moreover, there was a slight rise in nitrate concentration in control during 28 days. In Fig 2.6b the highest rate of ammonium oxidation occurred in the agricultural soil at week 3. Meanwhile, there was a slight different between the desert varnish and control. The levels of nitrate production found in agricultural soil are

almost 4 times higher than those found in desert varnish soil , whether covered by varnish or not .

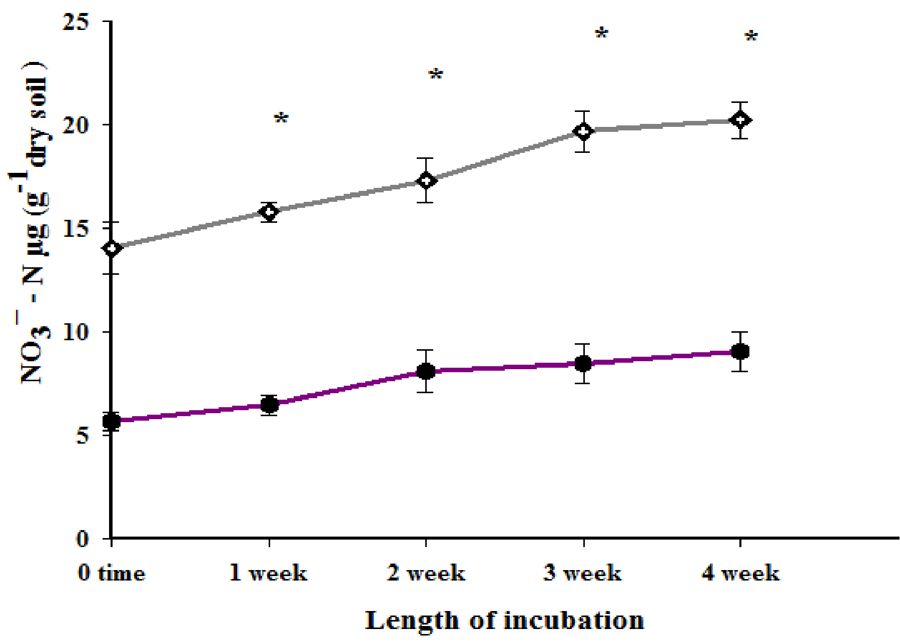


Figure 2.5 a; Nitrate production from the oxidation of ammonium in volcanic ash with plant cover (—◇—) (treatment) and (—●—) control

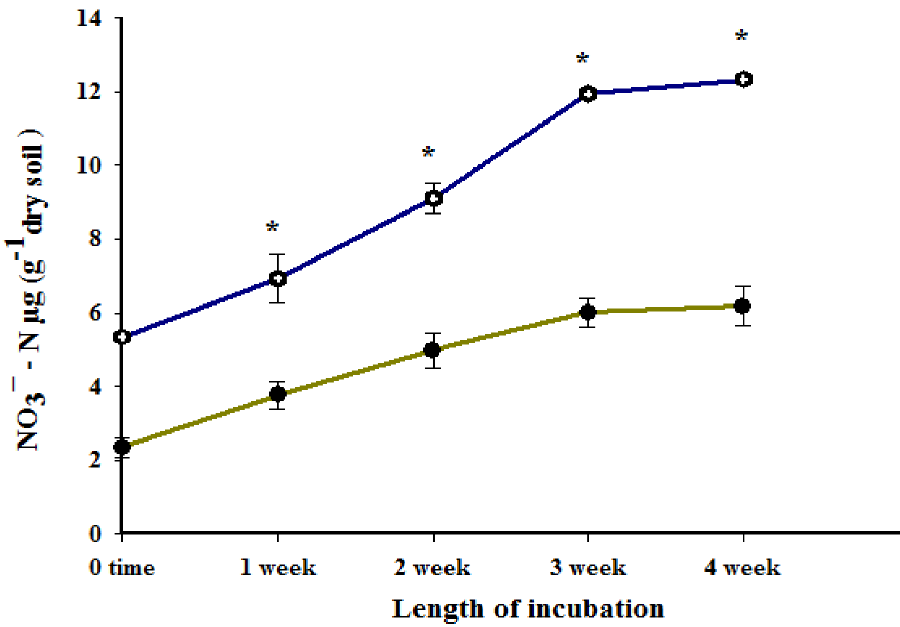


Figure 2.5 b; Nitrate production from the oxidation of ammonium in volcanic ash soil without plant cover (—◇—) (treatment) and (—●—) control.

Means of triplicates (±) standard error (SE).

*significant difference from control value, $P < 0.05$.

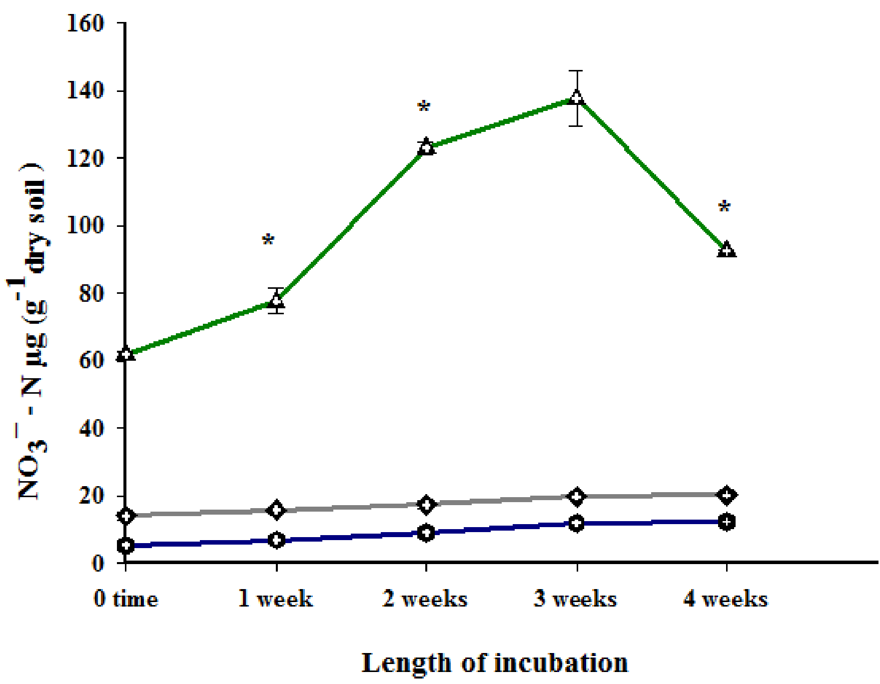


Figure 2.5 c; Nitrate production from the oxidation of ammonium in different soil; agricultural soil ( ) (treatment) volcanic ash with plant cover ( ) (treatment) and volcanic ash without plant cover ( ) (treatment).

Means of triplicates (±) standard error (SE).

*significant difference from control value, $P < 0.05$.

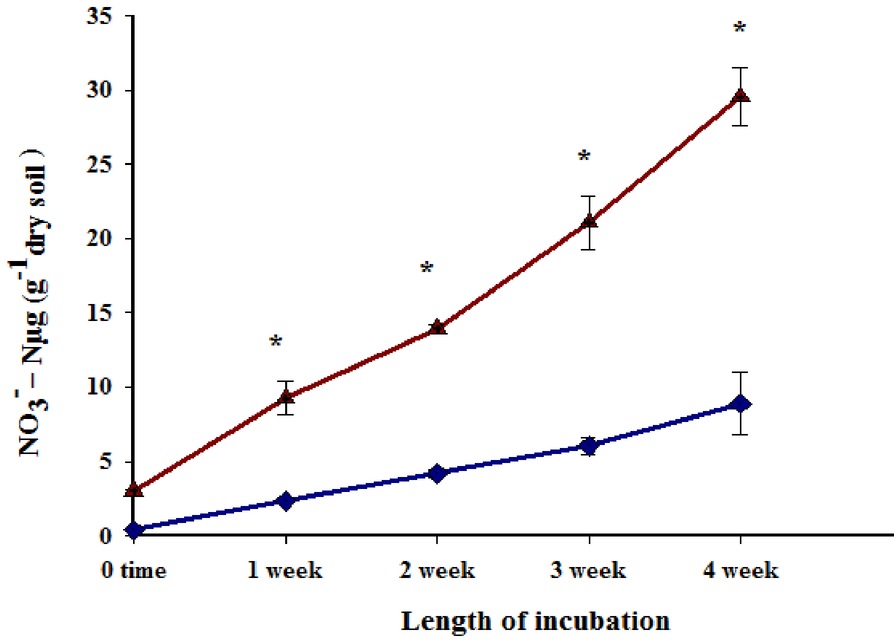


Figure 2.6 a; Nitrate production from the oxidation of ammonium in (▲) desert varnish soil and (◆) control.

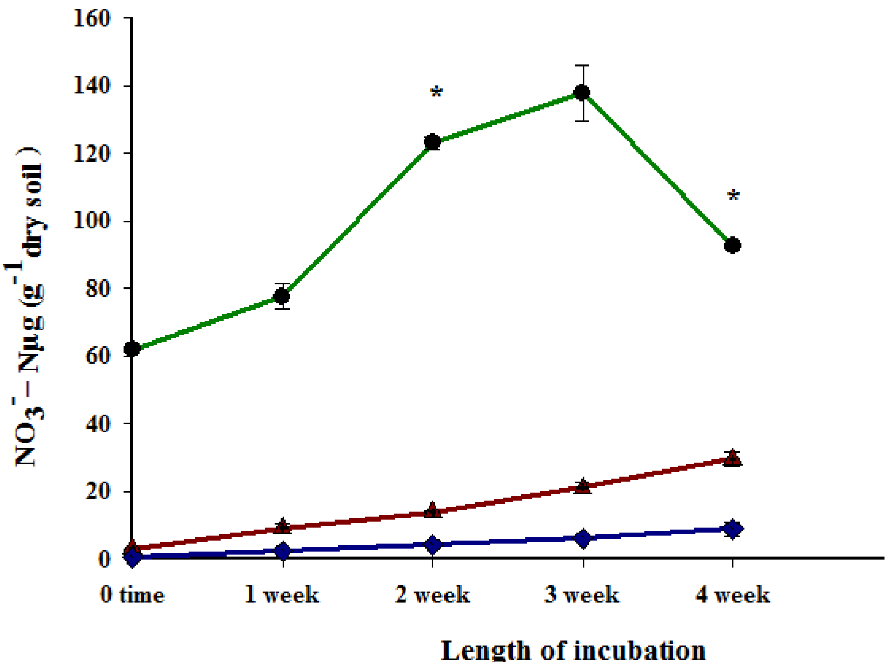


Figure 2.6 b; Nitrate production from the oxidation of ammonium in different soil; agricultural soil (●) (treatment), desert varnish (▲) and (◆) control. Means of triplicates (±) standard error (SE). *significant difference from control value, $P < 0.05$.

2.3.4. Ammonium production from the hydrolysis of urea

A whole range of heterotrophic bacteria gain energy and carbon by hydrolysing urea to form ammonium. Urea was added to soil samples, which is converted to carbon dioxide and ammonia by the enzyme urease in the soil. Ureases are formed by soil microbes, plants and animals.

Results from the hydrolysis of urea to ammonium in agricultural soil and desert surface soils with and without lichen cover (treatment) and control are shown in Fig 2.7a, b and c in addition; Fig 2.7d shows a comparison between all three soils (treatment) over the four week incubation period. Fig 2.7a shows the ammonium production from the hydrolysis of urea in agricultural soil (treatment) and control. It shows clearly that the levels of ammonium was substantially increased after 14 days and that the highest ammonium production was found at week 2 while there was no ammonium production in the control sample at some sampling points after 28 days. Fig 2.7b and 2.7c show that the amount of ammonium production in desert soil with lichen cover and without lichen cover after 1week and reached a peak at 2 week then the amount of ammonium produced decreased gradually. On the other hand, the control remained stable throughout the incubation period in both soils. Fig 2.7d compares the agricultural soil (treatment) with desert soil with and without lichen cover (treatment).It shows clearly, the amount of ammonium was found in the agricultural soil increased to 7 day and the highest ammonium production occurred at week 2. However, the highest hydrolysis of urea in desert soil with lichen cover and without lichen covers at the week 3 sampling point. The expected outcome was found, namely that the agricultural soil exhibits a higher rate of ammonium production then the desert soil having a lichen cover next , followed by the desert soil without lichen cover.

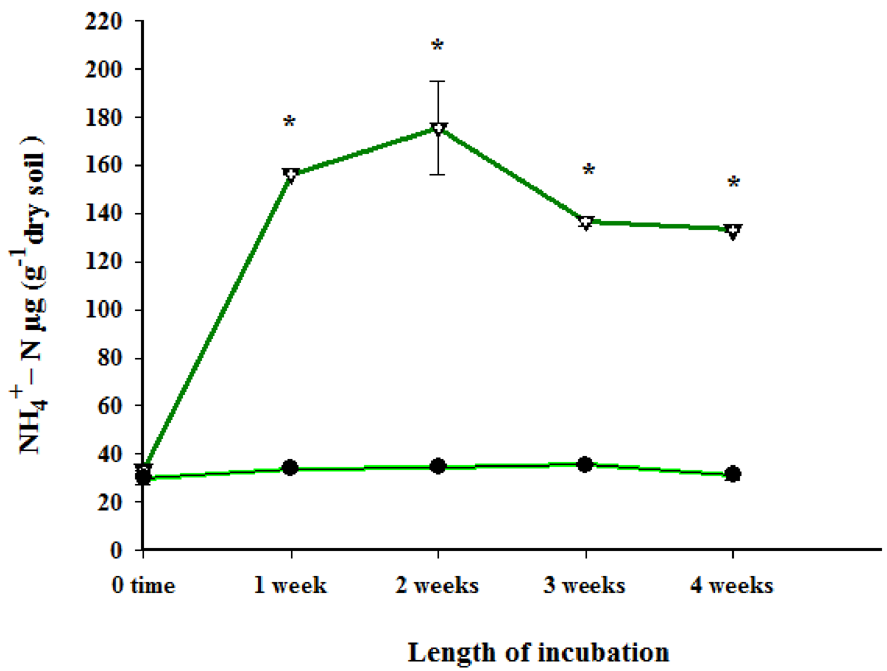


Figure 2.7 a; Ammonium production from the hydrolysis of urea in agricultural soil ( ) (treatment) and ( ) control.

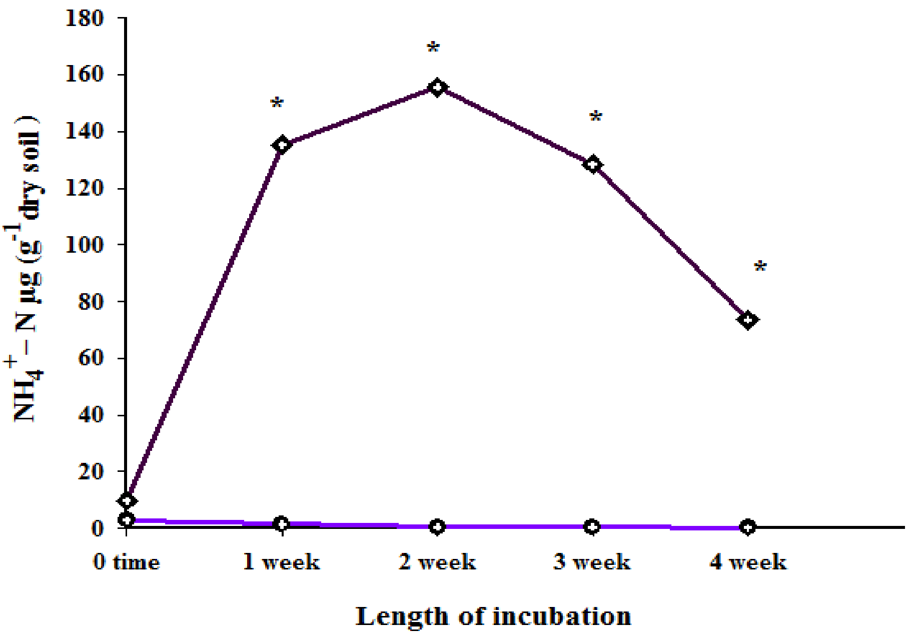


Figure 2.7 b; Ammonium production from the hydrolysis of urea in desert soil with lichen cover ( ) (treatment) and ( ) control.

Means of triplicates (±) standard error (SE).

*significant difference from control value, $P < 0.05$.

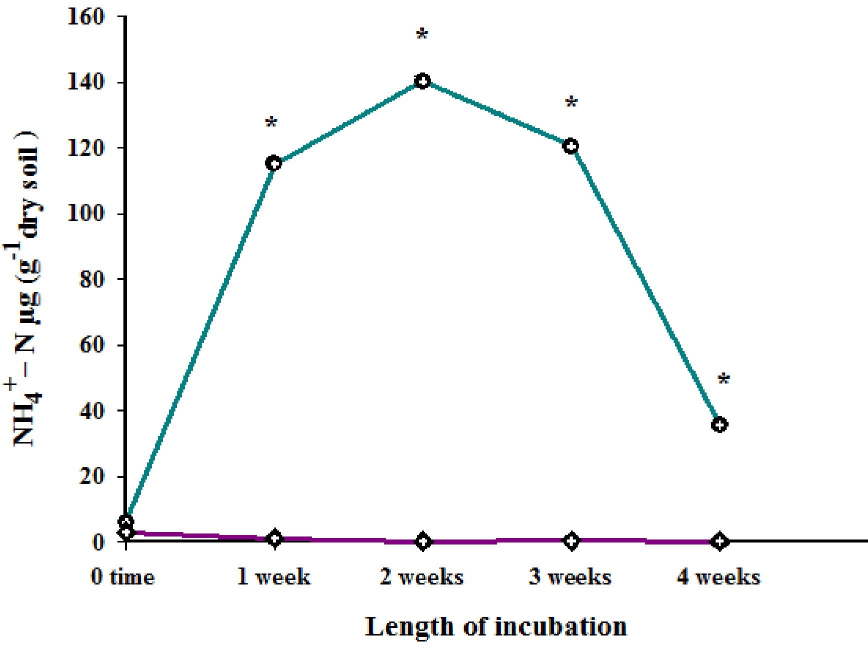


Figure 2.7c; Ammonium production from the hydrolysis of urea in desert soil without lichen cover ( ) (treatment) and ( ) control

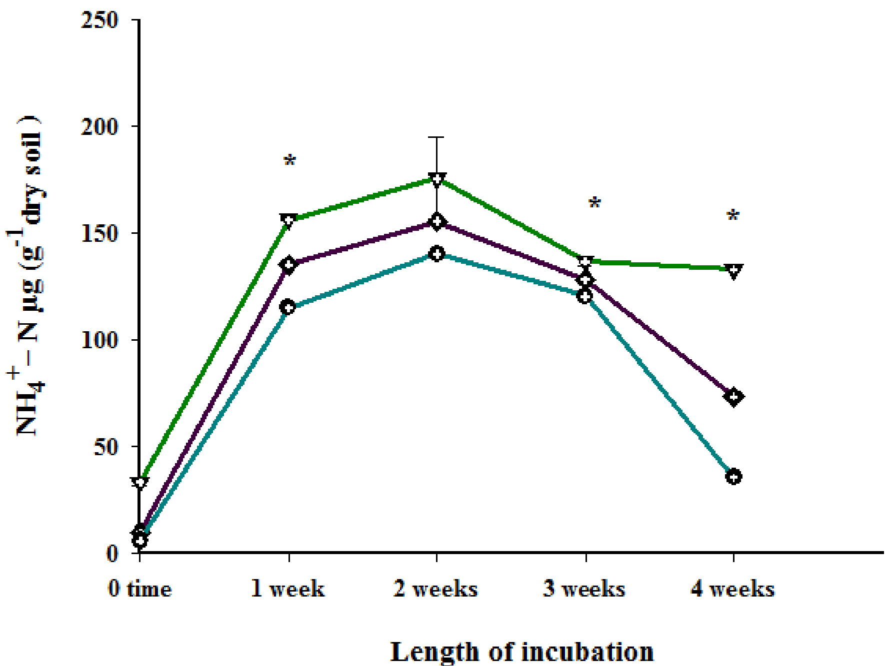


Figure 2.7 d; Ammonium production from the hydrolysis of urea in different soil; Agricultural soil ( ) (treatment) desert soil with lichen cover ( ) (treatment) and desert soil without lichen cover ( ) (treatment).

Means of triplicates (±) standard error (SE).

*significant difference from control value, $P < 0.05$.

The results provided in Fig 2.8a and 2.8b show that the concentration of ammonium in volcanic ash with and without plant cover (treatment) and control at four week incubation period. Fig 2.8a refers to the volcanic ash with plant (treatment) and the control. There was a gradual increase in ammonium production from the hydrolysis of urea throughout the whole treatment period and the ammonium production peaked at 28 days whereas there was a minimal increase of ammonium concentration in the control over the 28 days. Fig 2.8b shows the ammonium production in volcanic ash without plant cover (treatment) and control; it is noticeable that the concentration of ammonium fluctuated slightly for the period of 14 days then there was a gradual rise in ammonium production at week 3 and 4, whereas the concentration of ammonium in the control soil increased gradually to reach a peak at the 4 week sample. Fig 2.8c shows the comparison between the agricultural soil (treatment) and volcanic ash with and without plant cover (treatment). The results show that the concentration of ammonium in the agricultural soils is higher than in the volcanic ash with plant than in the volcanic ash without plant cover. Additionally, the levels of ammonium in agricultural soil are seen to be three times higher than in the volcanic ash without plants cover, while being two times greater than in volcanic ash with plant cover. Fig 2.9a and 2.9b show the hydrolysis of urea to ammonium in desert varnish soil and control. In Fig 2.9a; the amount of ammonium production increased gradually and the highest rate was found at week 4 in desert varnish. Moreover, there was a slight rise in ammonium concentration in the control during 28 days. In Fig 2.9b the highest rate of ammonium oxidation is seen to occur in the agricultural soil at week 2, and there was only a small difference between the desert varnish and control. The levels of

ammonium production found in agricultural soil are almost 3 times higher than those found in desert surface soil , whether covered by varnish or not .

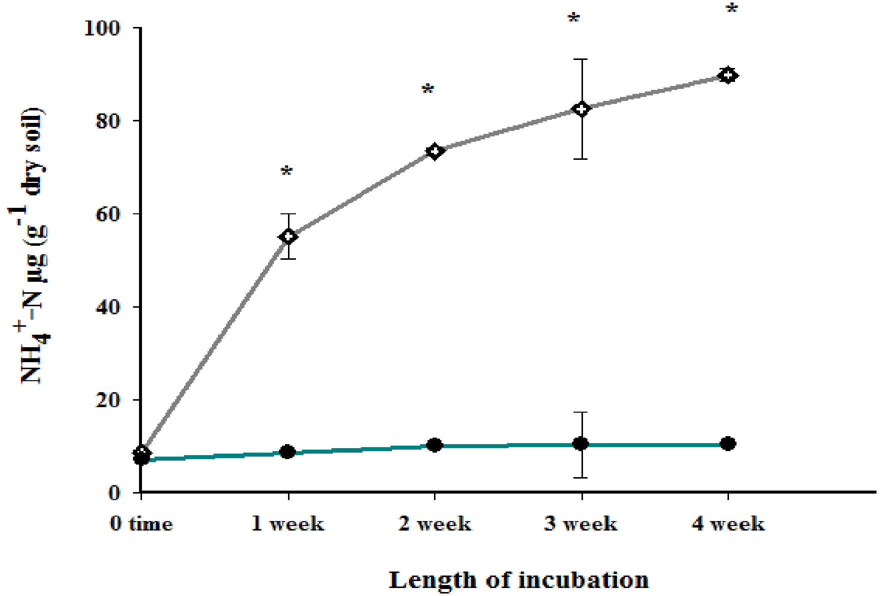


Figure 2.8 a; Ammonium production from the hydrolysis of urea in volcanic ash soil with plant cover ( ) (treatment) and ( ) control.

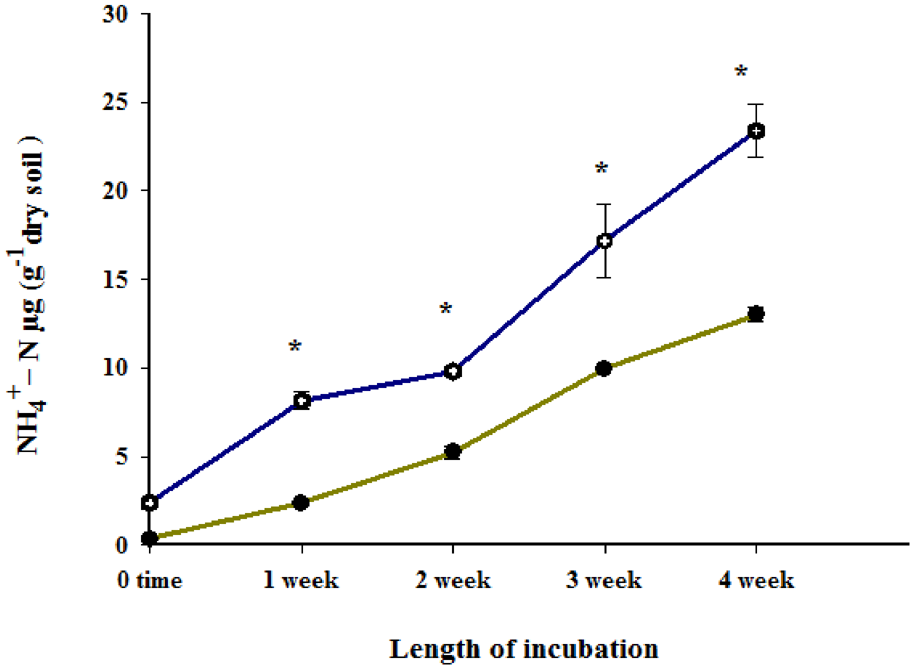


Figure 2.8 b; Ammonium production from the hydrolysis of urea in volcanic ash soil without plant cover ( ) (treatment) and ( ) control.

Means of triplicates (±) standard error (SE).

*significant difference from control value, $P < 0.05$.

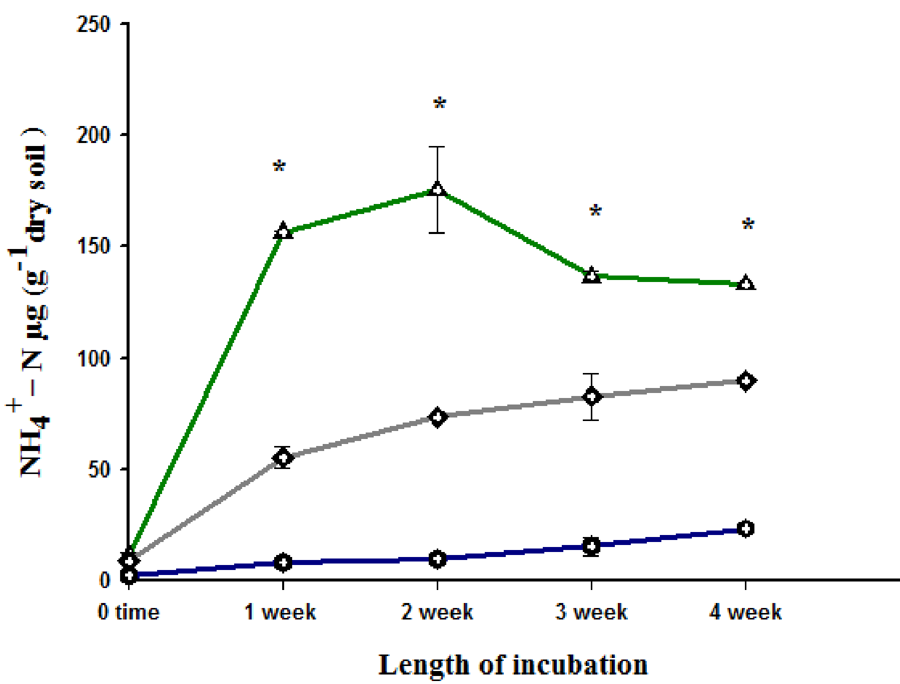


Figure 2.8 c; Ammonium production from the hydrolysis of urea in different soil; agricultural soil (green line with triangles) (treatment) volcanic ash with plant cover (grey line with diamonds) (treatment) and volcanic ash without plant cover (blue line with circles) (treatment).

Means of triplicates (±) standard error (SE).

*significant difference from control value, $P < 0.05$.

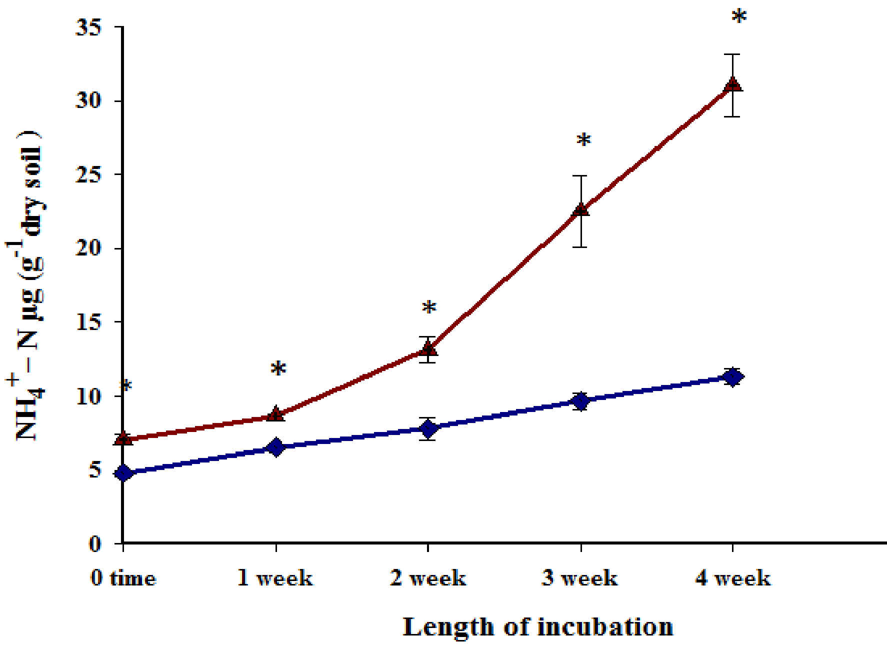


Figure 2.9 a; Ammonium production from the hydrolysis of urea in ( ) desert varnish soil and ( ) control.

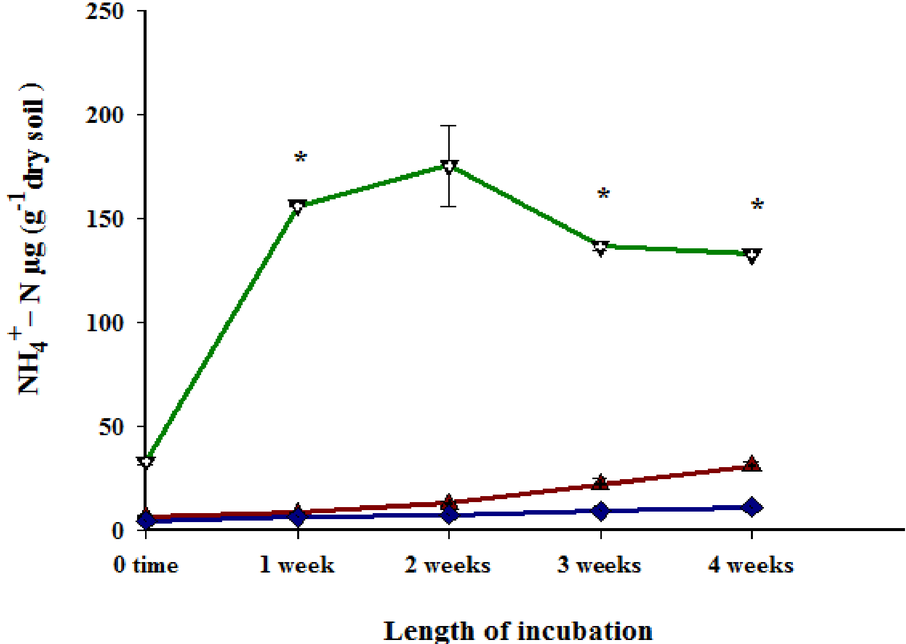


Figure 2.9 b; Ammonium production from the hydrolysis of urea in different soil; agricultural soil ( ) (treatment) desert varnish ( ) and ( ) control. Means of triplicates (±) standard error (SE). *significant difference from control value, $P < 0.05$.

2.3.5. Sulphate production from oxidation of elemental sulphur

Elemental sulphur was added to the soil samples; this activates S-oxidizing bacteria leading to the production of sulphate and hydrogen ions (i.e. acidity). Sulphur oxidation occurred in all sample soils and was not surprisingly greater in the soil amended with elemental sulphur compared to the control. Fig 2.10a, b, c and d show the oxidation of sulphur in agricultural soil and desert soil with and without lichen cover over the four week incubation period. Fig 2.10a shows the results for the agricultural soil (treatment) and control. The results show that microbial S-oxidation of sulphur increased from day 7 and continued increasing throughout 28 day incubation period while there was slight rise in sulphate concentration in the control at 28 days. Fig 2.10b the results show that the amount of S-oxidation of sulphur found in desert soil with lichen cover (treatment) and control. There was a considerable increase in sulphate production during 4 week (treatment); the control increased steadily through 28 days. Fig 2.10c shows results for the desert soil without lichen cover (treatment) and control. It can be clearly seen that there was a significant rise between treatment and control throughout the length of incubation. Moreover, oxidation of sulphur reached a peak of week 4. Fig 2.10d compares data between the agricultural soil (treatment) and the desert soil, with and without lichen cover (treatment). The results show that microbial oxidation of sulphur increased from day 7 and continued increasing throughout the 28 day incubation period. The highest oxidation in agricultural soil, then the desert soil with lichen cover and then desert soil without lichen cover.

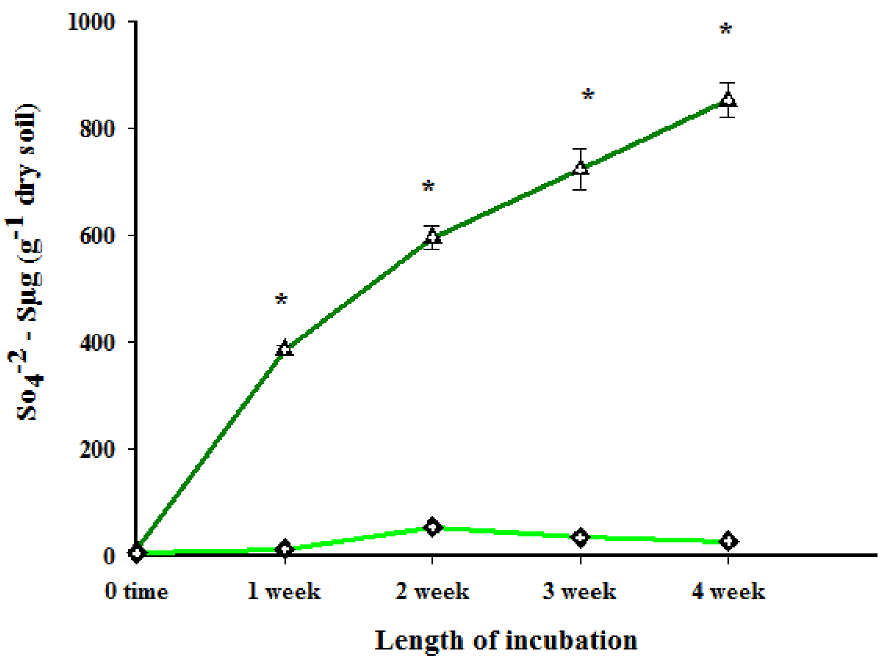


Figure 2.10 a; Sulphate production from oxidation of elemental sulphur in agricultural soil ( ) (treatment) and ( ) control.

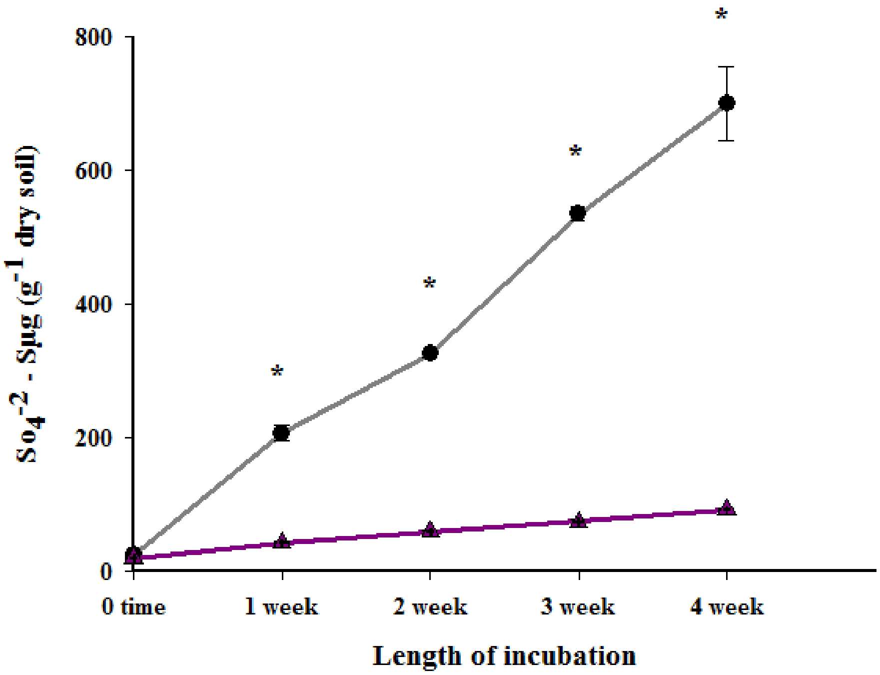


Figure 2.10 b; Sulphate production from oxidation of elemental sulphur in desert soil with lichen cover ( ) (treatment) and ( ) control

Means of triplicates (±) standard error (SE).

*significant difference from control value, $P < 0.05$.

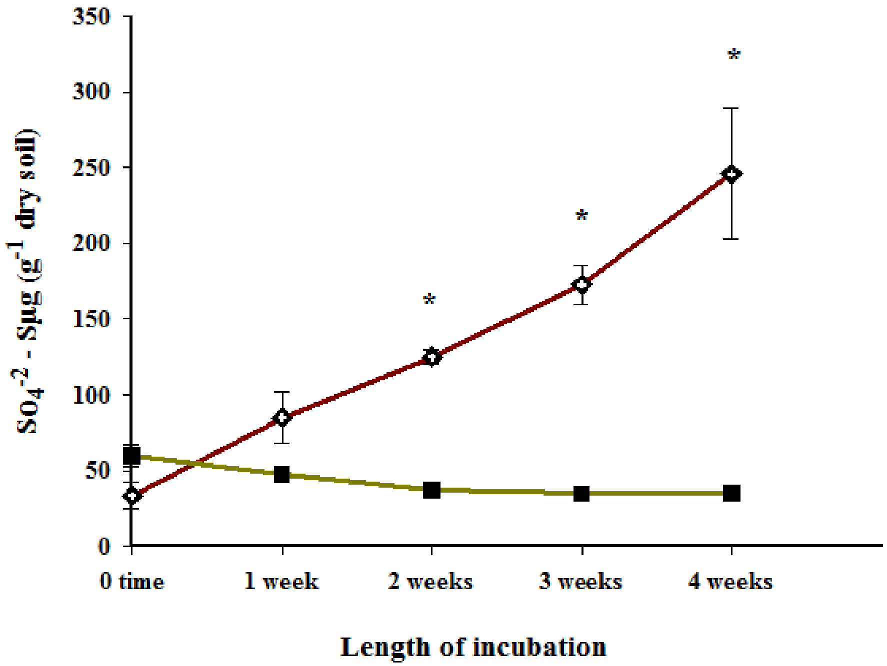


Figure 2.10 c; Sulphate production from oxidation of elemental sulphur in desert soil without lichen cover ( ) (treatment) and ( ) control.

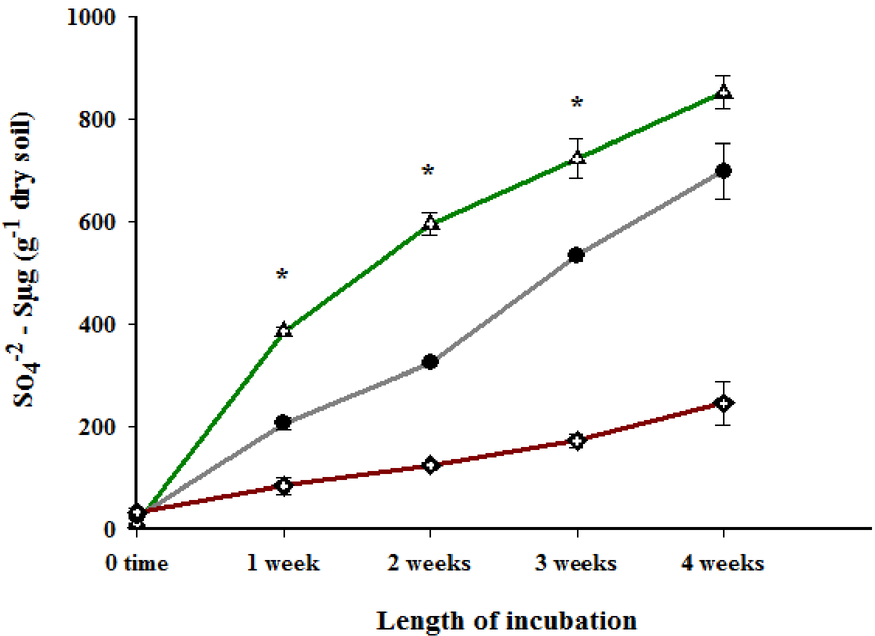


Figure 2.10 d; Sulphate production from oxidation of elemental sulphur in different soil; agricultural soil ( ) (treatment) desert soil with lichen cover ( ) (treatment) and desert soil without lichen cover ( ) (treatment). Means of triplicates (±) standard error (SE). *significant difference from control value, $P < 0.05$.

The results given in Fig 2.11a and 2.11b show that the concentration of sulphate in volcanic ash, with and without plant cover (treatment), and control at four week incubation period. Fig 2.11a illustrates the volcanic ash with plant (treatment) and the control. There was marked sulphate production at 7 day, followed by a large decrease at 14 day; the sulphate concentration then remained stable at 21and 28 days. In the same time the control has the minority sulphate production during 28 day.

Fig 2.11b shows the sulphate production in volcanic ash without plant cover (treatment) and control. It is noticeable that the highest concentration of sulphate occurred at week 1 and that there was a small decrease through week 2, 3 and 4. There was no change in sulphate production in the control in throughout the length of incubation. Fig 2.11c shows the comparison between agricultural soil (treatment) and volcanic ash with and without plant cover (treatment).

The results show that microbial oxidation of sulphur in agricultural soil increased from day 7 and continued increasing throughout the 28 day incubation Period. The highest rate of S-oxidation occurred in the agricultural soil, while there was only a small difference between the sulphate content of the volcanic ash with plant and without plant cover. The concentrations of S-oxidation found in agricultural soil are almost 4 times higher than those found in volcanic ash, whether covered by plants or not. Fig 2.12a, b shows the oxidations of sulphur in desert vanish soil and control. Fig 2.12a shows that the amount of sulphate production increased gradually after 7 day and the highest rate found at week 4 in desert varnish and control, the same trend being found in the control after 7 day. Fig 2.12b shows that the highest rate of sulphur oxidation occurred, over the 28 day period, in the agricultural soil. Meanwhile, there was a slight different between the desert varnish and control. The highest level of sulphate production was found in agricultural soil, while there was

some change in sulphate production in desert varnish and desert soil without varnish layer through 28 days.

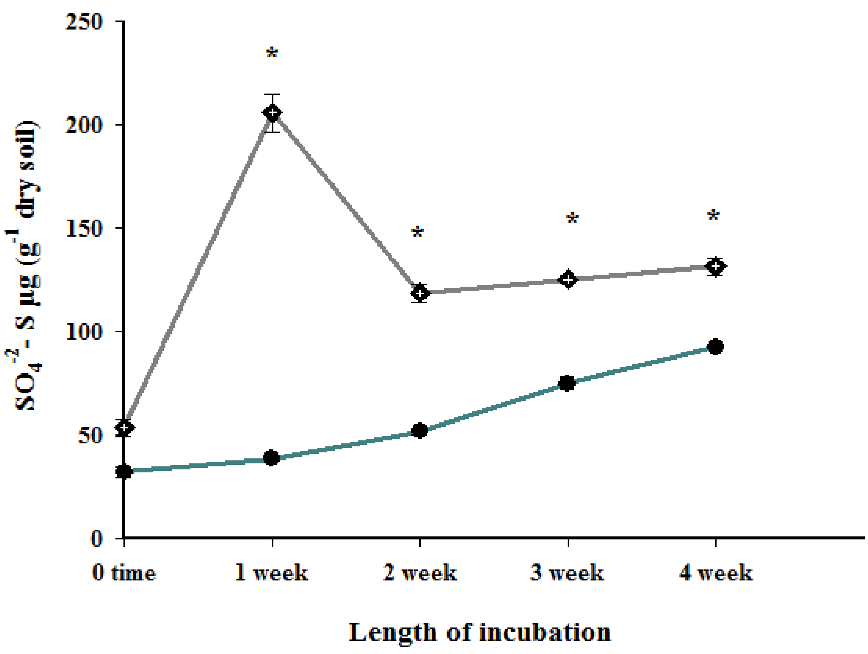


Figure 2.11 a; Sulphate production from oxidation of elemental sulphur in volcanic ash with plant cover ( ) (treatment) and ( ) control

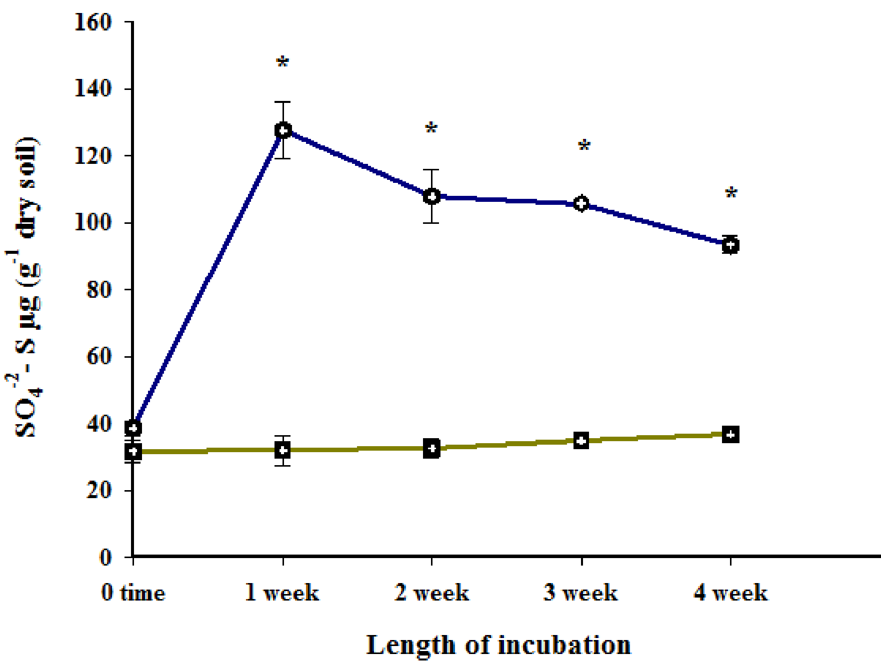


Figure 2.11 b; Sulphate production from oxidation of elemental sulphur in volcanic ash without plant cover ( ) (treatment) and ( ) control. Means of triplicates (±) standard error (SE).

*significant difference from control value, $P < 0.05$.

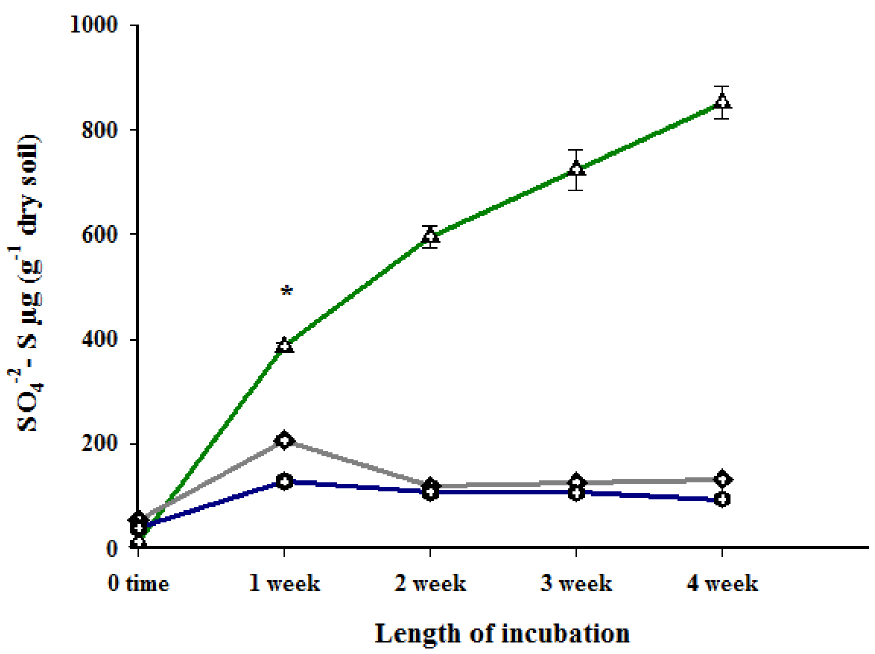


Figure 2.11 c; Sulphate production from oxidation of elemental sulphur in different soil; agricultural soil ( ) (treatment) volcanic ash with plant cover ( ) (treatment) and volcanic ash without plant cover ( ) (treatment).

Means of triplicates (±) standard error (SE).

*significant difference from control value, $P < 0.05$.

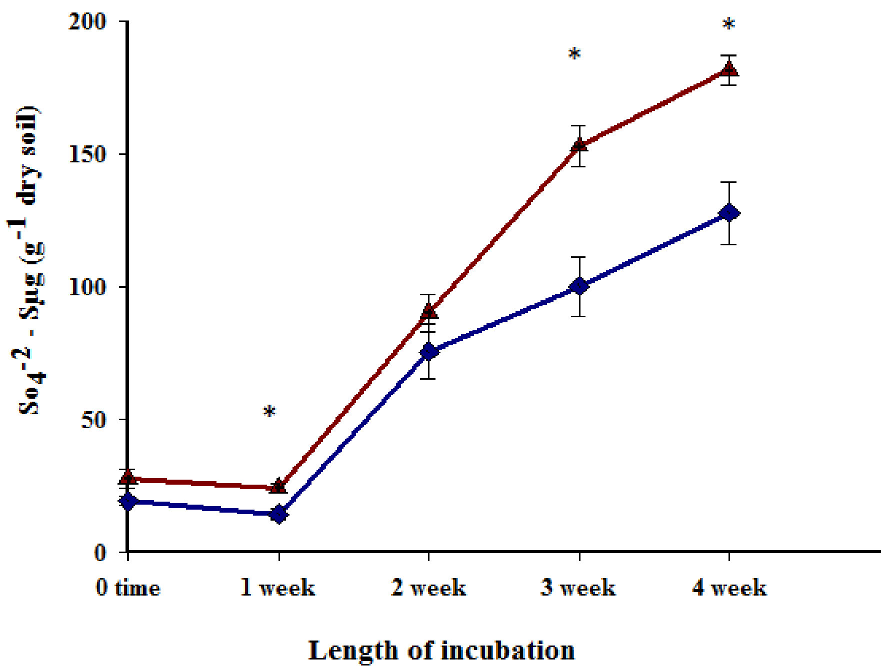


Figure 2.12 a; Sulphate production from oxidation of elemental sulphur in ( ) Desert varnish soil and ( ) control.

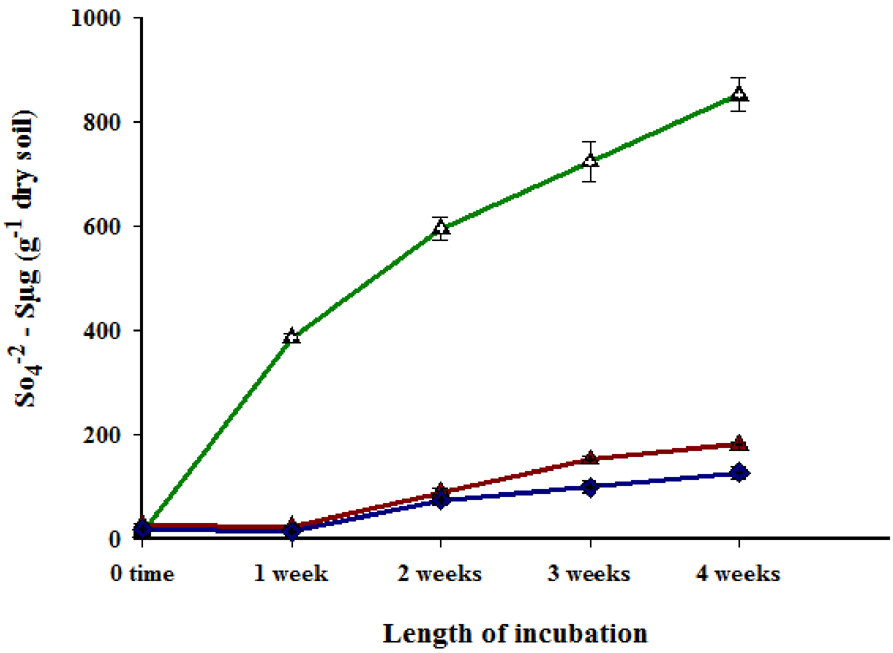


Figure 2.12 b; Sulphate production from oxidation of elemental sulphur in different soil; agricultural soil ( ) (treatment) desert varnish ( ) and ( ) control. Means of triplicates (±) standard error (SE). *significant difference from control value, $P < 0.05$.

2.3.6. Phosphate solubilisation

Fig 2.13a, b, c and d show the phosphate solubilisation over the four week incubation period. Fig 2.13a shows that microbial phosphorus solubilisation increased sharply after 7 day and then there was a gradual decrease at week 2, 3 and 4 in both treatment and control. This shows that microbial phosphorus solubilisation is associated with vegetation cover. Fig 2.13b shows the desert soil with lichen cover treatment and control. The highest phosphate solubilisation was established at week 4. However, there was a slight different between the treatment soil and the control during 21 day of incubation. Fig 2.13c shows the results for the desert soil without lichen cover treatment and control. It can be clearly seen that there was no change between treatment and control throughout the length of incubation; the highest solubilisation being found after four weeks incubation. Fig 2.13d compares the agricultural soil (treatment) with desert soil with and without lichen cover (treatment). It shows clearly that there was a significant difference in the rate of phosphate solubilisation in agricultural and desert soils, with highest rates being found in the agricultural soil at week 1. On the other hand, there was no change in phosphate solubilisation in desert soil with and without lichen cover through the entire incubation period.

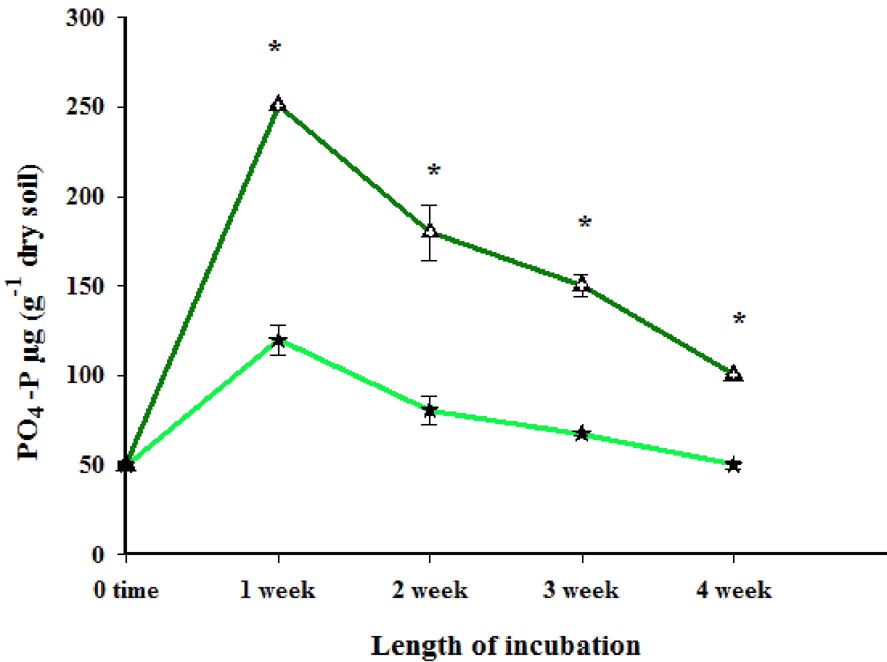


Figure 2.13 a; Phosphate solubilisation in agricultural soil (treatment) and control.

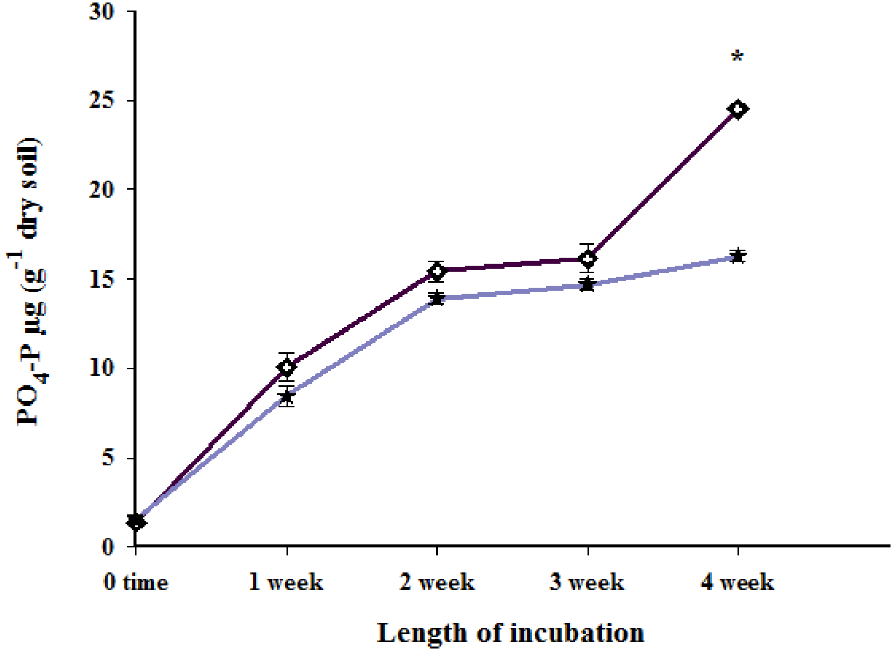


Figure 2.13 b; Phosphate solubilisation in desert soil with lichen cover (treatment) and control.

Means of triplicates (±) standard error (SE).

*significant difference from control value, $P < 0.05$.

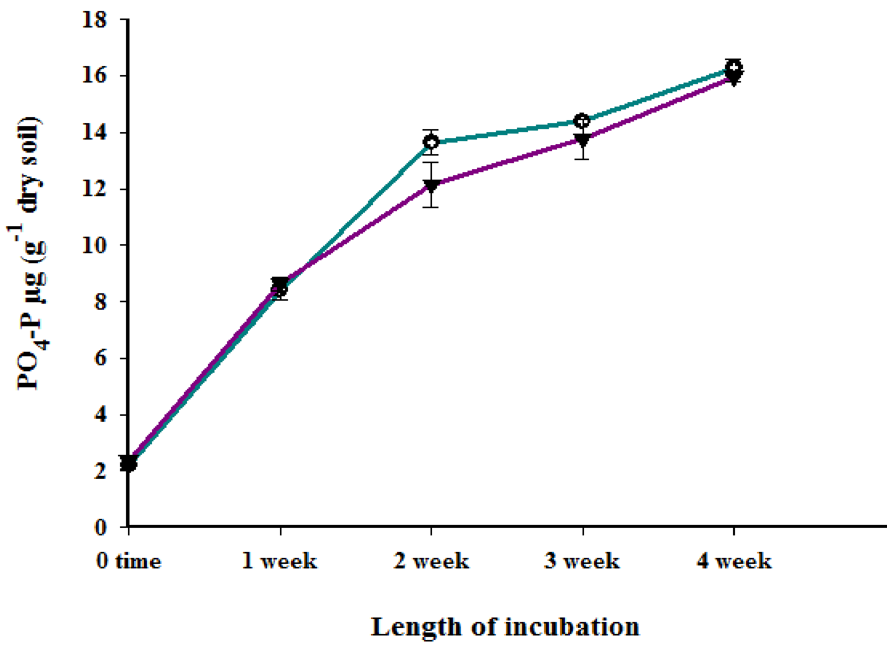


Figure 2.13 c; Phosphate solubilisation in desert soil without lichen cover (—○—) (treatment) and (—▼—) control.

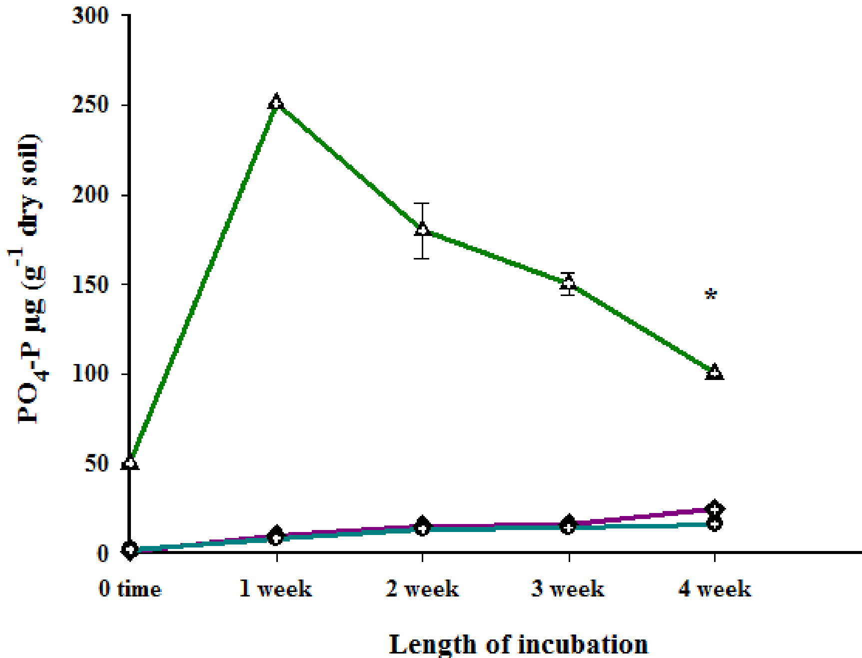


Figure 2.13 d; Phosphate solubilisation in different soil; agricultural soil (—△—) (treatment) desert soil with lichen cover (—○—) (treatment) and desert soil without lichen cover (—▼—) (treatment). Means of triplicates (±) standard error (SE). *significant difference from control value, $P < 0.05$.

The results given in Fig 2.14a and 2.14b show the phosphate solubilisation in volcanic ash with and without plant cover (treatment) and control at four week incubation period. Fig 2.14a refers to the volcanic ash with plant (treatment) and the control. It can be clearly seen that the concentration of phosphate solubilisation occur at week 1with the highest solubilisation and there was a small decrease through week 2, 3 and 4. There was no change in phosphate solubilisation production in the control throughout the length of incubation. Fig 2.14b shows that phosphate solubilisation in volcanic ash without plant cover (treatment) and control. The highest solubilisation was seen at week 2 then there was a slight decreased at week 3. However, the control remained constant during 4 week. Fig 2.14c shows the comparison between agricultural soil (treatment) and volcanic ash with and without plant cover (treatment). The results show that the concentration of phosphate solubilisation in the agricultural soils are higher than in the volcanic ash with plant then in the volcanic ash without plant cover. Meanwhile, there was a slight difference between the phosphate content of the volcanic ash with plant and without plant cover. The levels of phosphate solubilisation found in agricultural soil are almost 4 times higher than those found in volcanic ash, whether covered by plants or not. Fig 2.15a, b shows the phosphate solubilisations in desert vanish soil and control. In Fig 2.15a the amount of phosphate production increased gradually and the highest rate found at week 3and 4 in desert varnish. Moreover, there was a slight rise in phosphate concentration in control during 28 days and the control value remained steady through the length of incubation. In Fig 2.15b the highest rate of phosphate concentration occurred in the agricultural soil after 7 day. Meanwhile, there was a slight different between the desert varnish and control. The highest levels of phosphate production was found in agricultural soil and there was no change in phosphate production in desert soil

without varnish layer throughout the 28 day incubation period, while there was a slight increased in desert varnish during 28 days.

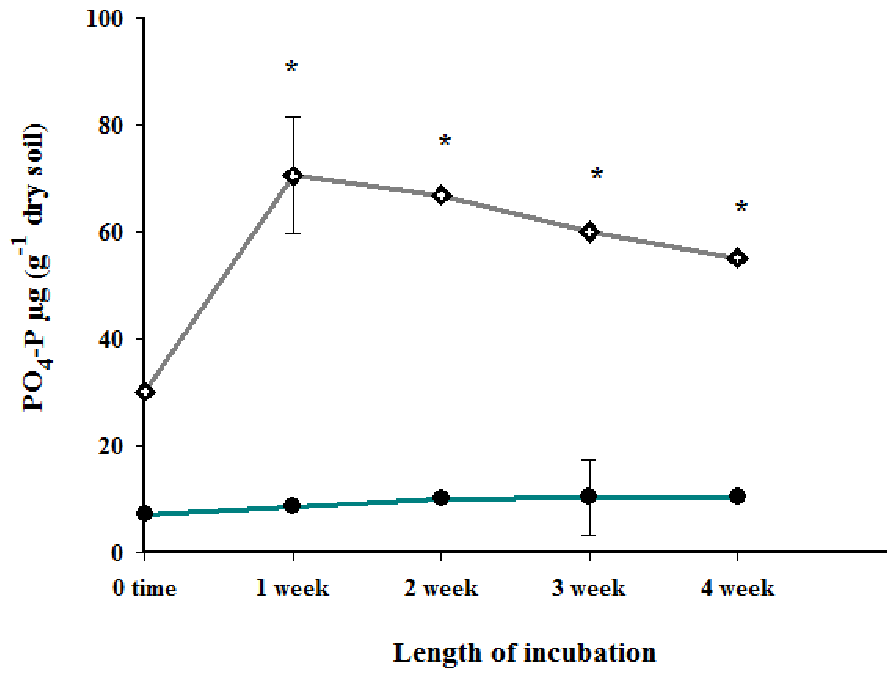


Figure 2.14 a; Phosphate solubilisation in volcanic ash soil with plant cover ( ) (treatment) and ( ) control.

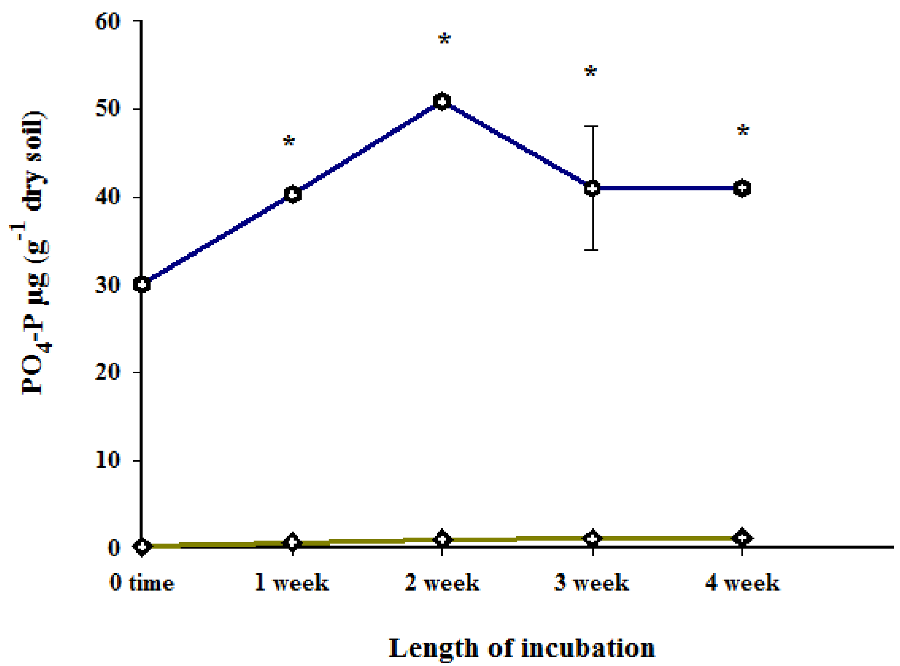


Figure 2.14 b; Phosphate solubilisation in volcanic ash soil without plant covers ( ) (treatment) and ( ) control.

Means of triplicates (±) standard error (SE).

*significant difference from control value $P < 0.05$.

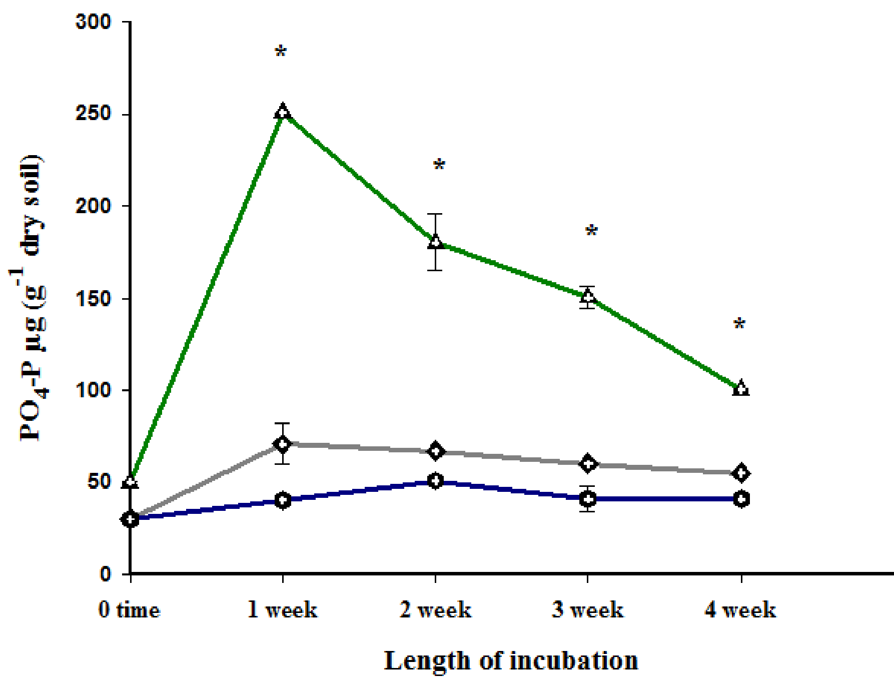


Figure 2.14 c; Phosphate solubilisation in different soil; agricultural soil (green line with triangle markers) (treatment) volcanic ash with plant cover (grey line with diamond markers) (treatment) and volcanic ash without plant cover (blue line with circle markers) (treatment)
Means of triplicates (±) standard error (SE).
*significant difference from control value, $P < 0.05$.

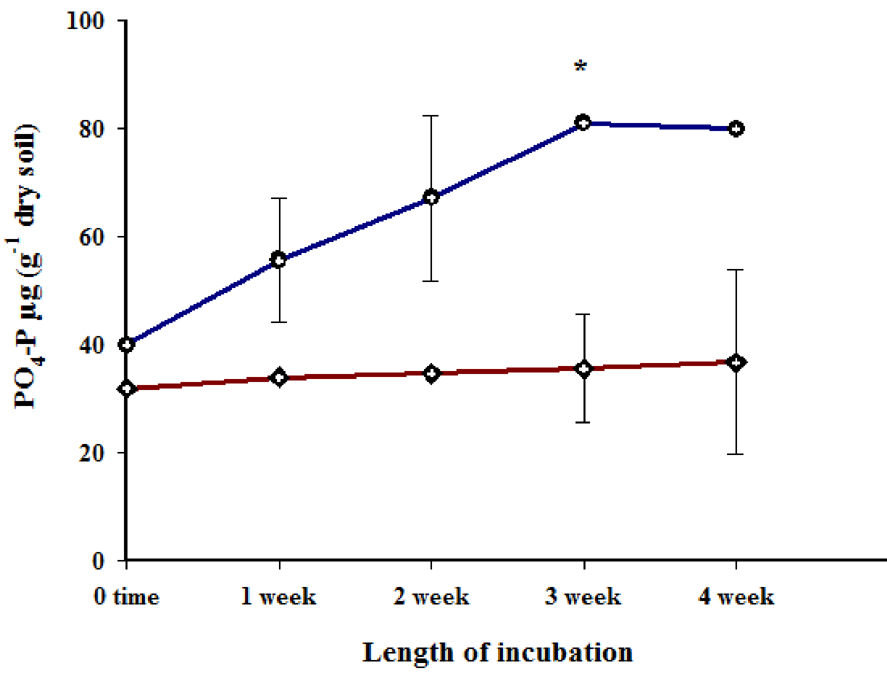


Figure 2.15 a; Phosphate solubilisation in ( ) desert varnish soil and ( ) control.

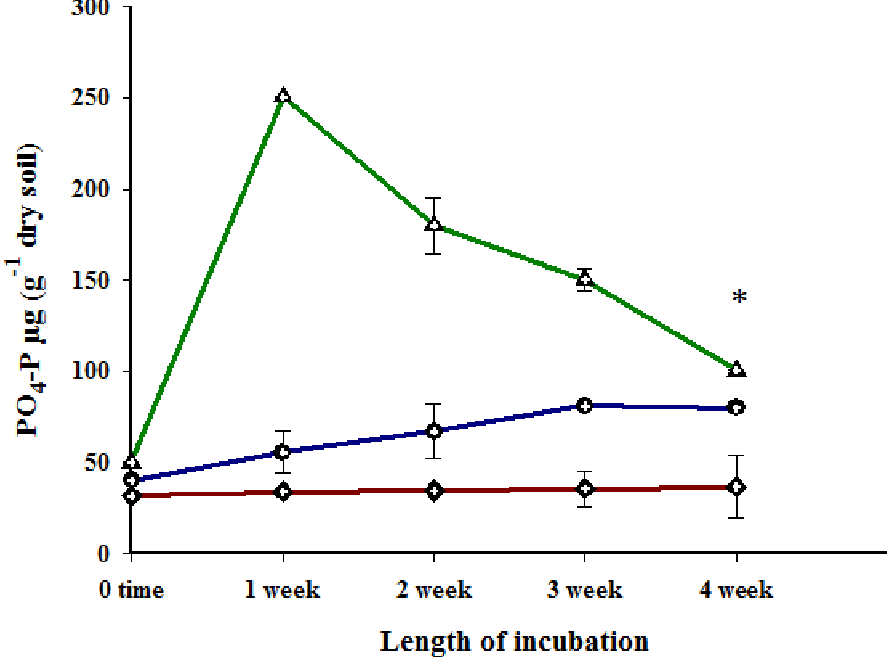


Figure 2.15 b; Phosphate solubilisation in different soil; agricultural soil ( ) (treatment) ( ) desert varnish and ( ) control. Means of triplicates (±) standard error (SE).

*significant difference from control value, $P < 0.05$.

2.4. Conclusions

The conclusion from Microbial Counts: Bacterial counts were found in the order: agricultural soil > desert soil with and without lichen cover > volcanic ash with and without plant cover > desert varnish and control (without varnish layers). This finding is what would be expected considering the relative organic matter contents of the three soils, which is expected to be in the same order as the bacterial counts. The decline in numbers of bacteria is assumed to be due to soil drying since the soil moisture content was not maintained constant over the incubation period.

The main conclusions from the study of the four microbial processes are: the highest level of microbial activity was found in agricultural soil and the lowest microbial activity was found in desert varnish. Ureases are produced by soil microbes and soils with low organic matter will posses low urease activity; microbial activity in desert soils was seen to be lower than in non-desert soils and increases in regions associated with lichen cover. The same trend was found in nitrification, urea hydrolysis and the oxidation of elemental sulphur to sulphate. The microbial activity as found in the order: agricultural soil > desert soil with and without lichen cover > volcanic ash with and without plant cover > desert varnish and control (without varnish layers). On the other hand, the highest level of phosphate solubilisation was found in the order: agricultural soil > volcanic ash with and without plant covers > desert varnish and control > desert soil with and without lichen cover.

CHAPTER 3

CHAPTER 3

MICROBIAL DIVERSITY ON DESERT SURFACES AND ROCK SAMPLES BY USING 16S AND 18S rRNA GENE SEQUENCING

3.1. Introduction

Microbial diversity has commonly been defined to include genetic diversity that is "the amount and distribution of genetic information within microbial species". Microbial diversity has been quantified by various counting techniques which include the plate count technique; in which colonies of unknown species are firstly isolated using specific media and then identified by using either 16S rRNA or 18S rRNA gene sequences (Nannipieri *et al.*, 2003). The molecular analysis of either 16S or 18S rRNA gene has recently become fundamental for research studies examining the diversity of prokaryotic and eukaryotic microorganisms in the environment. Modern molecular methods, such as PCR and sequencing techniques, have significantly provided important insights into the field of prokaryotic and eukaryotic diversity (Griffith *et al.*, 2000). The 16S rRNA gene has been chosen to study bacterial phylogeny because all bacteria have this gene, moreover the function of the 16S rRNA gene has not changed, over time, and it is a 1.5 Kb sized gene which is reasonably easily sequenced (Patel, 2001). The 18S rRNA gene is similarly one of the most important molecular markers used in phologenetic analyses and eukaryote biodiversity (Meyer *et al*., 2010). 18S rRNA gene has been used for molecular identification of eukaryotic microorganisms, such as fungi (Hejazi *et al*., 2010).

The aim of the work discussed in this Chapter was to determine the microbial diversity by using the molecular identification techniques which include both 16S and 18S rRNA gene sequencing of microbes isolated from various primitive desert surface soils and different rock samples.

3.2. Materials and Methods

General microbiology and specific molecular biology methods were used in the work described in this Chapter. All media and chemical reagents were prepared according to manufacture�given instructions by using dH_2O and stored at 4°C prior to use.

3.2. 1. Sites and descriptions of sampling

Various primitive desert surface soils were collected for microbiology and molecular identification studies. In addition to rock samples (See special methods for collecting rocks samples, Chapter 2 Section 2.2), surface soil desert samples were also used (e.g. from 0-5cm depth). All samples were stored in sterile autoclaved bags at room temperature. The samples studied were: desert surface soils with and without lichen cover from Tabernas in Spain, located in the north of Almerìa (37.05° N, 2.38° W).

Volcanic rocks from the French Indian Ocean Island of Reunion. Located off the southern coast of Africa east of Madagascar (21° 06′ S, 55° 36′ E). Rock samples from Ghar Al Hibashi caves from Saudi Arabia, located 300 km southeast of Makkah in the Nawasif / Buqum (21° 10′ N, 42° 10′ E), (Fig 3.1A). Desert varnish sample from Ashikhara in Oman, located 200 km south of Muscat (21° 51′ 14" N, 59° 34′ 50" E). And rock samples from Jarnan caves in Oman, located 120 km from Muscat (22°55'24"N 57°45'48"E) (Fig 3.1B).

Figure 3.1: Sites of samples (A) Ghar Al Hibashi caves, Saudi Arabia (B) Jarnan caves, Oman.

3.2.2. Initial isolation of desert surface soils and rock samples

Isolation of microorganisms from both soil and rock samples was carried out using LB medium, either liquid or solid forms. Luria- Bertani (LB) medium consisted of 10g tryptone, 10g NaCl and 5g yeast extract in 1L of dH_2O, dissolved and sterilised by autoclaving at 121°C for 15 minutes. For solid media 15g agar Oxoid No1 was added before autoclaving. The pH was then adjusted by addition of 1M NaOH or 1M HCl to pH 7.2.

The initial isolation of bacteria was carried out as follows: 10 g of each desert surface soil samples were added to a flask containing 90 ml of liquid LB medium, whereas, for desert varnish samples and rock samples; two sets of 1 g of each sample were added to 9 ml of liquid media, then, the samples been incubated at two different temperatures (e.g. 25°C and 37°C) with continuous shaking at (250 rpm) for 24 hours. The growth of bacteria was monitored by measuring the OD at 600 nm. Serial dilutions of each sample were prepared and used for plating. Portions of 0.1 ml of these samples were used for plating on solid LB medium and been incubated under similar conditions that for liquid samples. Observing colonies growth allowed the selection of single colonies of the isolated microorganisms, which were then, streaked onto new LB plates and incubated in order to obtain pure cultures; so as to ensure strain purity, the isolations were conducted in triplicate. After the third purification step, a single colony was used to inoculate 50 ml of LB medium, and this sample was then incubated under the similar conditions described above with continuous shaking (e.g. 250 rpm). The isolates were finally stored as stock cultures at 4°C until use. Desert varnish samples were harvested inside "the laminar flow cabinet" by scratching them using sterilised sharp scalpel. UV lamp was switched on for 2 hours prior to and during the experiment in order to reduce contamination.

All rock samples were broken into small pieces by using the cracking vessel (Figure 3.2), which consists of a thick glass-walled vessel, sealed with a metal cap. A plunger passed through the cup and touched the bottom of vessel. The top of the cap is covered with part of an autoclave bag. (Wainwright *et al*., 2009).

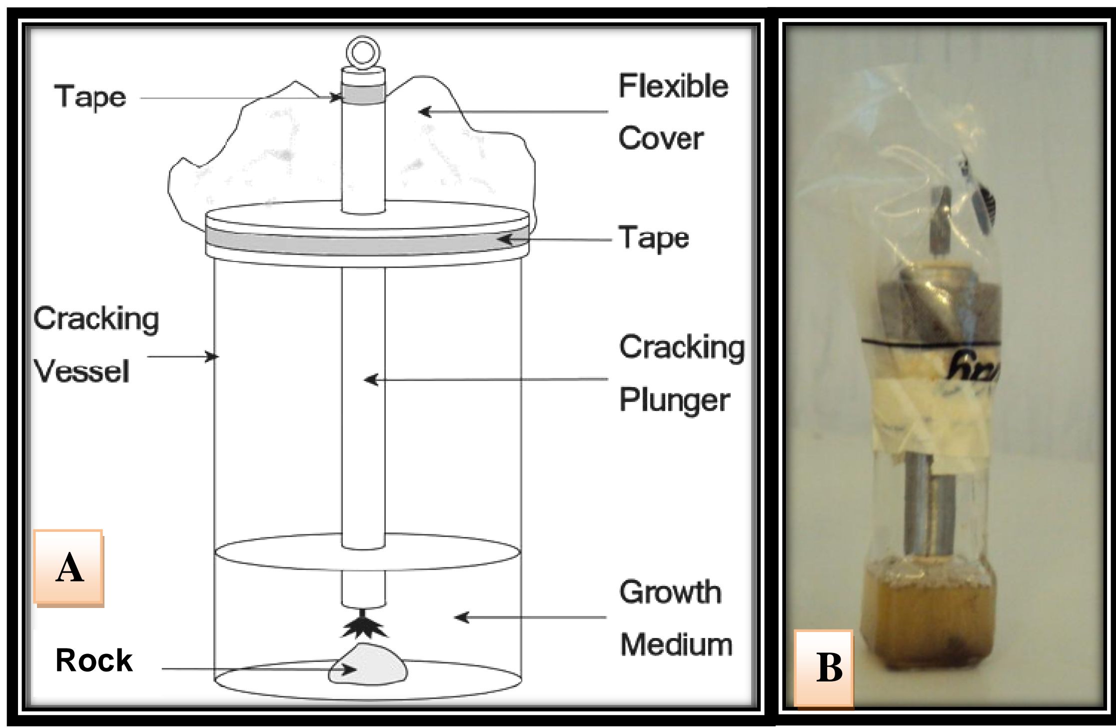


Figure 3.2: The explanatory scheme for cracking vessel (A) and the cracking vessel that used in this experiment (B).

3.2.3. Growth and isolation of oligotrophic fungi from desert varnish samples

Silica gel medium (Table 3.1) was used to isolate oligotrophic fungi from various desert varnish samples. The experiment was conducted as follows: a potion of 20 ml of solution (3) was mixed with 20 ml of the solution (1), followed by addition of 4 ml of solution (2), thoroughly mixed and then poured instantly into Petri–dish (Table 3.1). The gel set in around 15 min; and the plates were left overnight and any water of syneresis was poured off. The plates were then grouped into four based on different colours of varnish; in each plate one type of desert varnish samples was added and the plates were incubated at 25°C for 7-14 days (Parkinson *et al*., 1989).

Table 3.1: Composition silica gel medium

Components	Amount	Comments
1) **<u>Salts Solution</u>** KH_2PO_4 KCl $MgSO_4.7H_2O$ Fe $SO_4.7H_2O$ Milli Q water	 1.00 g 0.50 g 0.50 g 0.01 g 1.0 litre	* pH should be 5.2 *$FeSO_4.7H_2O$ should add after Autoclaving the salt solution at 120°C for 20min.
2)**<u>Orthophosphoric acid 20%</u>** Orthophosphoric acid Milli Q water	 20 ml 80 ml	The solution was autoclaved at 120°C for 20min
<u>3) Potassium silicate</u> KOH Milli Q water Silicic acid	 7.0 g 100 ml 8.0 g	KOH was dissolved in Mill Q water then added Silicic acid .It was autoclaved at 120°C for 20min

3.2.4. Determination of bacterial numbers (CFU)

Microbial counts were determined by plating and directly counting the number of viable microorganisms. (See further details in section 2.2.3). Portions (1.0 g) from each crushed rock sample was suspended in 10 ml of sterile Ringers solution (1/4 strength), shaken for 15 min on an orbital shaker (250 rpm), and then diluted serially with the same solution. An aliquot, 0.1 ml was withdrawn from each dilution series and was spread over the surface of Plate Count Agar (media preparation was described earlier in Chapter two Section 2.2.3); the experiment was conducted in triplicate for each sample and the plates were incubated at (25°C ±0.5°C) for1-3 days in order to enumerate the bacteria (Meintanis *et al.,* 2006).

3.2.5. Molecular identification techniques

3.2.5.1. Genomic DNA extraction for bacteria and fungi

Genomic DNA was extracted from each strain and then PCR was used with use of appropriate primers to produce sufficient quantities of the 16S rRNA or 18S rRNA gene. Genomic DNA was isolated by using (Key prep- Bacterial DNA Extraction Kit) (ANACHEM, labstore, UK) by following procedures prescribed for bacteria; and (Norgen Fungi Genomic DNA Isolation Kit) (GENEFLOW LIMITED, Labstore, UK) by following procedures prescribed for fungi. The genomic DNA was separated by gel electrophoresis on 1% agarose to check for purity.

(A) Procedures (Key prep- Bacterial DNA Extraction Kit)

1- Centrifugation: 3 ml of pure bacteria culture grown overnight in LB medium was collected by centrifugation at 6000×g for 2 minutes. The supernatant was then decanted completely.

2- Re-suspension of pellet: the pellet was re-suspended by adding 100 µl Buffer R1and pipetting it up and down.

3- Lysozyme treatment: 10 µl of lysozme (50mg/ml) was added to the suspension for Gram-negative bacteria but for Gram-positive bacteria should be 20µl of lysozyme. The suspensions were mixed completely and incubate at 37°C for 20 minutes.

4- Centrifugation: the sample was digested by Centrifugation at1000×g for 3 minutes then the supernatant was discarded immediately.

5- Protein denaturation: 180µl of Buffer R2 and 20 µl of Proteinase K were added to the pellet and mixed completely then incubated it in shaking water bath 65°C for 20 minutes.

6- Homogenization: 410 µl of Buffer BG was added to the sample and mixed it thoroughly then incubated at 65 °C for 10 minutes.

7- Addition of ethanol: 200 µl of absolute ethanol was added into the samples and mixed it carefully and immediately.

8- Loading to column: the sample was transferred into a column then centrifuge it at 10,000×g for 1 minute and discard the supernatant.

9- Column washing: 750 µl of wash buffer was gently washed the column and centrifuge at 10,000×g for 1 minute and discard flow through (Wash buffer was diluted by absolute ethanol before use). The column was centrifuged at 10,000×g for 1 minute again to removed residual ethanol.

10- DNA elution: a clean microcentrifuge tube was used to collecting DNA by adding 100 µl of preheated Elution Buffer directly into column membrane and standing for 2 minutes in room temperature then centrifuge at 10,000×g for 1 minute to elute DNA . DNA was stored at -20°C.DNA was set for gel electrophoresis (Section 3.2.5.2) and PCR reaction (section 3.2.5.3).

(B) Procedures (Norgen- Fungi Genomic DNA Isolation Kit)

1- Centrifugation: 50mg of pure fungi culture (wet weight) grown 3 days in Czapek Dox medium was collected by centrifugation at 14,000×g for 1 minute to pellet the cells. The supernatant was decanted carefully.

2- Lysate preparation: the pellet was re-suspended by adding 500 µl of lysis solution and the cells were re-suspended by gentle vortexing (optional RNase treatment) 10 KUnits of RNase was added to the suspension and mixed completely.

3- The samples were transferred into bead tube and vortex horizontally for 5 minutes on a flat bed vortex pad with tape then the samples were incubated at 65°C for 10 minutes.

4- The samples were transferred into a DNase-free microcentrifuge tube and centrifuge it at 14,000 xg for 2 minutes then transferred supernatant to a new microcentrifuge tube (note the volume)

5- Addition of ethanol: equal volume of absolute ethanol was added into the samples and vortex immediately then 300µl of binding solution was added into the samples and vortex.

6- Binding nucleic acids to column: 650µl of the lysate with ethanol was transferred into a column then centrifuge it at 6,000xg for 1 minutes then the supernatant was discard.

7- Column washing: 500 µl of wash solution was washed the column and centrifuge at 6,000×g for 1 minute and discard flow through (wash buffer was diluted by absolute ethanol before use). The column was centrifuged at 6,000×g for 1 minute again to removed residual ethanol. The samples were centrifuged at 14,000xg for 2 minutes and discard the collection tube.

8- DNA elution: Elution tube was used to collecting DNA by adding 100 µl of Elution Buffer directly into column membrane then centrifuge at 6,000×g for 2 minutes to elute DNA. DNA was stored at -20°C for a few days or -70°C for long term storage .DNA was set for gel electrophoresis (Section 3.2.5.2) and PCR reaction (section 3.2.5.3).

3.2.5.2. DNA quantification

There are several methods for examining DNA quantification. Quantification by using a spectrophotometer is one common method (Haque *et al*., 2003). In this method, 98.0 µl of elution buffer (EB) was added to 2.0 µl of the genomic DNA sample, mixed thoroughlly and filled in special UV cuvettes (UVette, eppendorf) and the optical density OD was measured at 260nm using spectrophotometer (Unicam, Hexios). Amount of DNA was calculated by following this equation:

$100 \div \mathbf{2} = 50$ (dilution factor).

$OD_{260} \times 50$ dilution factor = amount of DNA µg/ml.

3.2.5.3. Agarose gel electrophoresis

Separating of DNA fragments was achieved by using 1% agarose gel. These gels were prepared in the following manner. Because of the small size of the BioRad Subcell GT electrophoretic tank with just 6 or 14 well comb, 0.4 g of molecular biology grade agarose was dissolved in 0.8 ml of 50× TAE buffer and 40 ml dH_2O by heating in a microwave on a medium high power for approximately 3 minutes until the agrarose was dissolved and the solution was fairly cooled. Then, 2.5 µl ethidium bromide was added to visualise the DNA before setting the solution in gel tray, followed by pouring the gel in the gel rack. Afterwards, the comb was inserted at one side of the gel and kept at room temperature. The gel was, then, submerged in TAE buffer 1× and samples of 10 µl mixed with 2 µl loading dye were added to the wells. In order to determine the size of fragments, 6 µl of Hyper Ladder was used. The samples were then undergone electrophoresis for 40 min operated at 80V. The DNA was visualized on the gel and digital image was taken using UVitec ‘‘Uvidoc” attached to a digital camera. If there was smearing presence on the loading gel, 1.0 µl of RNAse was added to the whole extracted gDNA and incubated at 4°C overnight.

3.2.5.4. Polymerase chain reaction (PCR) amplification of 16S and 18S rRNA genes

After successful extraction of genomic DNA from unknown microorganisms, 16S and 18S rRNA genes were amplified from the whole DNA extraction by using polymerase chain reaction (PCR). Table 3.2 indicates the amount of components used for amplification of 16S and 18S rRNA genes.

Table 3.2: Components of the PCR reaction

Component	Quantity
Sterile Milli-Q water	35.0-39.0µl
10x Taq buffer	5.0 µl
$MgCl_2$ solution (50mM)	2.5 µl
Forward Primer (10 ppmole.I^{-1})	0.5 µl
Reverse Primer (10 ppmole.I^{-1})	0.5 µl
dNTPs mix (2.5 mM each)	1.0 µl
Genomic DNA (10-100 ng)	1.0-5.0 µl
Ampli Taq polymerase (5U/µl)	0.5 µl

Bacterial and fungal universal primers (Forward and Reverse) were used for DNA amplification in this experiment (Table 3.3). Primers were designed specifically to anneal to a certain region of the proposed DNA to amplify. Primers were purchased from Eurofins (mwg/operone).

Table 3.3: Oligonucleotide primers from Eurofins (mwg/operone) Germany.

Primer name	**Sequence(5`-3`)**	**Target gene**	**Reference**
16SUN1.FOR	CCGAATTCGTCGACAACAG AGGATCCTGGCTCAG (34)	Bacterial 16S rRNA	Weisburg *et al.*, 1991
16SUN1. REV	CCCGGGATCCAAGCTTACG GCTACCTTGTTACGACTT (37)	Bacterial 16S rRNA	Weisburg *et al.*, 1991
Nu-SSU-0817.FOR	TTAGCATGGAATAATRRAA TAGGA(24)	Fungal 18S rRNA	Borneman and Hartin, 2000
Nu-SSU-1196-39.REV	TCTGGACCTGGTGAGTTTC C(20)	Fungal 18S rRNA	Borneman and Hartin, 2000

The thermal cycling order which was used for the amplification of 16S rRNA gene in this experiment was as follows: initial denature at 94°C for 3 min to separate double stranded DNA into two single strands, followed by 30 cycles of DNA denaturation at 94°C for 1 min, and primer annealing at 60°C for 1 min. Afterwards, the temperature was decreased to allow primers to anneal. Strand elongation was conducted at 72°C for 5 min to allow the Taq polymerase to replicate the remaining single strand of DNA. Final elongation was done at 75°C for 5 min. Thermal cycling conditions for amplification of 16S rRNA gene are presented in Table 3.4.

Table 3.4: PCR amplification procedure for 16S rRNA

Steps	**Temperature and time**	**Number of cycle**
Initialization	94°C for 3 min	1
Denaturating	94°C for 1 min	30
Annealing	60°C for 1 min	
Elongation	72°C for 5 min	
Final elongation	75°C for 5 min	1
Hold	4°C	

Similarly, the thermal cycling order for amplification of 18S rRNA gene used in this experiment was as follows: initial denature at 94°C for 2 min, followed by 35 cycles of DNA denaturation at 94°C for 0 sec, primer annealing at 56°C for 10 sec, strand elongation at 72°C for 30 sec. Final elongation at 75°C for 2 min. The thermal cycling conditions for amplification of 18S rRNA gene are presented in Table 3.5.

Table 3.5: PCR amplification procedure for 18S rRNA

Steps	Temperature and time	Number of cycle
Initialization	94°C for 2 min	1
Denaturating	94°C for 0 sec	35
Annealing	56°C for 10 sec	
Elongation	72°C for 30 sec	
Final elongation	75°C for 2 min	1
Hold	4°C	

After finishing the amplification processes of both 16S and 18S rRNA genes, the PCR reaction was examined in electrophoresis on a 1% agarose gels. 10 µl of PCR product was mixed with 2 µl of Blue/Orange 6× loading dye and was run on a 1% agarose gel (as described in Section 3.2.5.3). In addition, 6 µl of 1 Kb Hyper ladder loading in the gel to confirm the correct sized product. Successful PCR products were immediately sent to the Medical School Core Genetics Unit, the University of Sheffield, for further sequencing. Phylogenetic analysis was performed as described below (Section 3.2.5.6).

3.2.5.5. Purification of PCR products

Occasionally, PCR products require clean up to purify single or double stranded DNA fragment from PCR and other enzymatic reactions. QiaQuick Gel Purification Kit (Qiagen Ltd.,UK) was used by following the protocol in manufacturer's instructions:

1- Five volume of buffer PE1were added to 1 volume of the PCR sample and mixed carefully.
2- To bind DNA: the samples were applied to the QiaQuick spin column, centrifuged for 30-60 seconds and the flow through were discarded and the QiaQuick spin column was placed again in the same tube.
3- The PCR product was washed by 750 µl of buffer PE and centrifuged for 30-60 seconds, then the flow through was discarded.
4- The QiaQuick spin column was place back in the same tube and centrifuged for an additional 1min.
5- The QiaQuick spin column was place in clean 1.5 ml microcentrifuge tube and then purified products were eluted by adding 50 µl of buffer EB (10mM Tris-Cl, pH 8.5) to the center of the QiaQuick membrane and centrifuged for 30-60 second. The product was run on 1% agrose gel and 6.0µl of 1Kb Hyberladder 1as a marker to confirm the correct sized product then the PCR product was stored at -20°C for a few days or -70°C for long term storage.

3.2.5.6. Phylogenetic analysis

For the phylogenetic determination of both 16S and 18S rRNA genes sequences were compared using The Basic Local Alignment Search Tool (BLAST) available from the website of National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) (Altschul *et al*., 1997). BLAST is very affirmative for comparing query sequence with the larger numbers of sequence held online at NCBI (Macrae, 2001).

Partial sequences, generated in this experiment, were assembled and the errors of consensus sequences were corrected manually by using Finch TV software (version 1.4) (Fig 3.3). In Finch TV software the unknown nucleotide represents as N, and it

could be either A, or T, or G, or C, according to the different colors appear (Mishra *et al*., 2010).

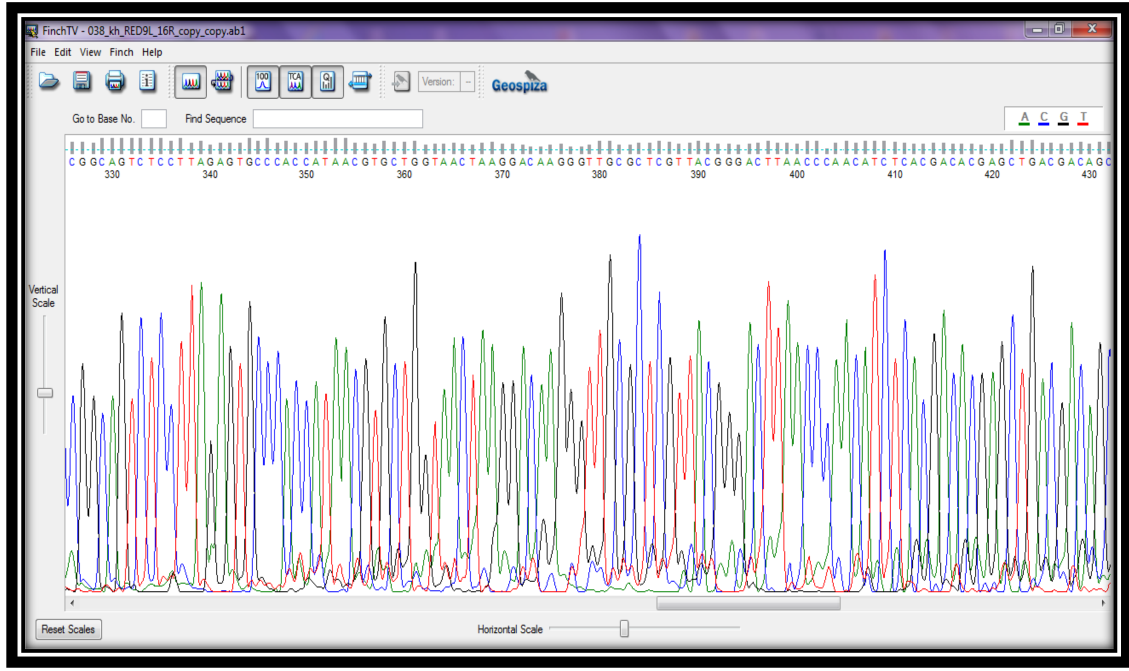


Figure 3.3: Finch TV software (Version 1.4) that manually corrects errors (N) of consent sequences and then exported into BLAST database.

3.3. Results and Discussion

3.3.1. Growth of fungi from rock samples

A range of fungi species were isolated which are able to grow on silica gel media, in which no carbon or nitrogen sources was added. Growth also occurred when the hyphae were transferred from silica gel to Czapek Dox medium, thereby showing that the isolated fungi are facultative oligotrophs, and could acclimatise to growth on nutrient-rich medium. This result is in agreement with the findings reported by Parikinson *et al* (1989), Al-Turk (1990), and Wainwright *et al* (1997).

3.3.2. Counting of colony forming units (C.F.U)

The total count of bacteria was determined in different rock samples by determining the number of culturable bacteria (viable) on the plate count agar obtained from a variety of rock samples as following:

(A) Volcanic rocks from the French Indian Ocean Island of Reunion (Fig 3.4), it is apparent that there were three types of volcanic rock-bacteria based on their rock colour (e.g. black, brown and green "quartz"). Also, there was a gradual decrease in numbers of the bacterial count in various samples of volcanic rocks. The highest counts were found observed in black volcanic rock samples. Meanwhile, quartz volcanic rock samples showed the least numbers of colony forming bacteria. There were no differences among the observed types of bacteria for different examined volcanic rocks samples. Most of the isolated bacteria were species of *Bacillus* (more details are discussed in Section 3.3.5).

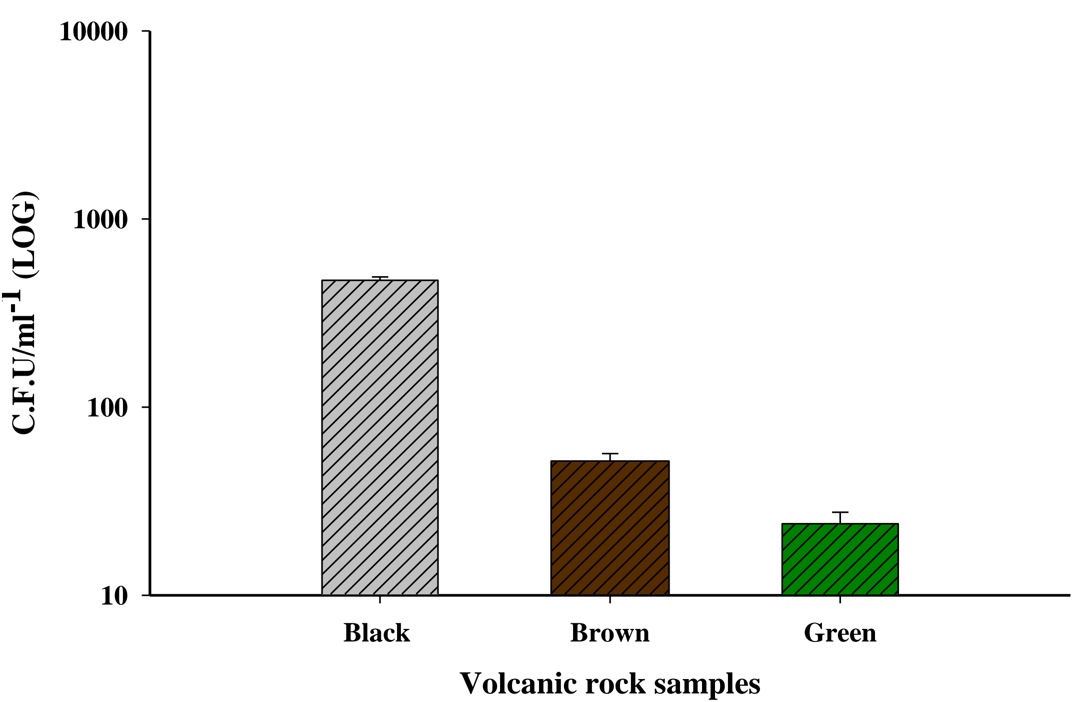


Figure 3.4: The total bacterial count from different volcanic rock sample from the French Indian Ocean Island of Reunion black (▨), brown (▨) and green (▨). (I) refers to Standard Error (SE).

(B) The total bacterial count of rock samples taken from Ghar- Al Hibashi caves in Saudi Arabia are presented in Fig 3.5. Referred to Johaj Pint studies (2006), the

author reported that: “the biological studies have never been carried out in Ghar- Al Hibashi caves in Saudi Arabia”, which means that this study is the first biological study on Ghar- Al Hibashi caves in Saudi Arabia. Fig 3.5 shows clearly that the number of colony forming units in different rock samples in the cave was almost similar. However, the highest bacterial count in the cave was found in the entrance, which was twice the number found for the cave floor.

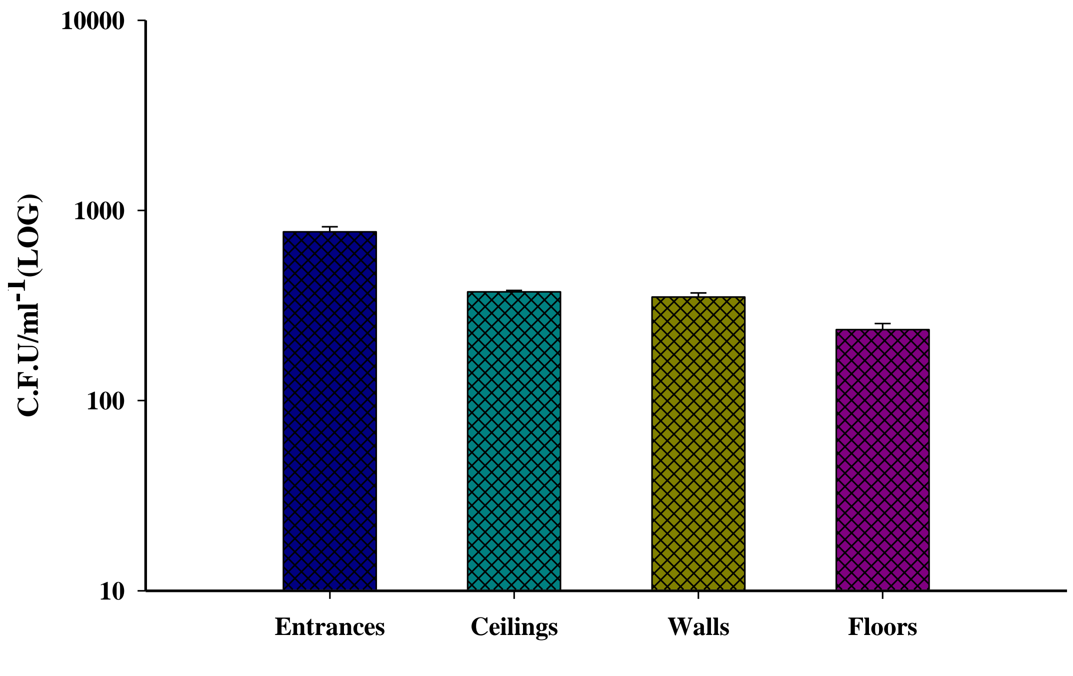


Figure 3.5: The total bacterial cells count from different rock sample from Ghar-Al Hibashi cave main entrance ( ), ceiling ( ), wall ( ) and floor ( ). (I) refers to Standard Error (SE).

(C) The total bacterial count of rock samples from Jarnan caves in Oman is shown in Fig 3.6. It can be clearly seen that the numbers of colony forming units in the cave entrance were higher than that for the walls and the floor. Moreover, the highest bacterial count found in the main entrance was three folds higher than that for the

cave floors and twice the numbers were observed for the cave walls. No literature has been reported for any biological studies conducted on Jarnan caves in Oman.

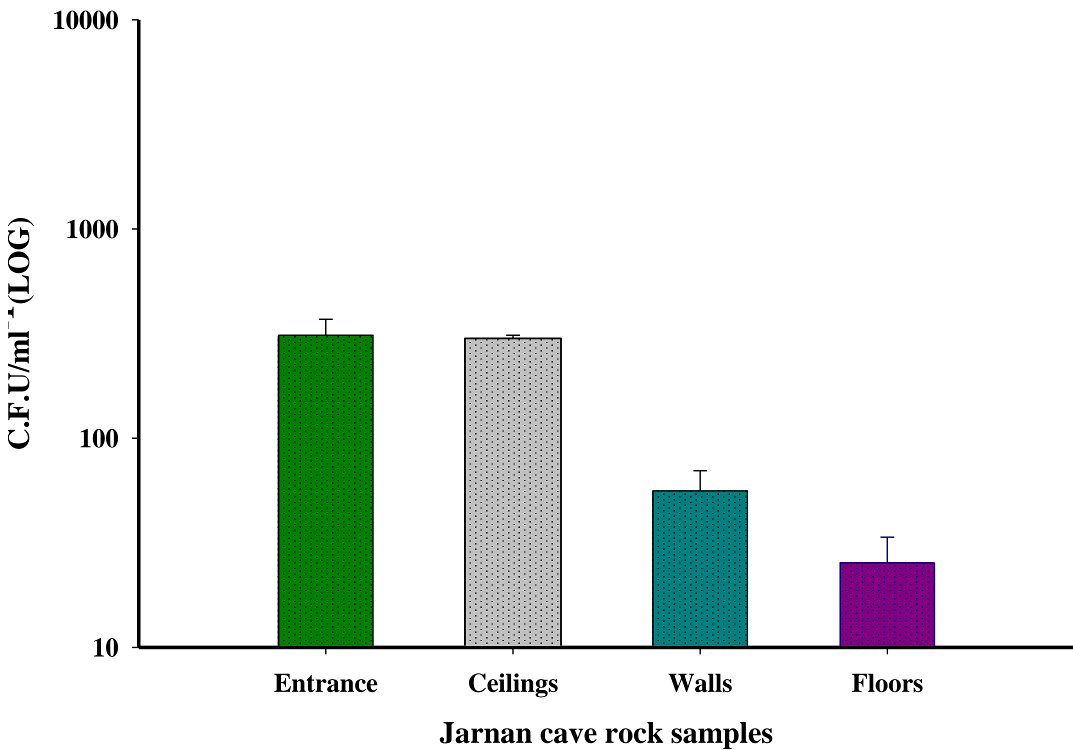


Figure 3.6: the total bacterial count from different rock sample from Jarnan cave in Oman main entrance ( ), ceiling ( ), wall ( ) and floor ( ). (I) refers to Standard Error (SE).

3.3.3. Extraction of genomic DNA

In this study, 35 bacterial strains have been isolated from different desert surface soils and rock samples. The whole genomic DNA has successfully been extracted from 15 strains by using (Key prep- Bacterial DNA Extraction Kit). Fig 3.7 shows an example of a high quality molecular weight of genomic DNA. Hyperladder 1 has been used in these studies to determine the size of DNA molecules (Fig 3.8). As for fungi three strains have been isolated from desert varnish, which were grown on silica gel medium. The isolated strains were transferred from silica gel to Czapek Dox medium,

and the whole genomic DNA has effectively been extracted from two strains by using Norgen- Fungi Genomic DNA Isolation Kit.

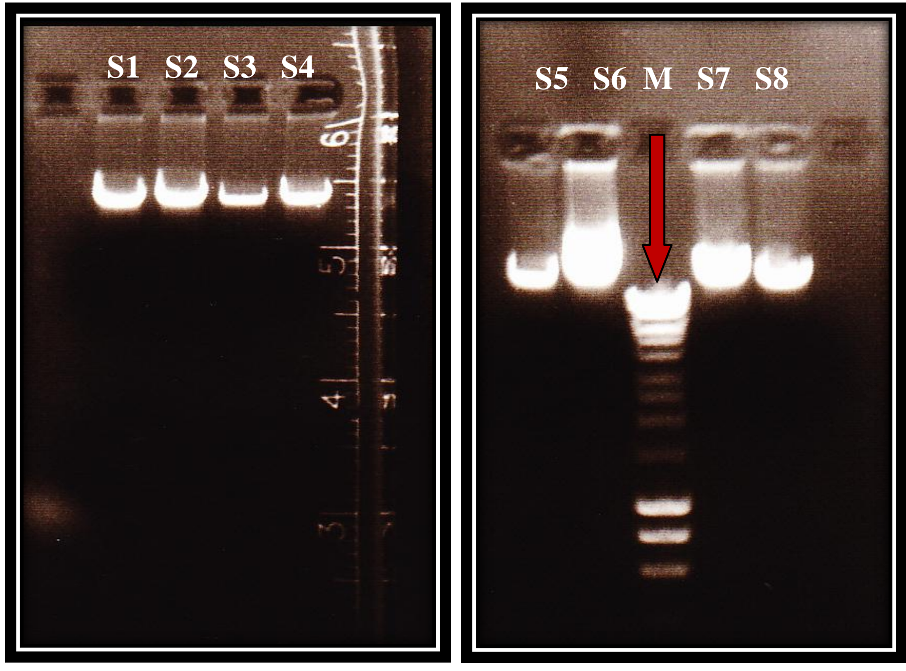


Figure 3.7: Examples of the total genomic DNA extraction (S1-S8) with band size over 1000 base pairs in agarose gel. Red arrow (M) shows the 1-Kb DNA ladder.

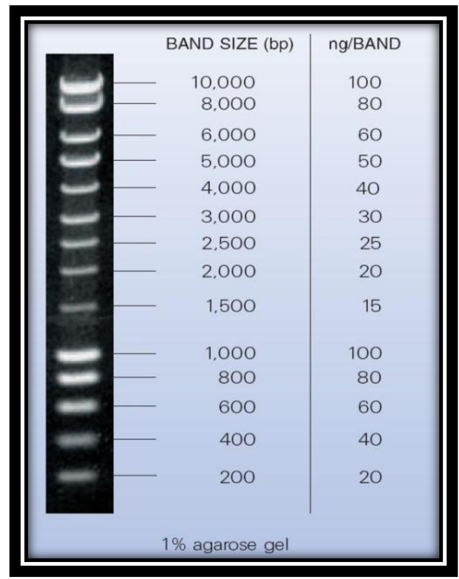


Figure 3.8: Standard Hyperladder I produces a pattern of 14 regularly spaced bands, 10,000-1000bp and each lane (5µl) provides 720ng of DNA.

3.3.4. PCR amplification of extracted DNA

16S rRNA gene sequences have hyper-variable regions where sequences have diverged over time and these are often flanked by strongly conserved regions. Primers targets are conserved regions and amplify variable regions. The DNA sequence gene of 16S rRNA has been determined for a large number of species, forming a readily accessible and extensive library. Amplified 16S rRNA genes are show in Fig 3.9 and the size was banded as expected around 1.5 Kb. On the other hand, the DNA sequence gene of 18S rRNA gene has been determined for two strains and the size was 1.5 Kb.

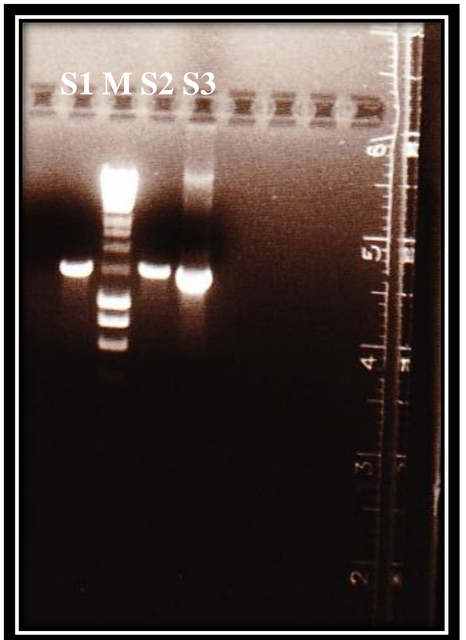


Figure 3.9: The polymerase chain reaction (PCR) on agarose gel (1%) electrophoresis with ethidium bromide indicates (S1-S6) an example for product of 16S rRNA gene (1.5 Kb). Red arrow (M) shows the 1-Kb DNA ladder at 1.5Kb.

3.3.5. Phylogenetic identification of unknown bacteria and fungi

16S and 18S rRNA sequences resulted from diverse strains were determined. The sequences data were used to produce a phylogenetic tree providing the basis for efficient phylogenetic investigation of each genus. Fig 3.10 is an example of phylogenetic analysis of *Bacillus licheniformis* (JCO2) strain whilst the rest of isolates are included in Appendix B. In addition, Table 3.6 shows 16S rRNA sequence and Table 3.7 shows 18S rRNA analyses representing the closest matches of all strains cultured from desert surface soils, desert varnish, volcanic rock samples and cave rock samples. Phylogenetic analysis of JCO2 by using the BLASTN algorithm at NCB1 indicated that a 99% identity to *Bacillus sp* particularly *B. licheniformis* (Fig 3.11) rRNA, (NCBL accession number EF113306.1).

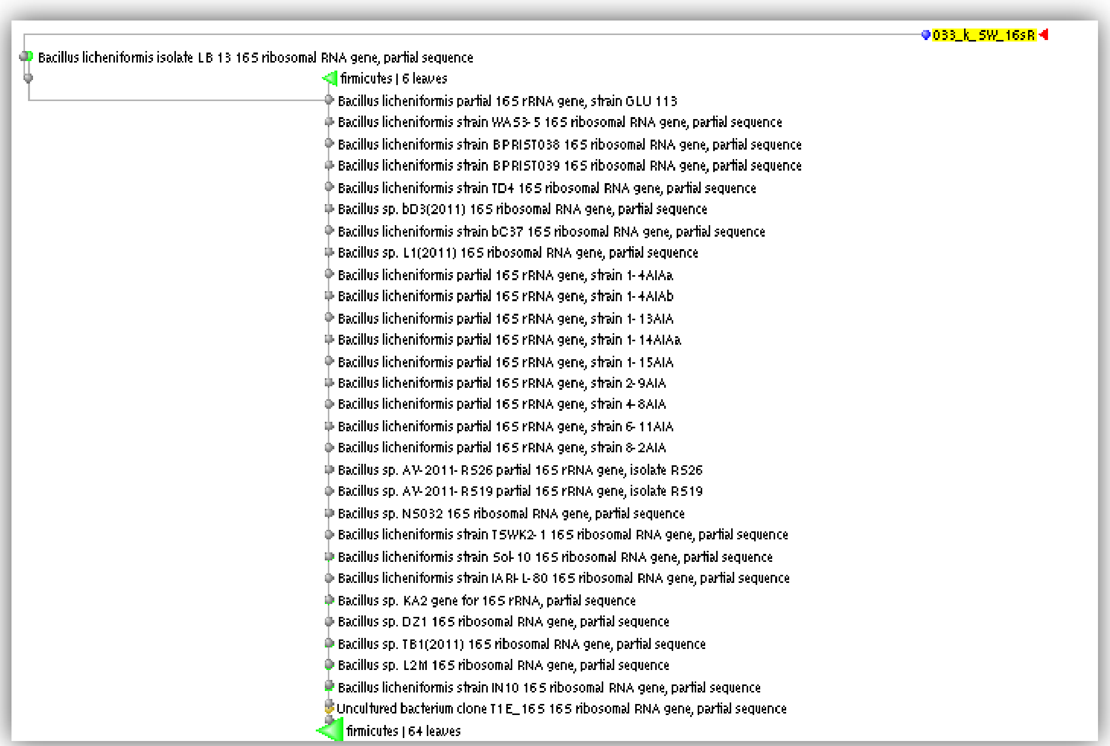


Figure 3.10: Neighbour joining phylogenetic tree of 16S rRNA gene of JCO2 strain Pair wise alignment with a maximum sequence difference of 0.05. Produced by using BLASTN at NCB1.

```
>gb|EF113306.1|  Bacillus licheniformis isolate LB 13 16S ribosomal RNA gene,
partial sequence
Length=1468

 Score =  969 bits (1074),  Expect = 0.0
 Identities = 542/545 (99%), Gaps = 0/545 (0%)
 Strand=Plus/Minus

Query  1     GGTGTTACCAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCAC  60
             |||||||| |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1412  GGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCAC  1353

Query  61    CGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1352  CGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACT  1293

Query  121   GCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAGCCTCGCGGCTTCGCTGCCCTT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1292  GCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAGCCTCGCGGCTTCGCTGCCCTT  1233

Query  181   TGTTCTGCCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGT  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1232  TGTTCTGCCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGT  1173

Query  241   CATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1172  CATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCT  1113

Query  301   GGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1112  GGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGA  1053

Query  361   GCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGAAAGCCCTATCTCTAG  420
             |||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||
Sbjct  1052  GCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGAAGCCCTATCTCTAG  993

Query  421   GGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACA  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  992   GGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACA  933

Query  481   TGCTCCACCGCTTGGGCGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTAC  540
             ||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  932   TGCTCCACCGCTTGGGCGGGCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTAC  873

Query  541   TCCCC  545
             |||||
Sbjct  872   TCCCC  868
```

Figure 3.11: The highest percentage identity matches following BLASTN comparison of *Bacillus sp* 16S rRNA and the NCBI nucleotide collection (nr/nt) database. The "Query " line refers to the input sequence, *Bacillus sp*. 16S rRNA gene, partial sequence whilst the " Subject " line refers to the matching sequence, in this instance *Bacillus licheniformis* small subunit rRNA.

Table 3.6: Summary of 16S rRNA sequence analyses of bacteria cultured from desert surface soils, desert varnish, volcanic rock samples and cave rock samples (representative BLASTN matches).

Samples	Representative sequence	Closest matches Identification	Sequence Identity	NBCl (Accession number)
Desert surface soils with lichen cover	DSL1	*Bacillus sp*	99.0 %	JQ23766672.1
Desert surface soils with lichen cover	DSL2	*Dermacoccus nishinomiyaensis*	99.0 %	NR044872.1
Desert surface soils without lichen cover	DSW1	*Streptomyces sp*	96.0%	GU220477.1
Desert surface soils without lichen cover	DSW2	*Staphylococcus lentus*	99.0%	FJ7955683.1
Desert varnish	DVB1	*Bacillus amyloliquefaciens*	100%	TQ267647.1
Desert varnish	DVS2	*Paenibacillus lautus*	99.0%	AB363733.1
Ghar Al Hibashi caves	GHC1	*Exiguobacterium mexicanum*	99.0%	JF505980.1
Ghar Al Hibashi caves	GHC2	*Bacillus mojavensis*	99.0%	HQ123468.1
Ghar Al Hibashi caves	GHC3	*Bacillus muralis*	99.0%	JQ271812.1
Jarnan caves	JCO1	*Bacillus subtilis*	99.0%	JN856456.1
Jarnan caves	JCO2	*Bacillus licheniformis*	99.0%	EF113306.1
Jarnan caves	JCO3	*Exiguobacterium indicum*	99.0%	JN644531.1
Volcanic rocks	VRB1	*Bacillus cereus*	99.0%	HM133938.1
Volcanic rocks	VRG2	*Bacillus sp*	99.0%	JQ237672.1
Volcanic rocks	VRI3	*Paenibacillus sp*	99.0%	JN650255.1

Table 3.7: Summary of 18S rRNA sequence analyses of oligotrophic fungi cultured from desert varnish (representative BLASTN matches).

Samples	Representative sequence	Closest matches Identification	Sequence Identity	NBCl (Accession number)
Desert varnish	DVO1	*Penicillium corylophilum*	93.0 %	JQ082506.1
Desert varnish	DVO2	*Mucor hiemalis*	98.0 %	HM172832.1

3.4. Conclusions

In general, desert and cave environments have been considered extreme environments, because of their low nutrient accessibility, reasonable temperature and moderately high humidity, in addition to no possible access of light into caves (Hathway, 2010).

The microbial diversity of caves located in the Arabic World has not yet received serious attention from researchers and authorities. In the past, there have been no published literature of any biological studies about the microbial diversity in the Saudi Arabia caves and Oman caves (Pint, 2006).

Methods were used based on the microbial genetic sequences of DNA and RNA and were employed here for the identification of cave microorganism in order to evaluate microbial diversity.16S rRNA gene was sequenced in order to determine the diversity within various samples investigated in this study. The majority of the isolated strains were very closely related to *Bacillus* species (e.g. 99% similarities) from desert surface soils, desert varnish, volcanic rocks, and cave rock samples. The results from cave studies emphasise the need for further investigations on microbial interactions with the cave environment, especially in the Middle Eastern regions.

CHAPTER 4

CHAPTER 4

MOLECULAR IDENTIFICATION AND PHYSIOLOGICAL CHARACTERISATION OF EXTREMOPHILIC BACTERIA FROM WEATHERED VOLCANIC ASH, A NON-EXTREME ENVIRONMENT

4.1. Introduction

In general, extremophiles have been isolated from extreme environments to which they were tailored to grow and survive. Various extremophilic microorganisms have been isolated from non-extreme environments. Extremely thermophilic microorganisms such as, for example *Geobacillus* Sp., have frequently been isolated from cool soil environments (Marchant *et al*., 2002; Banat *et al*., 2004; Marchant *et al.,* 2008). Naturally, the majority of microbiologists attempt to isolate alkaliphiles from alkaline environments; however, alkaliphiles have also been found in neutral and acidic environments (Grant *et al*., 1990). Relatively few reports have however, been published on the isolation of halophiles from non- saline environments such as garden and agricultural soils (Echigo *et al*., 2005;Usami *et al*., 2005).The aim of the work described in this Chapter is to identify and characterise extremophilic bacteria, isolated from non-extreme environments.

Classical microbiology and molecular techniques were employed to identify and characterise the bacteria isolated from volcanic ash soil samples, collected from the French Indian Ocean Island of Reunion (near Mauritius). The area of the volcano from which this bacterium was isolated, has not been recently active. Therefore, it was thought of interest to determine how these bacteria are adapted to grow, or just survive, in a mesophilic environment.

4.2. Materials and Methods

All chemicals were purchased from Sigma, Oxoid, Fisher, Bioline, DIFCO, and AnalaR. All media and reagents were prepared using dH_2O and autoclaved at 121°C for 15 minutes (Unless otherwise stated).

4.2.1. Isolation of microorganisms

Sample collection

A variety of volcanic ash soil samples were collected from the French Indian Ocean Island of Reunion (near Mauritius). The pH of the volcanic ash samples was measured giving a value of pH 7.5, and the temperature was 25°C at the location.

Growth media

Luria- Bertani (LB) medium: medium preparation was described previous in Chapter Three (Section 3.2.2).

M9 Minimal salt medium:(Sigma) was prepared by dissolving 11.3g of M9 (which consisted of 6.8g Na_2PO_4, 3g KPO_4, 0.5g NaCl and 1g NH_4Cl) in 900ml dH_2O. The pH was adjusted to 7.2 with 1M NaOH. The volume was then made up to 980ml with dH_2O and autoclaved.

Preparation of salts supplement: Each of the following was dissolved in water: 1.0 M of $MgSO_4.7H_2O$ in 50ml, 5g of NH_4Cl in 45ml, 15g glucose in 45ml, and 1.0ml of 1mM $CaCl_2.2H_2O$ in 50ml. Each solution was autoclaved and after cooling a 50°C was added to the M9 minimal media as follows: 1.0 ml of $MgSO_4.7H_2O$, 9.0 ml of glucose, 9.0 ml of NH_4Cl and 1ml of $CaCl_2.2H_2O$; this was then made up to a final volume of 1L.

Nutrient Broth: was prepared by suspending 28g containing (Lab-Lemco powder 1g, yeast extract 2g, peptone 5g and NaCl 5g) in 1L of dH_2O and boiled to dissolve completely. The pH was adjusted to pH 7.2 and autoclaved.

Plate Count Agar: media preparation was described earlier in Chapter Two (Section 2.2.3).

Horikoshi medium: consisted of 5g tryptone, 5g yeast extract, 0.2 $Mg_2SO_4.7H_2$ dissolved in 750ml of dH_2O and autoclaved. In addition three solutions were prepared separately; 10g of glucose was added to 100ml dH_2O, 10g Na_2CO_3 was added to 80ml $dH_2O$and 1g KH_2PO_4 was added to 50ml dH_2O.The solutions were autoclaved and cooled at 50°C and added to the Horikoshi medium. The pH was then adjusted to 7.2 by 1M NaOH or 1M HCl.

4.2.2. Initial isolation of TV1, EV2 and SV3

TV1: Approximately, 1g of volcanic ash soil sample was spread onto enrichment LB agar medium(1.5 % agar), and incubated at 70°C under aerobic conditions in a sterilized box, containing tissues moistened with dH_2O to maintain humidity.

EV2 and SV3: Approximately, 1g of volcanic ash soil sample was plated directly onto LB agar medium at different pH values, and incubated under aerobic conditions at 25°C, and 37°C for 24 and 48 hours. The pH values used were 3.0, 4.0, 5.0, 7.0, 0.9, 10.0, and 12.0; single colonies were then streaked onto new LB plates for (TV1). In order to obtain pure cultures, LB medium was used at the same varying pH values to that in EV2 and SV3, followed by incubation under the same conditions. In order to ensure strain purity, these steps were repeated three times. After the third purification step, a single colony was used to inoculate 50ml of LB medium and this was incubated in the same conditions with shaking at (250rpm).The strains were stored as stock cultures at 4°C until used. For long term storage, 800µl of overnight culture in was added to 200µl sterile glycerol; this was mixed thoroughly using a vortex mixer and frozen at -80°C.When the strains were required, they were slowly thawed on the ice for revival; 100µl of thawed stock was then used to inoculate 5ml

of LB broth and left to grow at optimal temperature, with shaking at 250rpm. This step was repeated twice for strain re-establishment and stored at 4°C, until required. Every strain TV1, EV2 and SV3 was sub-cultured once every three weeks in order to reduce the risk of contamination.

4.2.3. Molecular identification techniques

4.2.3.1. Genomic DNA extraction

Genomic DNA was extracted from TV1, EV2 and SV3 using the ANACHEM (Key prep-Bacterial DNA Extraction Kit) the procedure is described in Chapter Three (Section 3.2.5.1). This kit is designed for the quick and efficient purification of genomic DNA from both, Gram-positive and Gram-negative bacteria. The genomic DNA was separated by gel electrophoresis on 1% agarose to check for purity, as described in Chapter Three (Section 3.2.5.3).

4.2.3.2. Polymerase chain reaction (PCR) amplification of 16S rRNA gene

16S rRNA obtained from a genomic DNA extraction was PCR amplified. The procedure was carried out as described in Chapter Three (Section 3.2.5.4). Bacterial universal primers (Forward and Reverse) were used for amplification Table 4.1.Primers were designed specifically to anneal to a certain region of DNA that we wish to amplify. Primers were purchased from Eurofins (mwg/operone)

Table 4.1: Oligonucleotide primers from Eurofins (mwg/operone) Germany.

Primer	Sequence(5`-3`)	Reference
16SUN1.FOR	CCGAATTCGTCGACAACAGAGGATCCT GGCTCAG (34)	Weisburg e*t al.*, 1991
16SUN1. REV	CCCGGGATCCAAGCTTACGGCTACCTTG TTACGACTT (37)	Weisburg e*t al.*, 1991

10µl of PCR product was mixed with 2µl of the 6X Blue/Orange loading dye and separated on 1% agarose. Hyper ladder 1Kb was used to confirm the correct size. The strains were sent to the Medical School Core Genetics Unit, the University of Sheffield for sequencing.

16SrRNA gene sequences were compared using The Basic Local Alignment Search Tool (BLAST) available from the website of National Centre for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov. All sequences were corrected by the Finch TV software and exported into (BLAST) to identify matches, with existing characterised sequences.

4.2.4. Morphological characters

Colony morphology: of TV1, ET2 and SV3 was determined by using overnight cultures grown on agar plates for 24 hours at optimal temperature for each strain.

Gram stain: was preformed then checked using a light microscope (Romano *et al.*, 2005).

Motility assay: TV1, EV2 and SV3 were tested for motility by using semisolid motility test media (LB medium 0.5% agar) in glass test tube. The media was inoculated with each strain by sterile straight needle to roughly one-have the depth of the tube. The tube was incubated at optimal temperature for each strain for 24 and 48 hours (DeFlaun *et al.*, 2007).

4.2.5. Physiological characteristics

4.2.5.1. Response to oxygen

In order to investigate the ability to grow in conditions of oxygen starvation, LB medium plates were streaked with each strain, and placed in an anaerobic jar. This was carried out for each strain followed by incubation at the optimum temperature for 24-48 hours.

4.2.5.2. Catalase and oxidase tests

TV1, EV2 and SV3 strain was tested for catalase by pouring 1ml of hydrogen peroxide over the surface of overnight culture. A positive test for catalase was indicated by bubbles rising over the surface. Oxidase substrate (tetramethyl-p-phenylenediamine hydrochloride 1%) was poured over the surface of each overnight culture. Oxidase positive colonies were indicated by the development of a pink colour which became black after 10 to 30 minutes.

4.2.5.3. Starch, gelatine and fat hydrolysis

Starch hydrolysis: Each strain was inoculated in a plate of starch agar (nutrient agar with the addition of 1% soluble starch). After a 24 hour incubation period, the plates were flooded with iodine solution. Hydrolysis was indicated by the presence of a clear zone around the culture.

Gelatine hydrolysis: Deep stab cultures in nutrient gelatine were made from every strain and incubated at optimal temperature for 24 hours. Cultures presenting liquefaction were refrigerated before being recorded as a positive result.

Fat hydrolysis: sterilised olive oil (5%) was added to the molten nutrient agar. Each strain was inoculated on this agar, and incubated at optimal temperature for 24 hours.

The culture was flooded by Copper (II) sulphate (20%). A deep zone surrounding the microbial growth indicated a positive reaction.

4.2.5.4. Antibiotic resistance profile

Effect of antibiotics on growth of TV, EV2 and SV3: The LB agar plates were inoculated with 1m of overnight culture. The subsequent antibiotics were used at the following concentration in µg/ml Amoxycillin (25), Ampicillin (25), Cefoxitin (30), Chloramphenicol (30), Erythromycin (15), Gentamicn (30), Imipenem (10), Lincomycin (15), Methicillin (10), Penicillin (10), Penicillin (6), Streptomycin (10),

Tetracycline (30) and Vancomycin (30). Each antibiotic disc was placed on agar along with the strain and incubated at the optimum temperature for 24 to 48 hours; this procedure was repeated for each strain.

4.2.5.5. Response to temperature shock

The effect of different temperature (25°C, 37°C, 45°C, 55°C, 65°C and 70°C) on the growth of TV1, EV2 and SV3 was determined by using overnight cultures of LB and M9 media with rotary shaking (250 rpm). Overnight cultures (1ml) from every strain were used to inoculate 50ml of media. Three replicates were set up from LB and M9 media.M9 culture was prepared by using 15ml of overnight LB culture, centrifuged followed by decanting of supernatant. This process was performed three times to make sure that M9 medium was free of remnants of the LB medium. The OD was measured at 600nm.

4.2.5.6. Effects of different temperatures on growth curve of TV1, EV2 and SV3

For TV1 the temperatures at which the organism grew: 45°C, 55°C, 65°C and 70°C. The different temperature for EV2 and SV3 were: 25°C, 37°C, and 45°C. Growth curves were prepared by using overnight culture which was growing at optimal temperature, and measured at OD_{600}.50ml of LB broth was inoculated overnight cultures of each strain at a starting optical density of, 0.200.The OD of the first inoculation was then measured hourly; cultures were maintained on a shaker 250rpm at different temperatures. Growth curves were plotted after 10 hours. (The flasks were returned to shaker as soon as possible measuring the OD in between measurements).

4.2.5.7. Response to pH shock

The effects of different pH (pH 3.0, pH 4.0, pH 5.0, pH 7.0, PH9.0, pH 10.0 and pH12) on the growth of TV1, EV2 and SV3 were determined using overnight cultures in LB. Each strain was used to inoculate LB, Horikoshi and M9 media, and

incubated at optimal temperature with rotary shaking (250rpm).50ml of media at varying pH was inoculated with 1ml of culture from every strain. Three replicates were set up from LB, Horikoshi and M9 media at different pH values. The same procedure as (section 4.2.2.5) was used for M9 media. All the flasks were incubated for 24 hours at the optimal temperature, and then the OD was measured at 600 nm.

4.2.5.8. Effect of different pH on growth curve of TV1, EV2 and SV3.

The effect of the following pH values: pH 5.0, pH 7.0, and pH9.0 on growth curves of TV1, was measured. For the strain EV2 the effect of the following pH values 4.0, pH 5.0, pH 7.0, pH9.0 and pH10.0 on growth curves was measured, and for SV3 the pH range was pH 5.0, pH 7.0, and pH9.0and pH10.0. Growth curves were prepared by using overnight culture growing at the optimal temperature and measured at OD_{600}. 50 ml of LB medium inoculated with overnight cultures of each strain at starting OD of 0.200. As a control, one extra flask at a different pH values was incubated under the same temperature, and aerobic conditions. The pH was amended accordingly over a period of time by the addition of either NaOH 1M or HCl 1M to all the flasks. The OD of the first inoculation was measured immediately, then every hour. The cultures were maintained on a shaker 250rpm at different temperatures. Growth curves were then plotted after 10 hours.

4.2.5.9. Response to salinity shock

The effect of different salinities concentration on the growth of isolated strains TV1, EV2 and SV3, was determined at different concentrations (0.17, 0.50, 1.0, 1.50, 2.0, 2.5and 3.0 M NaCl). Cultures for each strain were shaken overnight at optimum temperature, to provide an OD_{600}. 1ml of culture was used to inoculate 50 ml LB medium at different concentration of salinity in three replicates. Each flask was incubated for 24 hours at the optimal temperature, with shaking (250 rpm); the OD was then measured at 600nm.

4.2.5.10. Adaptation of TV1, EV2 and SV3 at different concentration of (0.17, 0.50, 1.0, 1.50 and 2.0 M NaCl) using M9 minimal salt medium.

The effect of adaptation to salinity on the growth of isolated strains TV1, EV2 and SV3, were determined at different concentration of NaCl. Actively growing culture (1ml) was used to inoculate 50 ml of M9 medium (0.17 M NaCl), followed by incubation at optimal temperature, with shaking for 24 hours. The OD was recorded for each strain, then another 1ml of culture was used to inoculate a further 50ml of M9 medium (0.50 M NaCl), and incubated using the same conditions. Once again, the OD_{600} was measured for each strain and then 1ml from the M9 culture (0.50 M NaCl) was transferred into another 50ml M9 medium (1.0M NaCl). This was again incubated at the same conditions. The same procedure was repeated to grow all strains in M9 medium of (1.50 M and 2.0 M NaCl).

4.2.5.11. Effect of ultraviolet radiation (UV-B and UV-C) on TV1, EV2 and SV3

The UV equipment used included three types of UV lamps (UV-A, UV-B and UV-C).The distance between the sample holder and the lamp was 12cm. The J-2b25 meter was used for controlling the wavelength of radiation during exposure time Fig 4.1. The 8W UV was provided by a commercial low-pressure mercury lamp (model UVGL-25; UV Products, San Gabriel, CA) see (Appendix C). The UV apparatus was used in the attempt to reduce contamination. Overnight cultures for each strain were grown with shaking. These cultures were then serially diluted from $10^{-1}$to 10^{-6} in dH_2O.100µl of the 10^{-5}dilution was transferred to the surface of a sterile fused quartz glass cover slip (2.5cm ×2.5cm).

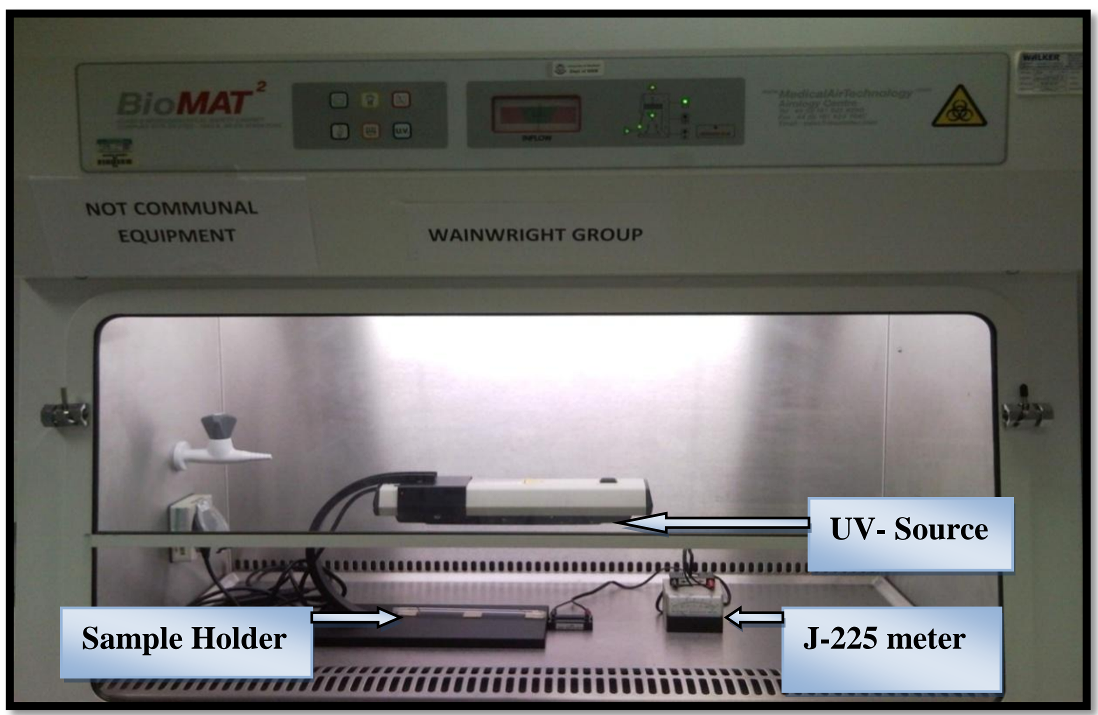


Figure 4.1: The UV exposure apparatus inside the laboratory hood.

After air drying the slide, it was exposed to UV-B or UV-C. The cover slip was washed with 900µl of dH_2O; the resulting aliquot was spread onto agar and incubated at optimal temperature for 24 hours; this procedure was then repeated three times. At time zero, without exposing the strain to UV-B or UV-C, 1ml from the same dilution directly plated onto agar media. The time points for this test were: 30 min, 20 min, 15 min, 10 min, 5 min and 2 min. The other time points used 60 second, 50 sec, 40 sec, 30 sec, 20sec, 10 sec and 5 sec. The result show the number of colony forming units and the ability of these strains to survive when exposed to UV-B and UV-C.

4.2.5.12 Scanning electron microscopy (SEM) and Transmission electron microscopy (TES) studies on morphology of TV1, EV2 and SV3

The procedure was performed by centrifuging 15 ml of overnight culture TV1, EV2 and SV3 at 5,000 g for 30min. The resulting cells pellets were washed using sterilised MilliQ water twice.

For preparing samples to scanning electron microscopy: harvested cells were fixed in 3% Glutaraldehyde in 0.1 M phosphate buffers for 4 hours at 4°C. The specimens were washed twice in 0.1M phosphate buffer 15 intervals at 4°C.Sample fixation was carried out in 2% osmium tetroxide aqueous for 1hour at 25°C.This step was then repeated. Cells were dehydrated in ethanol using the following volumes: 75%, 95% and 100% three times. A final step of 100% ethanol dried over anhydrous copper sulphate for 15 min. For hexamethyldisilazane (HMDS) drying, the samples were absorbed in 100% hexamethyldisilazane for 30 min after the next 100% ethanol stage. The samples were then allowed to air dry overnight and then mounted on 12.5mm diameter stubs and attached with Carbon-Sticky Tabs. This was followed by coating in an Edwards S150B sputter coater with approximately 25nm of gold. The samples were finally examined in a Philips XL-20 Scanning Electron Microscope at an accelerating voltage of 20Kv.

For preparing samples to transmission electron microscopy: harvested cells were fixed in 3% glutaraldehyde in 0.1M phosphate buffer for 4 hours at 4°C,and the specimens washed twice in 0.1 M phosphate buffer with 15 intervals at 4°C. The sample fixation was carried out in 2% osmium tetroxide aqueous for 2 hours at 25°C.This step was repeated and the cells were dehydrated as described above. The samples were then placed in an intermediate solvent, propylene oxide, for two changes of a 15 min period. Infiltration was accomplished by placing the cells in a 50/50 mixture of propylene oxide/Araldite resin. The samples were then left in this 50/50 mixture overnight at room temperature. Once this incubation was finished, the samples were transferred to full strength Araldite resin, and left for 6-8 hours at room temperature, followed by embedding in fresh Araldite resin for 48-72 hours at 60°C. Semi-thin sections, approximately 0.5µm thick were cut on a Reichert Ultracut E

ultramicrotome, and stained with 1% toluidine blue in 1% borax. Ultrathin sections, approximately 70-90nm thick, were then cut using on a Reichert Ultracut E ultramicrotome and stained for 25 minutes with 3% uranyl acetate in 50% ethanol followed by staining with Reynold's lead citrate for 25 minutes. The sections were examined using a FEI Tecnai Transmission Electron Microscope at an accelerating voltage of 80Kv (Alnaimat, 2011).

4.3. Results and Discussion

4.3.1. 16S rRNA sequencing

Three strains of unknown bacteria were isolated from volcanic ash soil samples obtained from the French Indian Ocean Island of Reunion (near Mauritius)), they were designated TV1, EV2 and SV3. Each strain was grown at optimal temperature with shaking at (250 rpm) for 24 hours. The extraction of genomic DNA from the strains using (Key prep- Bacterial DNA Extraction) and 16S rRNA gene amplification by PCR was confirmed by electrophoresis Fig 4.2.

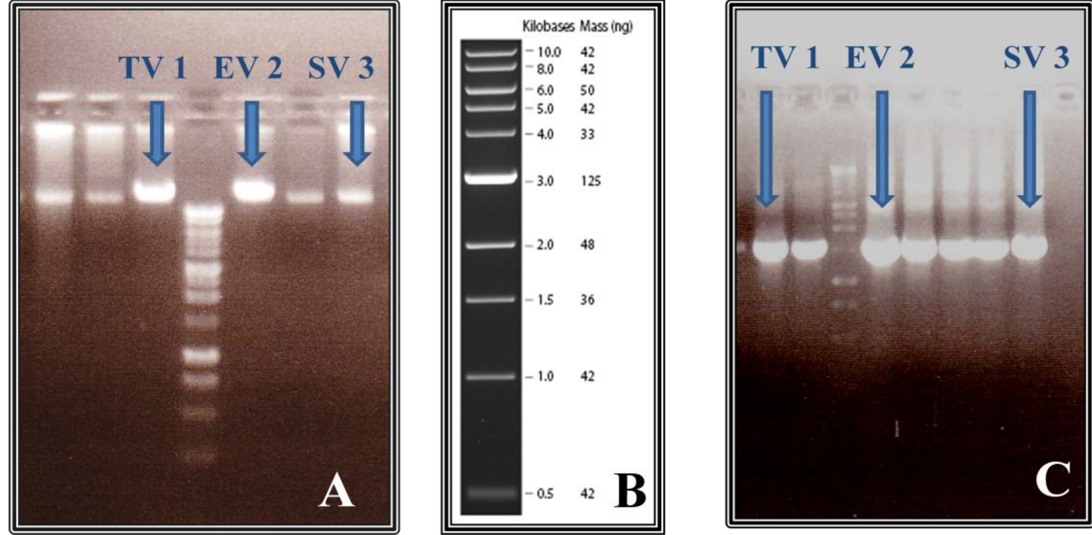


Figure 4.2: (A) Successful extraction of complete genomic DNA of unknown bacteria (B) 1Kb DNA ladder (C) product of 16S rRNA gene (1.5 kb) from (PCR).both figure (A and C) on agrose gel (1%) electrophoresis and detected by staining with ethidium bromide.

4.3.2. Phylogenetic identification of unknown organisms

16S rRNA sequences from TV1, EV2 and SV3 were determined and the sequence data was used to produce a phylogenetic tree providing the basis for efficient phylogenetic investigation, of each genus. Fig 4.3 is an example of phylogenetic analysis of *Geobacillus thermoleovorans* (TV1) strain whilst *Enterobacter mori* (EV2) and *Pseudomonas putida* (SV3) are plotted in Appendix B. In addition (Table 4.2) shows 16S rRNA sequence analyses representing the closest matches of TV1, EV2 and SV3 cultured from volcanic ash soil.

Generally, the phylogentic tree, identified by using ribosomal RNA sequences, established the closeness of phenotypically defined TV1 strain, an exception was thermophilic bacterium. Phylogenetic analysis of TV1 by using the BLASTN algorithm at NCB1 indicated a 99% to *Geobacillus sp* particularly *G. thermoleovorans* Fig 4.4 rRNA ,(NCBL accession number AJ564614.1)

Table 4.2: 16S rRNA sequence analyses of TV1, EV2 and SV3 cultured from volcanic ash soil.

Samples	**Representative sequence**	**Closest matches Identification**	**Sequence Identity**	**NBCl (Accession number)**
Volcanic ash	TV1	*Geobacillus thermoleovorans*	99.0 %	AJ564614.1
Volcanic ash	EV2	*Enterobacter mori*	99.0 %	EU721605.2
Volcanic ash	SV3	*Pseudomonas putida*	100%	AF291048.1

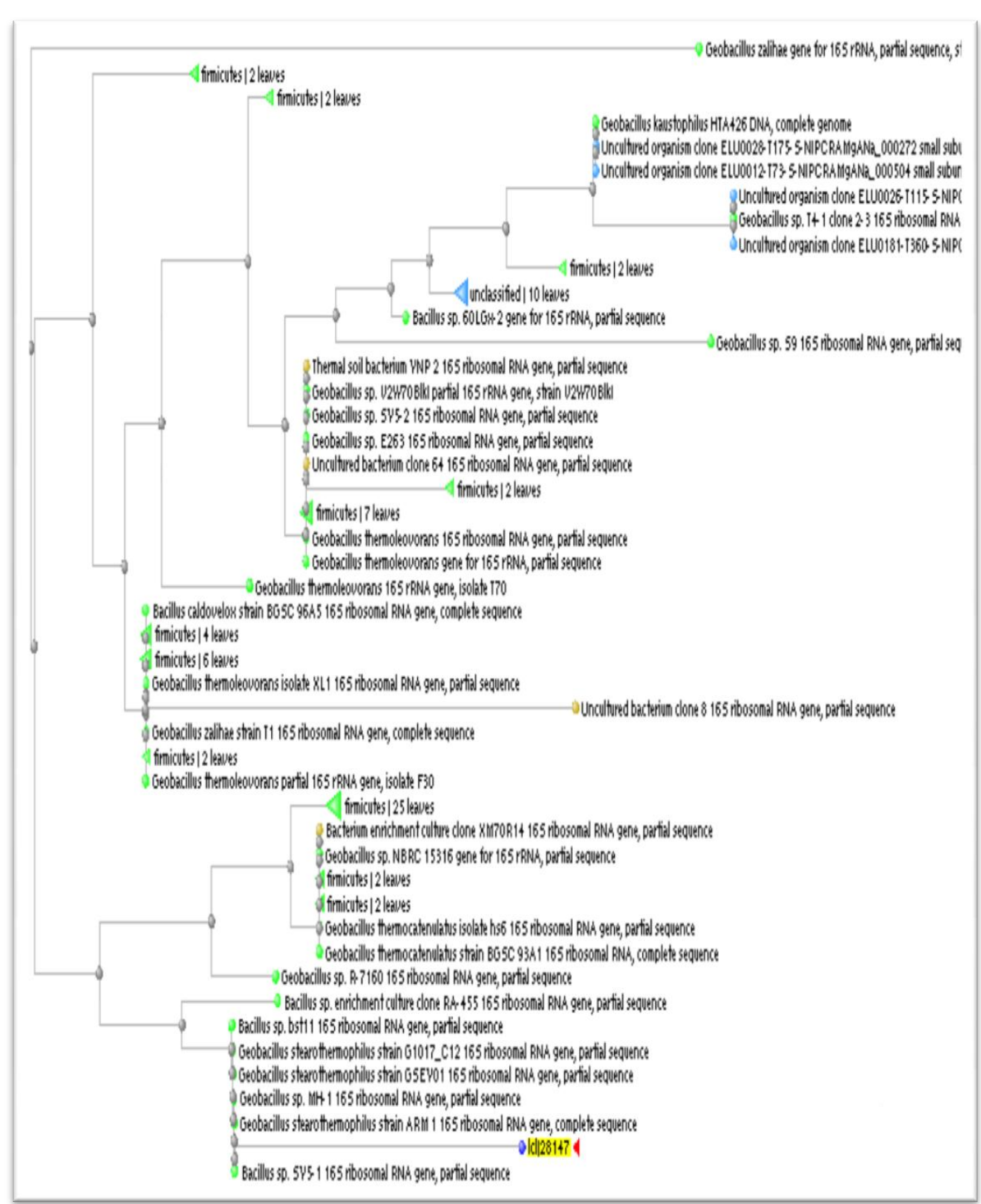


Figure 4.3: Neighbour joining phylogenetic tree of 16S rRNA gene of TV1 strain

Pair wise alignment with a maximum sequence difference of 0.05. Produced by using BLASTN at NCB1.

```
>emb|AJ564614.1|  Geobacillus thermoleovorans partial 16S rRNA gene, isolate T7
Length=1521

 Score =  961 bits (520),  Expect = 0.0
 Identities = 524/526 (99%), Gaps = 0/526 (0%)
 Strand=Plus/Minus

Query  1     GCGGCTGGCTCCCTTGCGGGTTGCCTCACCGACTTCGGGTGTTGCAAGCTCTCGTGGTGT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1475  GCGGCTGGCTCCCTTGCGGGTTGCCTCACCGACTTCGGGTGTTGCAAGCTCTCGTGGTGT  1416

Query  61    GACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1415  GACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTA  1356

Query  121   CTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGAGCGGCTT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1355  CTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGAGCGGCTT  1296

Query  181   TTTGGGATTCGCTCCCCCTCGCGGGTTCGCAGCCCTTTGTACCGCCCATTGTAGCACGTG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1295  TTTGGGATTCGCTCCCCCTCGCGGGTTCGCAGCCCTTTGTACCGCCCATTGTAGCACGTG  1236

Query  241   TGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGACTTG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1235  TGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGACTTG  1176

Query  301   TCGCCGGCAGTCCCTCTAGAGTGCCCAACTTCGTGCTGGCAACTAGAGGCGAGGGTTGCG  360
             ||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  1175  TCGCCGGCAGTCCCTCTAGAGTGCCCACCTTCGTGCTGGCAACTAGAGGCGAGGGTTGCG  1116

Query  361   CTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1115  CTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACC  1056

Query  421   TGTCACCCTGTCCCCCGAAGGGGAACGCCCAATCTCTTGGGTTGTCAGGGGATGTCAA  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1055  TGTCACCCTGTCCCCCGAAGGGGAACGCCCAATCTCTTGGGTTGTCAGGGGATGTCAA  996

Query  481   GAACTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTC  526
             || |||||||||||||||||||||||||||||||||||||||||||
Sbjct  995   GACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTC  950
```

Figure 4.4: The highest percentage identity matches following BLASTN comparison of *Geobacillus sp* 16S rRNA and the NCBI nucleotide collection (nr/nt) database. The “Query “ line refers to the input sequence, *Geobacillus sp*. 16S rRNA gene, partial sequence whilst the “ Subject “ line refers to the matching sequence, in this instance *Geobacillus thermoleovorans* small subunit rRNA.

4.3.3. Morphological and physiological characteristics

Various biochemical tests were carried out on the three strainsTV1, EV2 and SV3, isolated from volcanic ash soil samples. Standards methods were used to characterise the strain morphologically and physiologically, the results are given in Table 4.3.

Table 4.3: A summary of the basic characteristics of *G. thermoleovorans* (TV1), *E. moir* (EV2) and *P. putida* (SV3).

Characteristic	***Geobacillus thermoleovorans* (TV1)**	***Enterobacter moir* (EV2)**	***Pseudomonas putida* (SV3)**
Morphology			
Cell-shape	Long-rod	Rod	Rod
Colony-colour	Cream	Light yellow	Colourless
Cell width(µm)	0.7-1.0	0.3-1.0	0.5-1.0
Cell length(µm)	1.5-3.5	0.8-2.0	1.0-5.0
Gram staining	Positive	Negative	Negative
Motility	Non-motile	Motile	Motile
Response to oxygen	Aerobic	Facultative	Facultative
Catalase test	Positive	Positive	Positive
Oxidase test	Positive	Negative	Positive
Starch hydrolysis	Positive	Negative	Negative
Gelatine hydrolysis	Positive	Positive	Negative
Fat hydrolysis	Positive	Negative	Negative
Maximum growth temperatures (°C)	70	55	45
Minimum growth temperatures (°C)	45	25	25
pH	5.0-9.0	4.0-10.0	5.0-9.0
NaCl M	0.17-1.0	0.17-1.5	0.17-1.0
UV-B resistant	50 second	1minuets	1 minuets
UV-C resistant	Non-resistant	20 second	20 second

The physiological characteristic of the antibiotic resistance profile for *G. thermoleovorans* (TV1), *E. mori* (EV2) and *P. putida* (SV3) are given in Table 4.4, which shows the antibiotics and the concentration used and the inhibition zone obtained for each strain.

Table 4.4: The effect of antibiotics on growth of *G.thermoleovorans, E. mori* and *P.putida* on LB medium.

Antibiotic	**Abbreviation, concentrations**	***G.thermoleovorans* zone of inhibition (mm)**	***E. mori* Zone of inhibition (mm)**	***P.putida* Zone of inhibition (mm)**
Amoxycillin	AML 25µg	-	-	-
Ampicillin	AMP 25 µg	-	-	2.5mm
Cefoxitin	FOX 30 µg	2.8mm	-	-
Chloramphenicol	C 30 µg	3.3mm	1.3mm	-
Erythromycin	E 15µg	2.4mm	-	0.5mm
Gentamicn	CN 30µg	2.0mm	1.3mm	1.3mm
Imipenem	IPM 10µg	3.5mm	2.0mm	-
Lincomycin	My 15µg	0.6mm	-	-
Methicillin	M 10µ	-	-	-
Penicillin	P10µg	-	-	-
Penicillin	P 6µg	-	-	-
Streptomycin	S 10µg	2.6mm	-	-
Tetracycline	TE 30µg	3.0mm	1.0mm	1.0mm
Vancomycin	VA 30µg	2.5mm	-	-

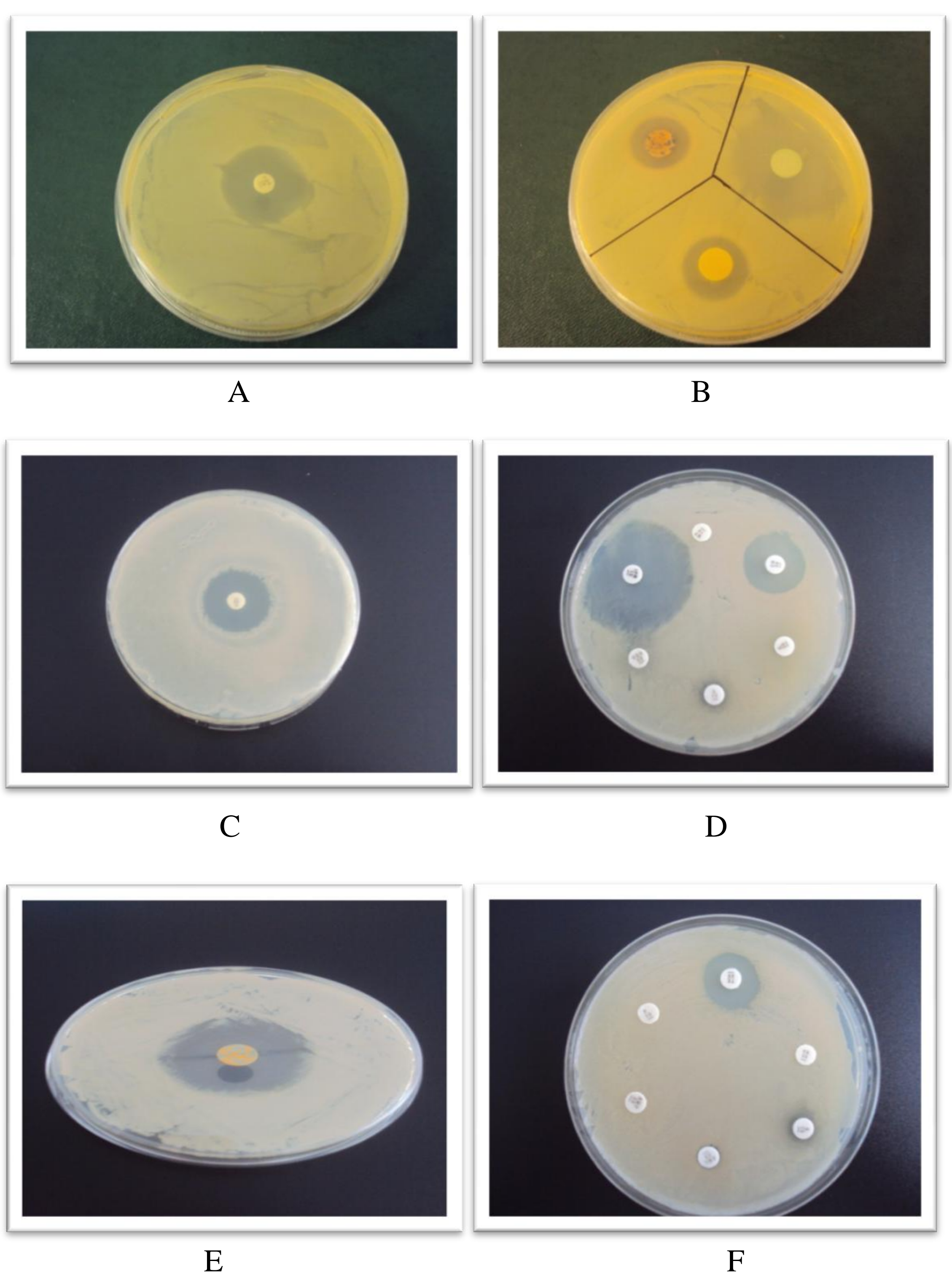


Figure 4.5: The effect of antibiotics (A) Fox 30µg (B) VA 30 µg, C30µg and E15µg on *G. thermoleovorans* (C) C30µg (D) CN30µg and IPM 10µg on *E. mori* (E) IPM 10µg and (F) TE 30µg on *P. putida*; all strains grown on LB medium for 24 hours.

4.3.4. Physiological characteristics of *Geobacillus thermoleovorans* (TV1)

4.3.4.1. Growth response of *G. thermoleovorans* (TV1) to different temperature and different media

The effect of a range of temperatures and different media on the growth of *G. thermoleovorans* is shown in Fig 4.6. *G. thermoleovorans* was grown for 24 hours with rotary shaking (250 rpm) and the resultant optical density was measured at 600nm. The result shows that *G. thermoleovorans* was able to grow on rich LB medium and minimal salts media, M9. The strain was grown at 45°C to 70°C with 55°C being optimal in both media. This result agrees with findings of Sunna *et al*., (1997) and Nazina *et al.,* (2001). No growth was observed at 25°C and 37°C in LB or M9 media for 24 hours and 48 hours. In conclusion, *G. thermoleovorans* is a thermophilic bacterium, isolated from a non-extreme temperature environment and is unable to grow at temperatures lower than 45°C.

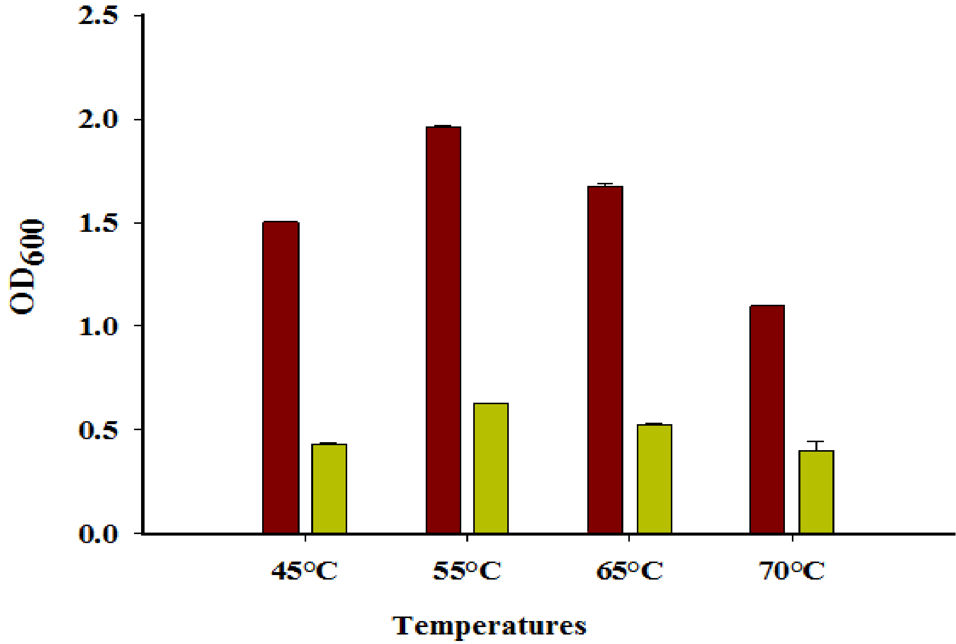


Figure 4.6: The effect of different temperatures (45, 55, 65 and 70°C) on the growth of *Geobacillus thermoleovorans* using LB ( ) and M9 ( ) minimal salt media. (I) refers to Standard Error (SE).

4.3.4.2. Growth curve of *G. thermoleovorans* (TV1) at different temperatures (45°C, 55°C, 65°C and 70°C) in LB medium at pH 7.0 and 0.17M NaCl.

It can be clearly seen from Fig 4.7 that temperature determined the rate of the growth of the bacterium, with the highest growth observed at 55°C. The lowest growth was shown at 70°C after 10 hours. However, there was good growth after 24 hours at 70°C and the optical density was (1.244). *G. thermoleovorans* demonstrated the ability to grow at range (45°C -70°C). It can be concluded that *G. thermoleovorans* is as thermopile since it requires a temperature of around 55°C for optimum growth and was unable to grow outside the temperatures range of 45°C-70°C (Graham *et al*., 2006). These findings are in agreement with those of Souza and Martins (2001).

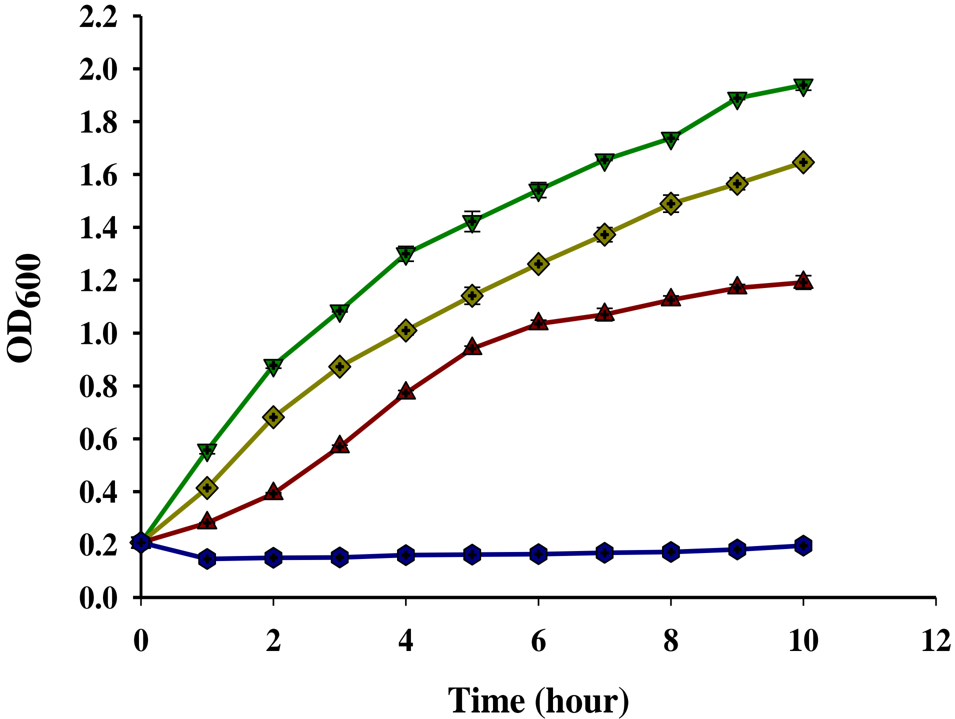


Figure 4.7: Growth curves of *Geobacillus thermoleovorans* grown at different temperature; 45 °C ( ), 55°C ( ), 65°C ( ) and 70°C ( ) measured at optical density of 600 nm. (I) refers to Standard Error (SE).

4.3.4.3. Growth response of *G. thermoleovorans* (TV1) to different pH and different media

The effect of different pH values on the growth of *G. thermoleovorans* on different media is presented in Fig 4.8. *G. thermoleovorans* was grown for 24 hours and the OD_{600} measured. *G. thermoleovorans* was capable of growth on rich LB medium as well as Horikoshi medium. It also grew on minimal salts medium M9. The strain was grown at pH 5.0 to pH 9.0 with pH 7.0 being optimal in each media. There was no observable growth under alkaline conditions (pH10.0 and pH 12.0) or acidic conditions (pH 3.0, pH 4.0) in LB, Horikoshi or M9 media for 24 hours or 48 hours.

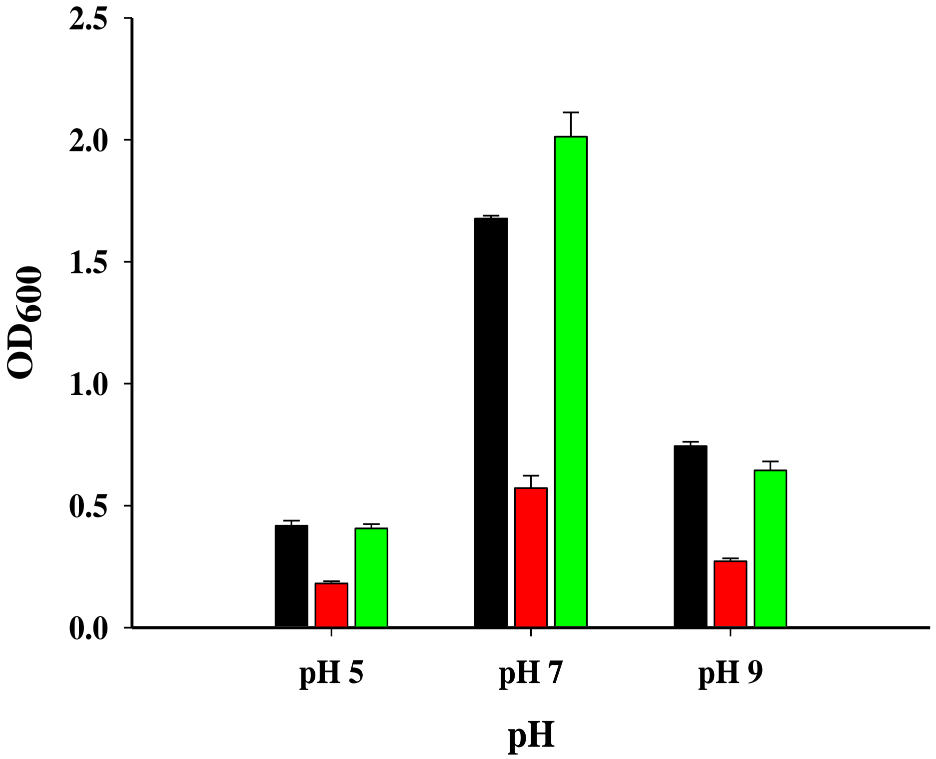


Figure 4.8: The effect of different pH (pH 5.0, pH 7.0, and pH 9.0) on the growth of *Geobacillus thermoleovorans* using LB ( ), Horikoshi ( ) and M9 minimal salts media ( ). (I) refers to Standard Error (SE).

4.3.4.4. Growth curve of *G. thermoleovorans* (TV1) at different pH values (pH 5.0, pH 7.0 and pH 9.0) in LB medium at 55°C and 0.17M NaCl.

The result shows that the optimal growth of the isolate occurred at neutral (pH7.0) conditions (Fig 4.9). Moreover, there was good growth at alkaline conditions pH 9.0 after 5 hours and steady growth at pH 5.0 during 10 hours. *G. thermoleovorans* can survive at pH 5 after 24 hours. In conclusion, *G. thermoleovorans* can grow in media at neutral pH and can tolerate pH values between 5.5 and 8.0. (Romano,*et al*., 2005)

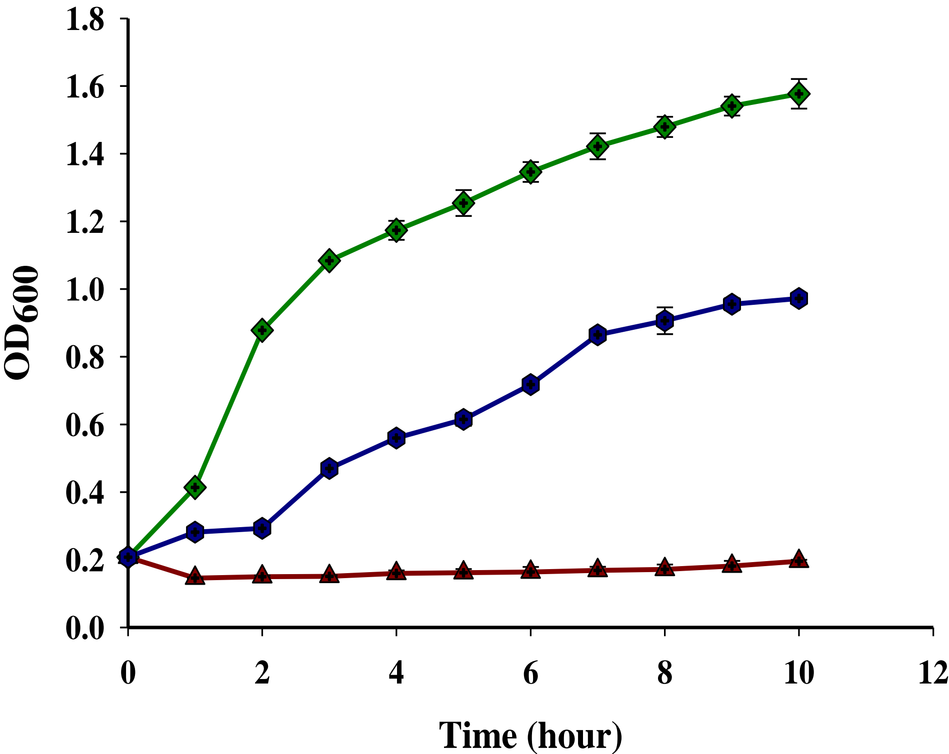


Figure 4.9: Growth curves of *Geobacillus thermoleovorans* grown at different pH values; pH 5.0 ( ), pH 7.0 ( ) and pH 9.0 ( ) measured at optical density of 600 nm. (I) refers to Standard Error (SE).

4.3.4.5. The effect of different NaCl concentration (0.17, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 M) on the growth of *G. thermoleovorans* (TV1) using LB and M9 media

The result shows that there was a considerable growth of the isolate at 0.17 M NaCl, and good growth over the concentration range of 0.5M to 1.0M NaCl (Fig 4.10), in both media. On the other hand, *G. thermoleovorans* shows a dramatic decrease in growth at salinities from 1.5 M to 3.0M NaCl. It can be concluded that *G. thermoleovorans* does not require NaCl for growth however; it was able to grow in a NaCl concentration of 1.0M NaCl. The optimum growth was detected at 0.4M NaCl (Romano,*et al.*, 2005); *G. thermoleovorans* has been shown to tolerate NaCl concentrations from 0.0 to 2.1 M (DeFlaun *et al.*, 2007).

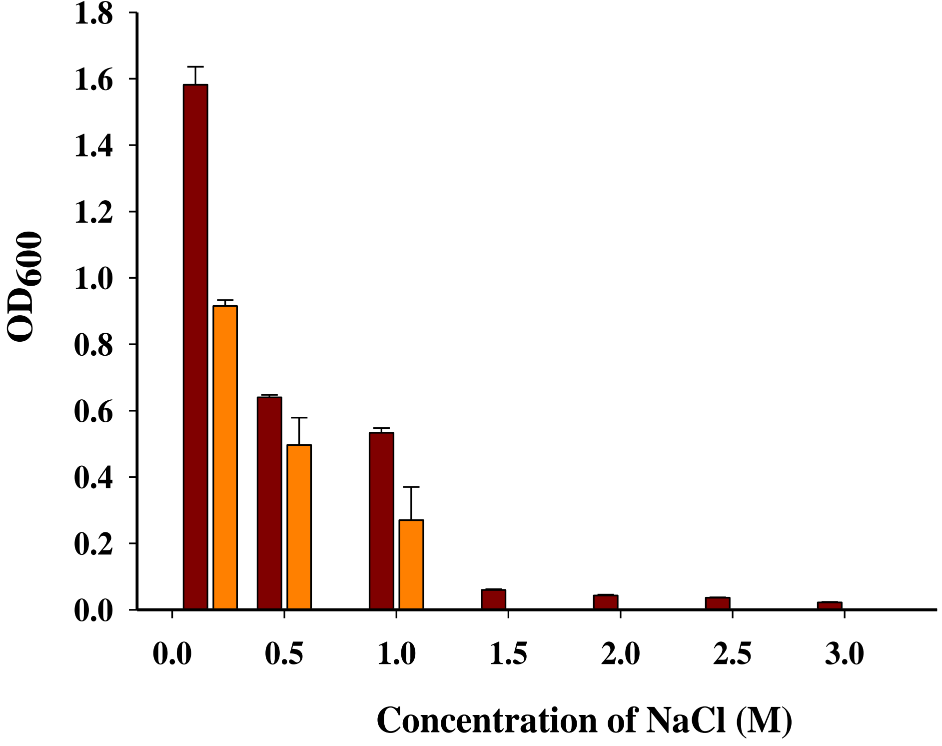


Figure 4 .10: The effect of different NaCl concentrations (0.17, 0.5, 1.5, 2.0, 2.5 and 3.0 M) on the growth of *Geobacillus thermoleovorans* using LB ( ) and M9 ( ) media. (I) refers to Standard Error (SE).

4.3.4.6. The effect of UV (B and C) on the number of colony forming units (CFU) of *G. thermoleovorans* (TV1).

The number of colony forming units of *G. thermoleovorans* found after exposure to UV-B and UV-C, along with the total count before radiation exposure to UV-B or UV-C, is presented in Fig 4.11. It can be clearly seen that *G. thermoleovorana* cannot survive when exposed to UV-C radiation from 5 to 60sec. However, *G. thermoleovorana* CFU decreased with increasing doses of UV-B radiation and was not resistant to 50sec. exposure. In conclusion, the effect of UV-C significantly reduced CFU, while UV-B decreased the CFU with increasing length of exposure.

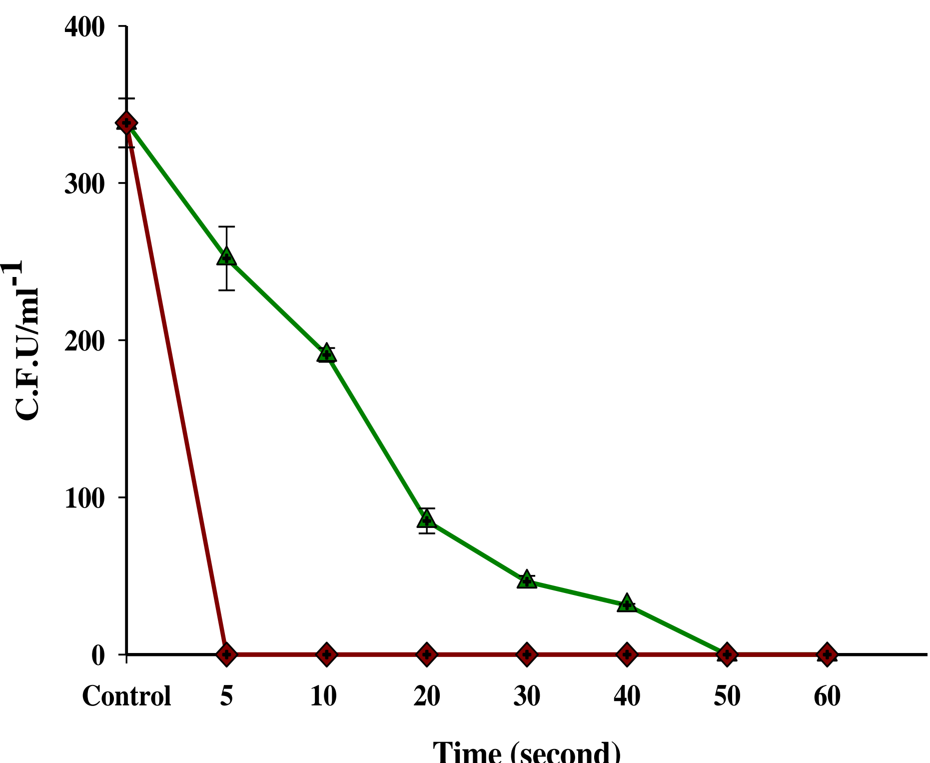


Figure 4.11: Effect of UV-B ( ) and UV-C ( ) on the number of colony forming units of *Geobacillus thermoleovorans.* (I) refers to Standard Error (SE).

4.3.4.7 External morphology of *G. thermoleovorans* (TV1) observed by scanning electron microscopy (SEM).

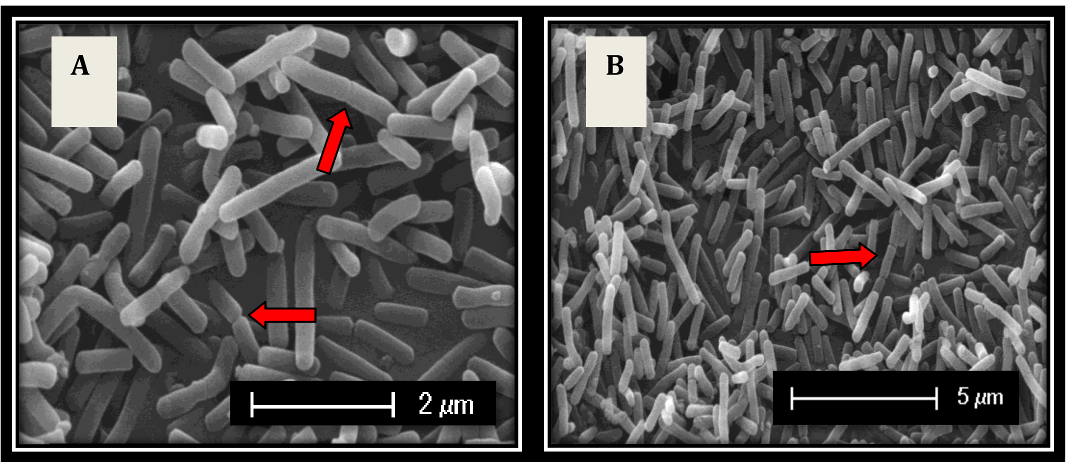


Figure 4.12: Scanning electron micrograph showing the morphology of the *Geobacillus thermoleovorans* (A) scale marker 2µm. (B) scale marker 5µm. Note red arrows show long rods undergoing division cells.

4.3.4.8 Internal morphology of *G. thermoleovorans* (TV1) observed by transmission electron microscopy (TEM).

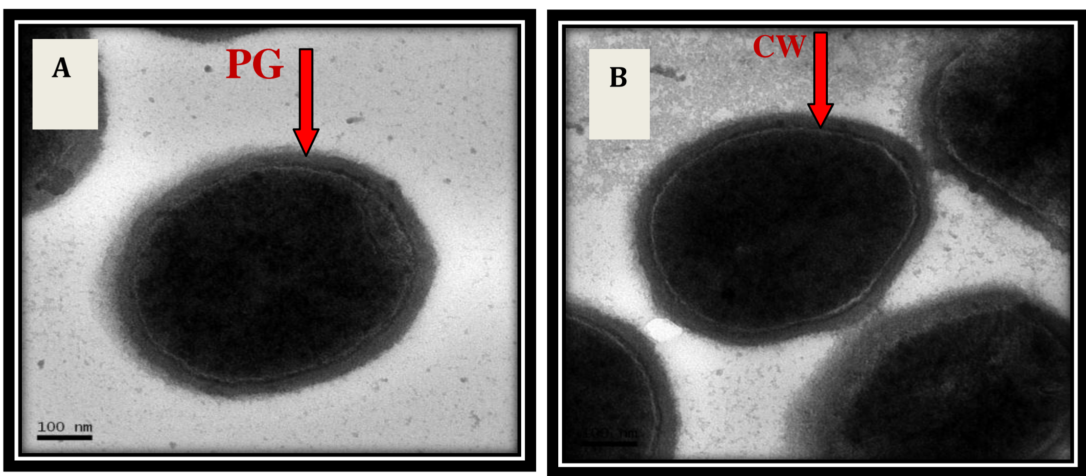


Figure 4.13: Transmission electron micrograph showing the morphology of the *Geobacillus thermoleovorans* (A) and (B) scale marker 100nm. Note red arrows (A) showing (PG) peptidoglycan layer, (B) showing (CW) cytoplasmic membrane.

4.3.5. Physiological characteristics of *Enterobacter mori* (EV2)

4.3.5.1. Growth response of *E. mori* (EV2) to different temperatures and different media

The effect of a range of temperatures and media on the growth of *E. mori* is shown in Fig 4.14. *E. mori* was grown for 24 hours with rotary shaking (250rpm) and measured at OD of 600nm. The result shows that *E. mori* was able to grow on rich media such as LB and minimal salts media, M9. The strain was grown at 25°C to 55°C, with 37°C being optimal in both media. There was no observed growth at 15°C and 65°C in LB or M9 media for 24hours and 48 hours.

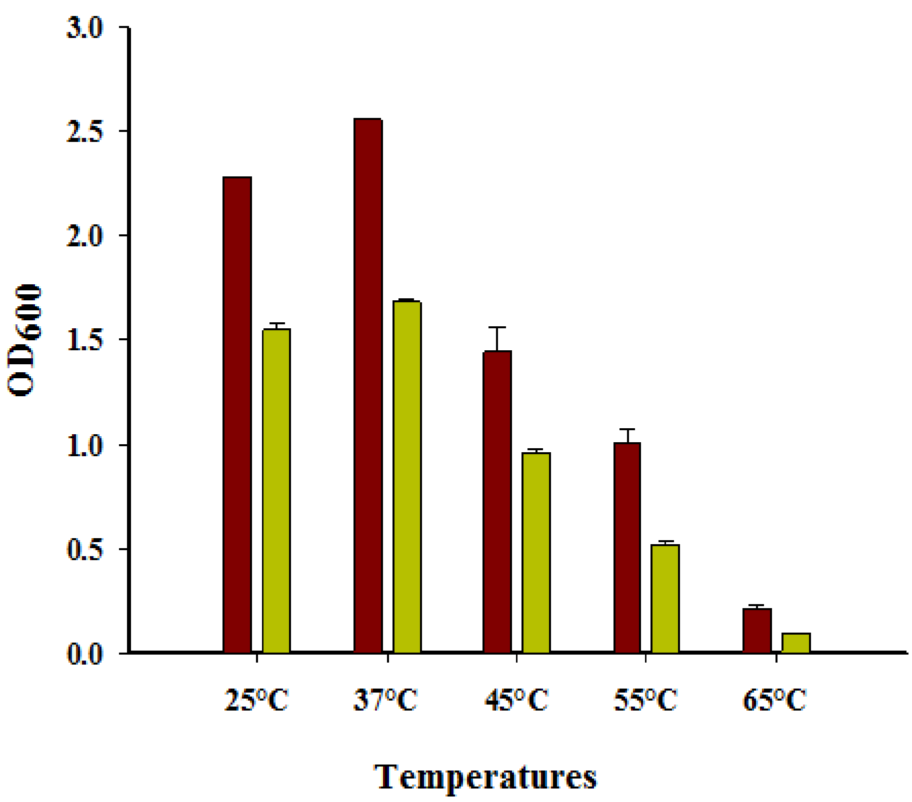


Figure 4.14: The effect of a range of temperature (25, 37, 45, 55 and 65°C) on the growth of *Enterobacter mori* using LB ( ) and M9 ( ) minimal salts media (I) refers to Standard Error (SE).

4.3.5.2. Growth curve of *E. mori* (EV2) at different temperatures (25°C, 37°C and 45°C) in LB medium at pH 7.0 and 0.17M NaCl.

It can be clearly seen from Fig 4.15 that temperatures determined the rate of the growth of the bacterium, with the highest growth observed at 37°C. The lowest growth was shown at 45°C after 10 hours. However, there was good growth after 24 hours at 45°C and the optical density was (1.060). *E. mori* was shown to be capable of growing over the temperature range 25°C -55°C.

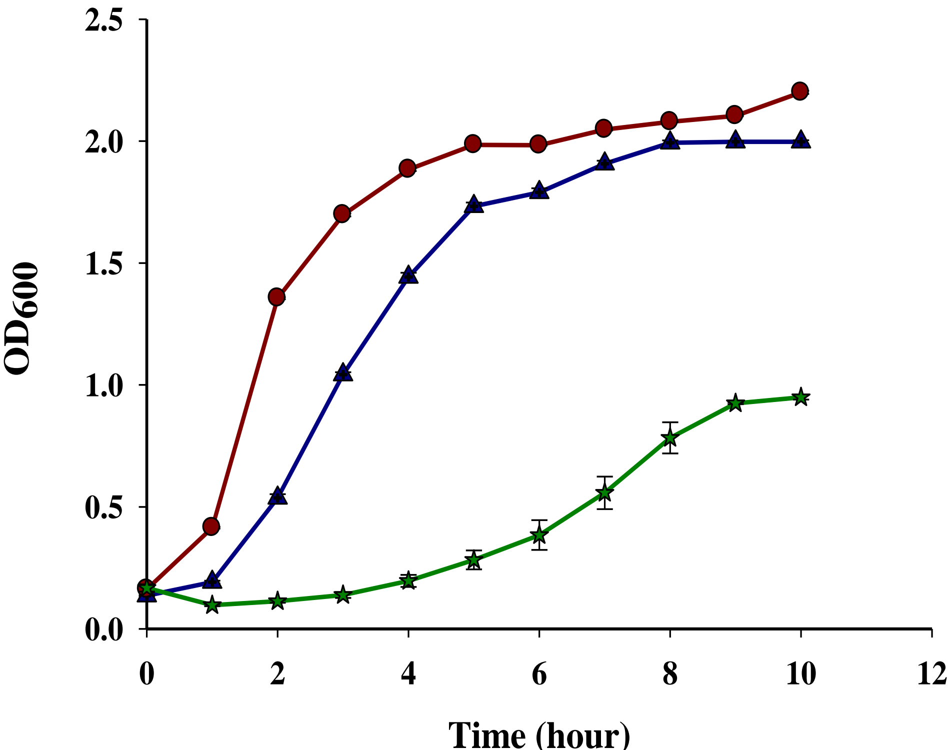


Figure 4.15: Growth curves of *Enterobacter mori* grown at different temperature; 25 °C (—▲—), 37°C (—●—) and 45°C (—★—) measured at optical density of 600 nm. (I) refers to Standard Error (SE).

4.3.5.3. Growth response of *E. mori* (EV2) at different pH and different media

The effect of a range of pH on the growth of *E. mori* at different media is shown in Fig 4.16. *E. mori* was grown for 24 hours and the OD_{600} measured. *E. mori* was capable of growth on rich LB medium and Horikoshi medium. It was also able to grow on minimal salts media M9, the strain was grown at pH 4.0 to pH 10.0 with pH 7.0 being optimal in each media. There was no observable growth under highly alkaline conditions (pH12.0) or highly acidic conditions (pH 3.0) in LB, Horikoshi and M9 media for 24hours or 48 hours. Therefore, it can be concluded that *E. mori* can grow and survive in both alkaline and acidic conditions.

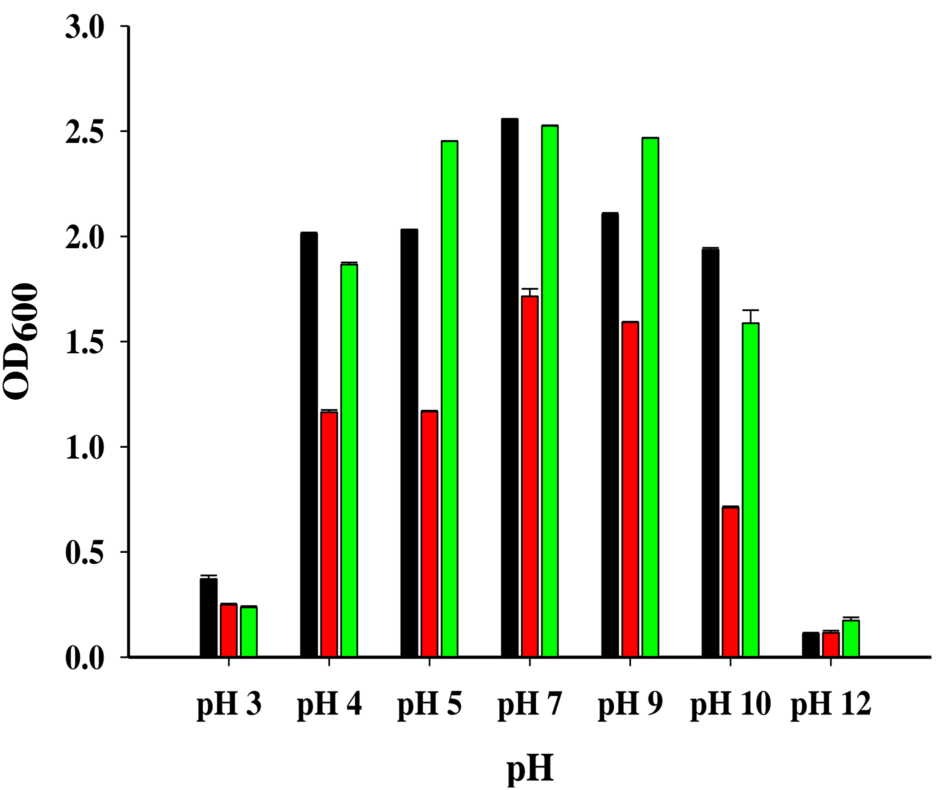


Figure 4.16: The effect of different pH (pH 5.0, pH 7.0, and pH 9.0) on the growth of *Enterobacter mori* using LB (black), Horikoshi (green) and M9 minimal salts media (red). (I) refers to Standard Error (SE).

4.3.5.4. Growth curve of *E. mori* (EV2) at different pH values (pH 4.0, pH 5.0, pH 7.0, pH 9.0 and pH 10.0) in LB medium at 37°C and 0.17M NaCl.

The results show that optimal growth of the isolate occurred at neutral (pH 7.0) conditions (Fig 4.17). Moreover, there was a good growth at alkaline conditions pH 9.0 and pH 10.0. This bacterium grew well at acidic conditions (pH 4.0). In conclusion, *E. mori* can grow in media at neutral pH but it also survives at pH values between pH 4.0 and pH 10.0.This is in agreement with the results obtained by Zhu *et al.,* (2011).

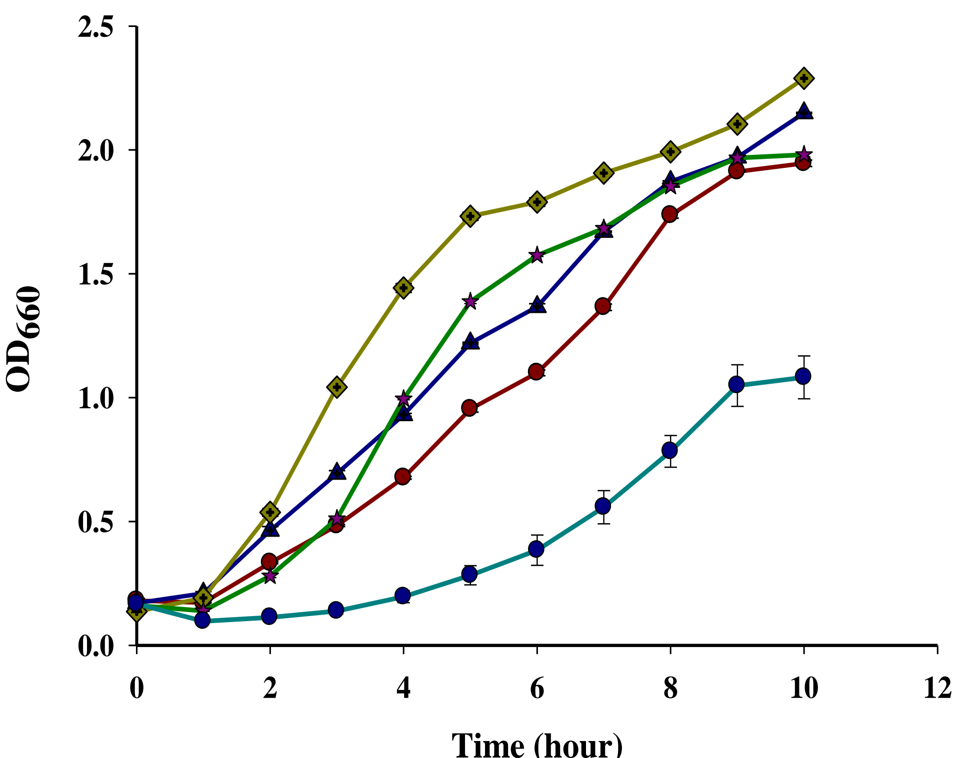


Figure 4.17: Growth curves of *Enterobacte mori* grown at different pH values; pH 4.0 ( ), pH 5.0 ( ), pH 7.0 ( ), pH 9.0( ) and pH 10.0 ( ) measured at optical density of 600 nm. (I) refers to Standard Error (SE).

4.3.5.5. The effect of different NaCl concentration (0.17, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 M) on the growth of *E. mori* (EV2) using LB and M9 media

The result shows that there was a significant growth of the isolate at 0.17, 0.5 and 1.0 M NaCl and there was a good growth at 1.5M NaCl Fig 4.18 in LB medium. On the other hand, *E. mori* shows a dramatic decrease in growth at salinities from 2.0 M to 3.0M NaCl in LB medium. There was a gradual decrease in growth at 0.17 to 1.0 M NaCl in M9 media. We can conclude therefore that *E. mori* does not require NaCl for growth but can tolerate salt concentration more than 1.0M NaCl, and NaCl concentration at 1.5M in LB medium.

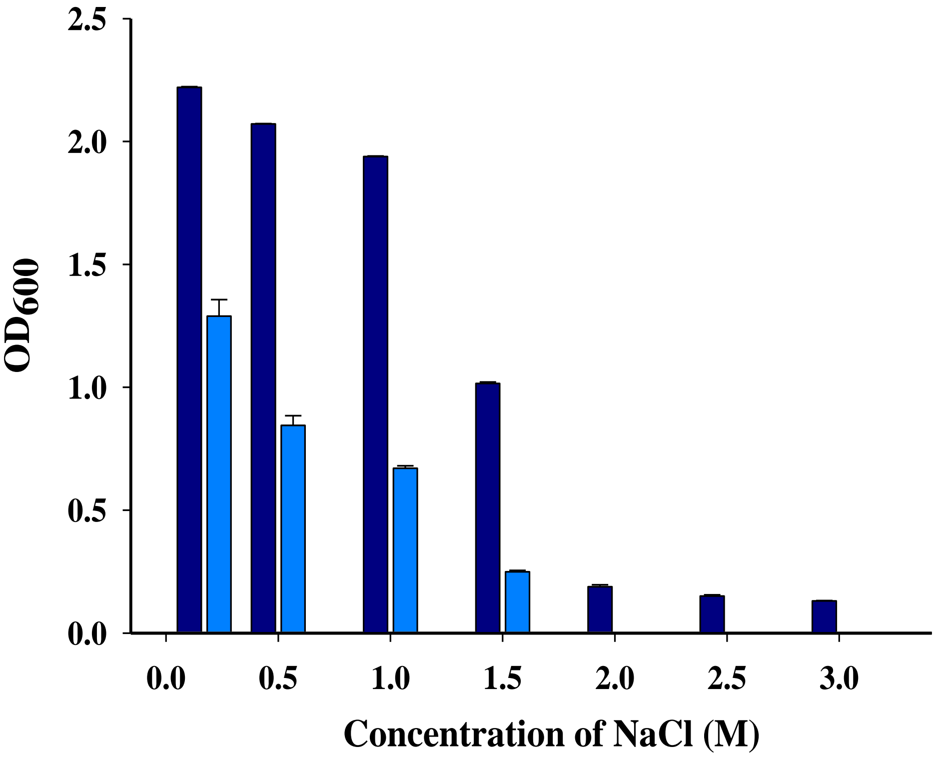


Figure 4 .18: The effect of different NaCl concentrations (0.17, 0.5, 1.5, 2.0, 2.5 and 3.0 M) on the growth of *Enterobacter mori* using LB (▬) and M9 (▬) media. (I) refers to Standard Error (SE).

4.3.5.6. The effect of UV (B and C) on the number of colony forming units (CFU) of *E. mori* (EV2).

The number of colony forming units of *E. mori* following exposure to UV-B and UV-C are shown in Fig 4.19, together with the total count before radiation exposed to UV-B or UV-C. It can be clearly seen that *E. mori* survives when exposed to UV-C radiation at 5 to 20sec and it cannot survive after 30sec. However, *E. mori* colony forming units decreased with increasing dose of UV-B radiation and it could not resist a 1 min exposure.

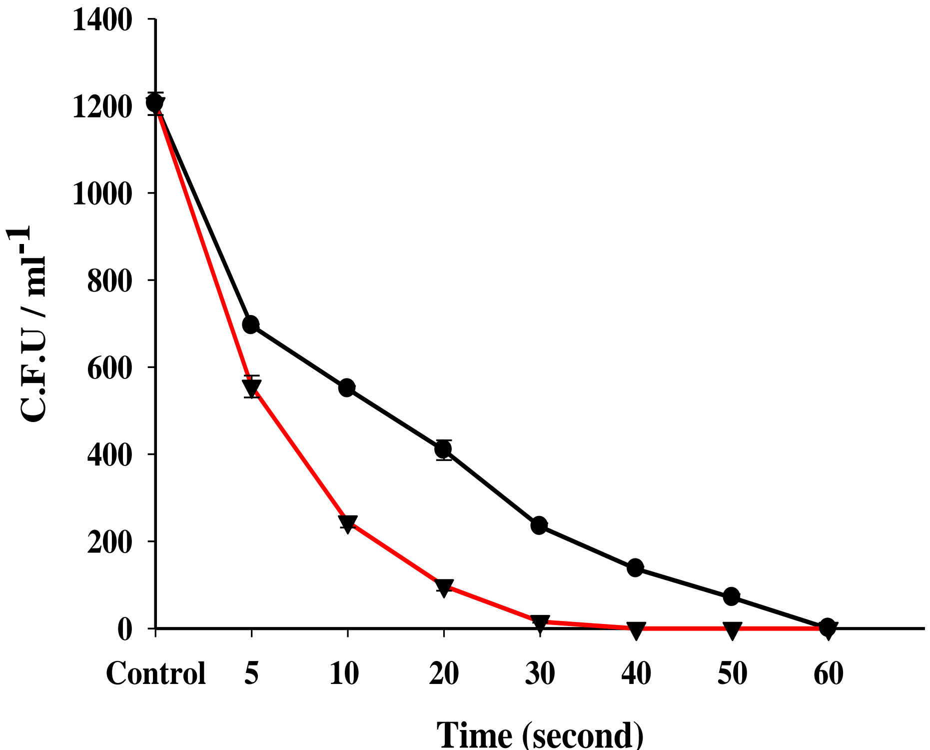


Figure 4.19: Effect of UV-B ( ) and UV-C ( ) on the number of colony forming units of *Enterobacter mori.* (I) refers to Standard Error (SE).

4.3.5.7. External morphology of *E. mori* (EV2) observed by scanning electron microscopy (SEM).

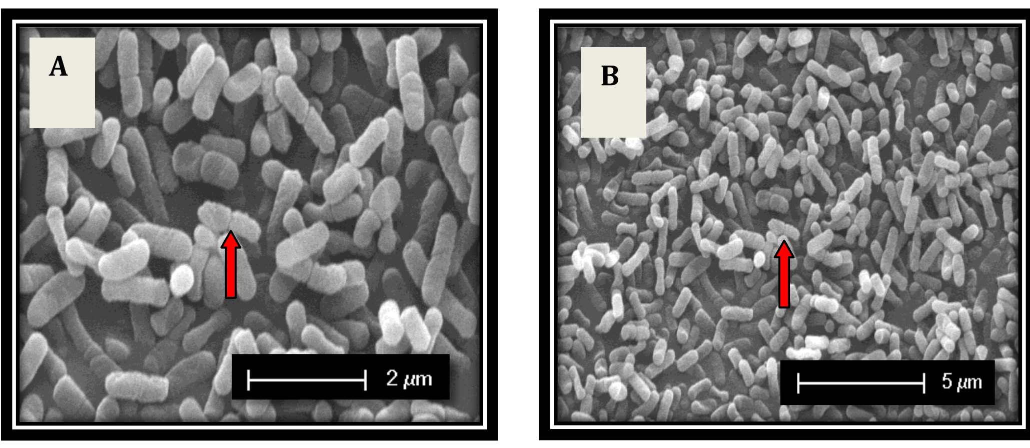


Figure 4.20: Scanning electron micrograph shows the morphology of the *Enterobacter mori* (A) scale marker 2µm. (B) scale marker 5µm. Note red arrows showing short- rod undergoing division cells.

4.3.5.8. Internal morphology of *E. mori* (EV2) observed by transmission electron microscopy (TEM).

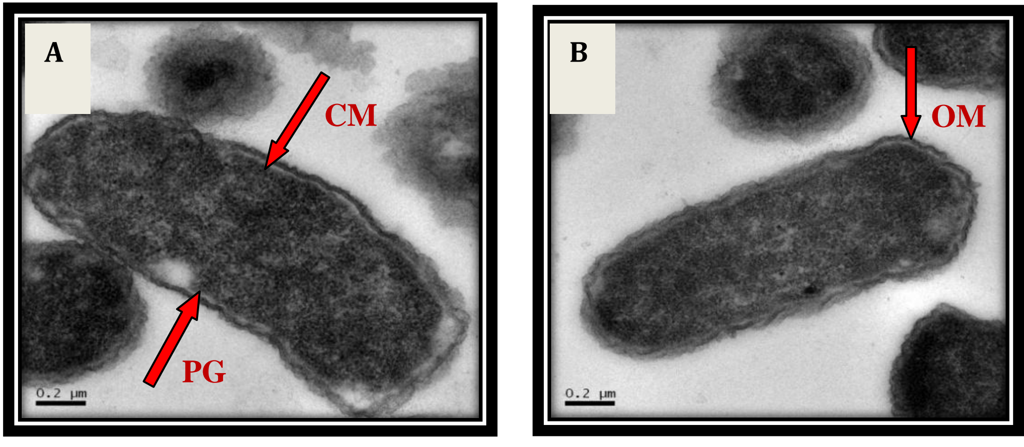


Figure 4.21: Transmission electron micrograph shows the morphology of the *Enterobacter mori* (A) and (B) scale marker 0.2µm.Note red arrows (A) showing (PG) peptidoglycan layer and (CM) cytoplasmic membrane, (B) showing (OM) outer membrane.

4.3.6. Physiological characteristics of *Pseudomonas putida* (SV3)

4.3.6.1. Growth response of *P. putida* (SV3) at different temperature and different media

The effect of a range of temperatures and different media on the growth of *P. putida* is presented in Fig 4.22. *P. putida* was grown for 24 hours with rotary shaking (250rpm) and measured at OD of 600nm.The results show that *P. putida* was able to grow on rich media as LB and minimal salts media M9. The strain was grown at 25°C to 45°C, 25°C being optimal in both media. This result agrees with the findings of Li *et al.,* (2010). There was no growth observed at 15°C, 55°C and 65°C in LB or M9 media for 24 hours and 48 hours.

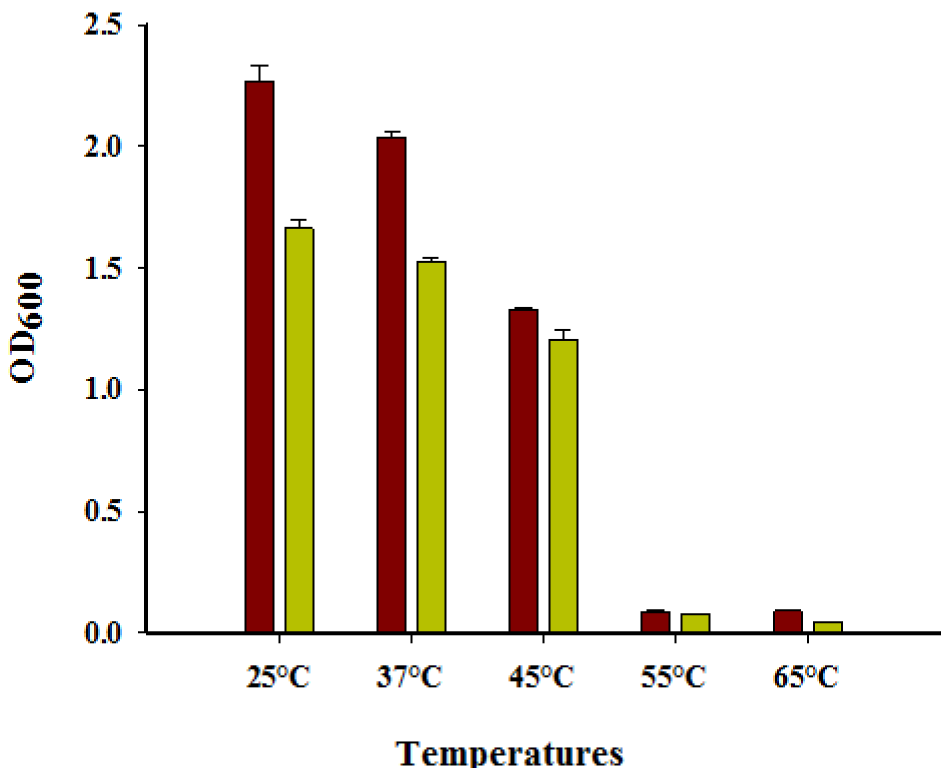


Figure 4.22: The effect of different temperature (25, 37, 45, 55 and 65°C) on the growth of *Pseudomonas putida* using LB ( ) and M9 ( ) minimal salts media. (I) refers to Standard Error (SE).

4.3.6.2. Growth curve of *P. putida* (SV3) at different temperatures (25°C, 37°C and 45°C) in LB medium at pH 7.0 and 0.17M NaCl.

It can be clearly seen from Fig 4.23 that temperatures determined the rate of the growth of the bacterium, with the highest growth observed at 25°C. The lowest growth was shown at 45°C after 10 hours; however, there was good growth after 24 hours at 45°C and the OD was (1.333). *P. putida* grew over the range 25°C -45°C, so it can be concluded that *P. Putida* cannot grow outside the temperatures range of 25°C-45°C and is considered a mesophilic bacterium.

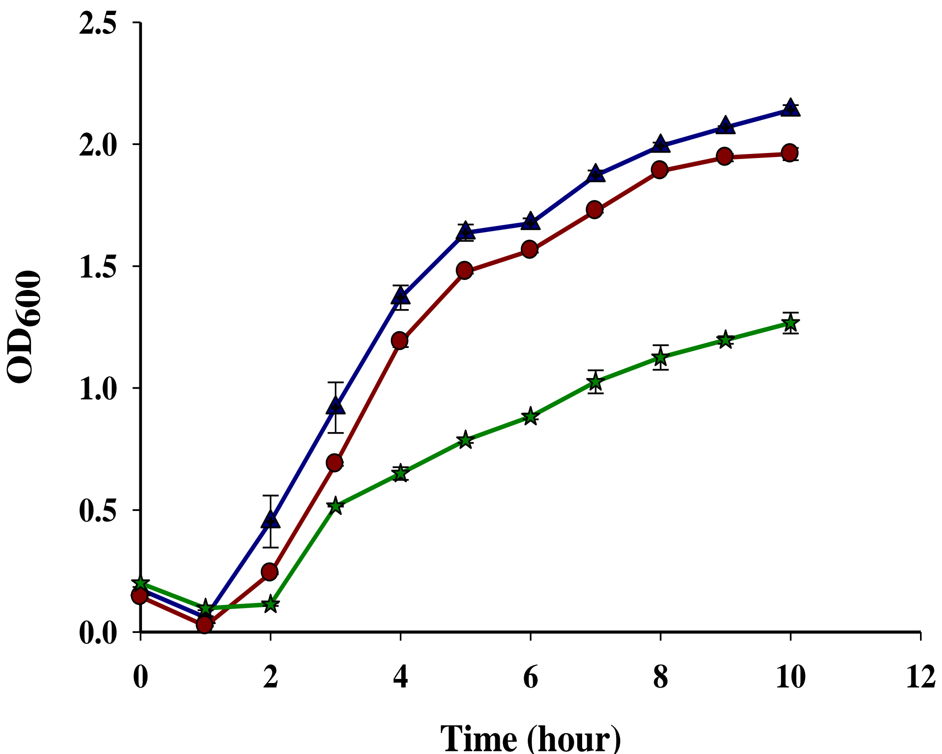


Figure 4.23: Growth curves of *Pseudomonas putida* grown at different temperature; 25 °C (▲), 37°C (●) and 45°C (★) measured at optical density of 600 nm. (I) refers to Standard Error (SE).

4.3.6.3. Growth response of *P. putida* (SV3) at different pH and different media

The effect of a range of pH on the growth of *P. putida* at different media is shown in Fig 4.24. *P. putida* was grown for 24 hours and the OD_{600} measured. *P. putida* was able to grow on rich media such as LB and Horikoshi media. It was also able to grow on minimal salts media, M9. The strain was grown at pH 5.0 to pH 9.0, with pH 7.0 being optimal in each medium. There was no observable growth under alkaline conditions (pH 10.0 and pH 12.0), or acidic conditions (pH 3.0, pH 4.0) in LB, Horikoshi and M9 media for 24 hours or 48 hours.

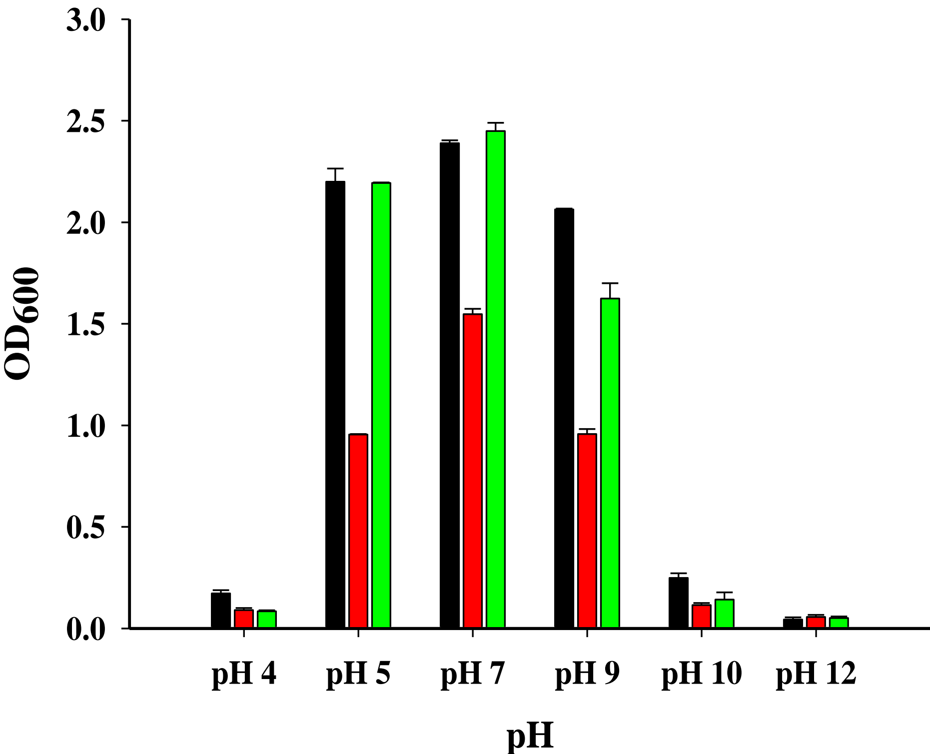


Figure 4.24: The effect of different pH (pH4.0, pH 5.0, pH 7.0, pH 9.0, pH10.0 and pH 12.0) on the growth of *Pseudomonas putida* using LB (black), Horikoshi (green) and M9 minimal salts media (red). (I) refers to Standard Error (SE).

4.3.6.4. Growth curve of *P. putida* (SV3) at different pH values (pH 5.0, pH 7.0, pH 9.0 and pH 10.0) in LB medium at 37°C and 0.17M NaCl.

The result shows that the optimal growth of the isolate occurred at neutral (pH 7.0) conditions (Fig 4.25). There was however, good growth at alkaline conditions pH 9.0 and at acidic conditions (pH 5.0). In conclusion, *P. putida* can grow in media at neutral pH but it also survives at pH values between pH 5.0 and pH 10.0. These findings are in agreement with those of Zhu *et al.,* (2011).

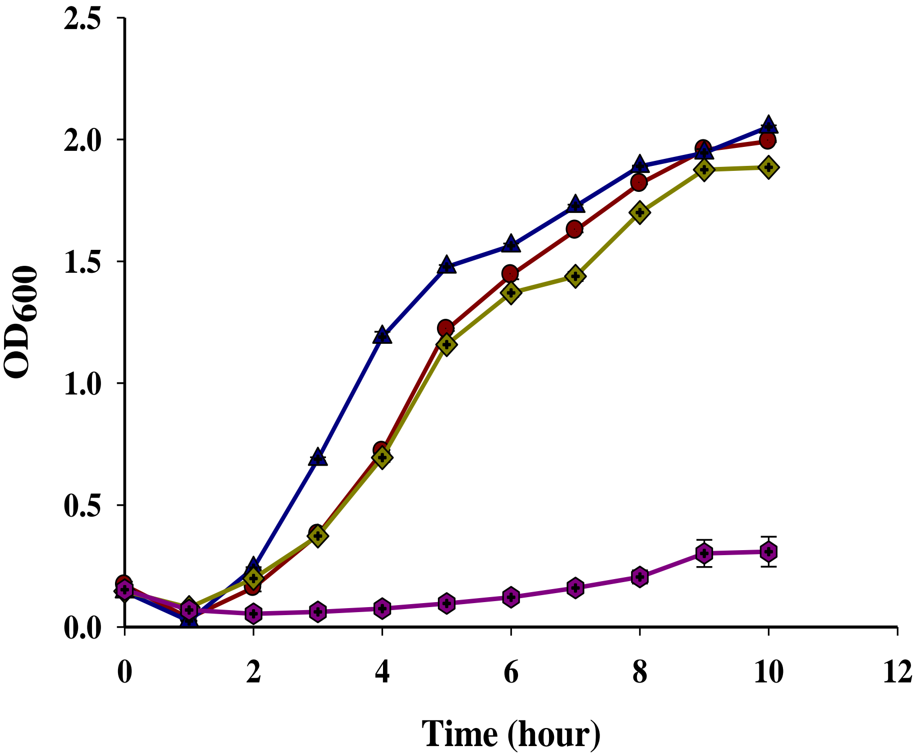


Figure 4.25: Growth curves of *Pseudomonas putida* grown at different pH values; pH 5.0 ( ), pH 7.0 ( ), pH 9.0 ( ) and pH 10.0 ( ) measured at optical density of 600 nm. (I) refers to Standard Error (SE).

4.3.6.5. The effect of different NaCl concentration (0.17, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 M) on the growth of *P. putida* (SV3) using LB and M9 media.

The results show that there was a considerable growth of the isolate at 0.17, 0.5 and 1.0 M NaCl (Fig 4.26) in LB medium. On the other hand, *P. putida* shows a dramatic decline in growth at salinities from 1.5 M to 3.0M NaCl, in LB medium. There was a gradual decrease in growth at 0.17 to 1.0 M NaCl in M9 media. It can be concluded therefore that *P. putida* does not require NaCl for growth but can tolerate a salt concentration of, 1.0M NaCl.

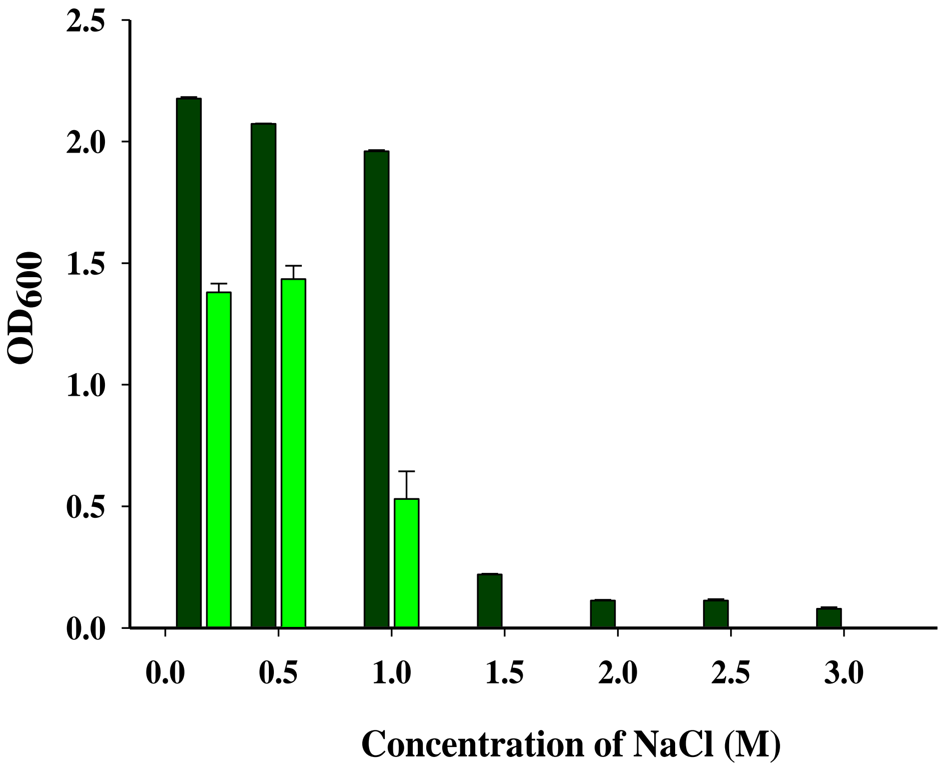


Figure 4 .26: The effect of different NaCl concentrations (0.17, 0.5, 1.5, 2.0, 2.5 and 3.0 M) on the growth of *Pseudomonas putida* using LB ( ) and M9 ( ) media. (I) refers to Standard Error (SE).

4.3.6.6. The effect of UV (B and C) on the number of colony forming units (CFU) of *P. putida* (SV3).

The number of colony forming units of *P. putida* following exposure to UV-B and UV-C are presented in Fig 4.27, as well as the total count before radiation exposure to UV-B or UV-C. It can be clearly seen that *P. putida* survives when exposed to UV-C radiation at 5 to 20sec, but that it cannot survive 30sec of exposure. However, *P. putida* colonies forming units decreased with an increasing dose of UV-B radiation and were killed after 1 min exposure.

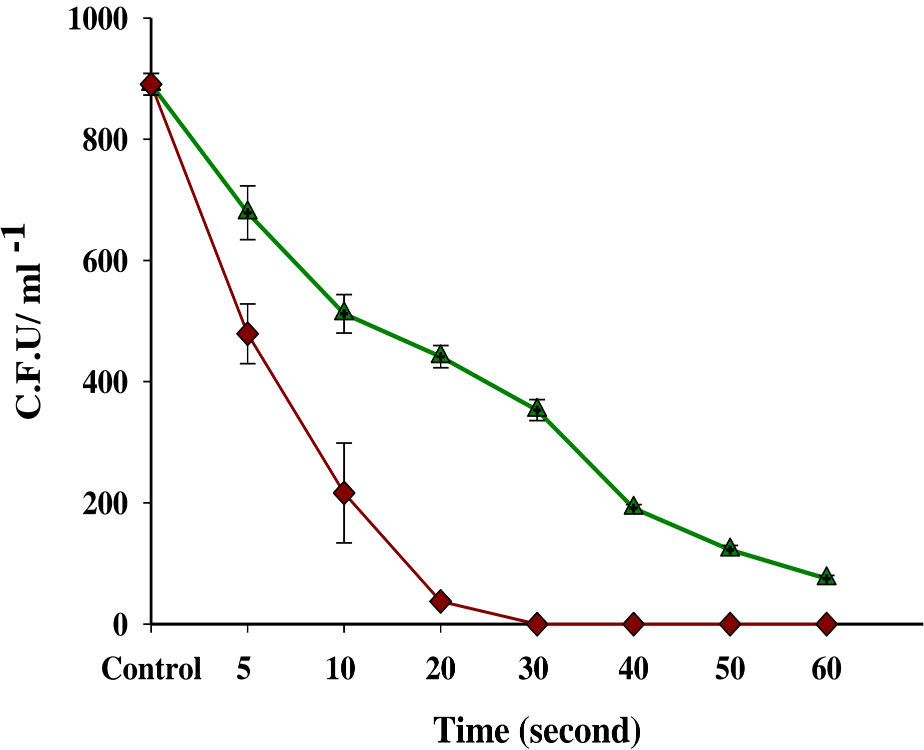


Figure 4.27: Effect of UV-B (green line with triangles) and UV-C (red line with diamonds) on the number of colony forming units of *Pseudomonas putida.* (I) refers to Standard Error (SE).

4.3.6.7 External morphology of *P. putida* (SV3) observed by scanning electron microscopy (SEM).

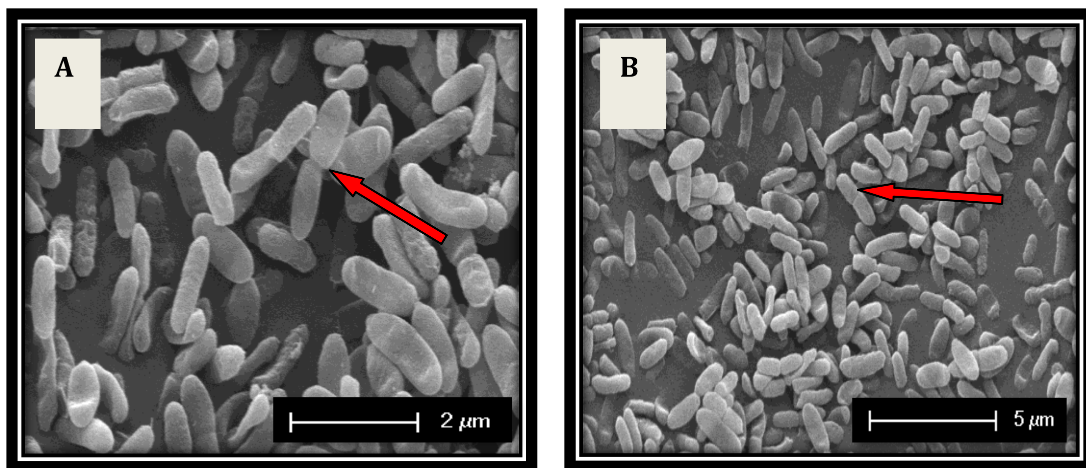


Figure 4.28: Scanning electron micrograph shows the morphology of the *Pseudomonas putida* (A) scale marker 2µm. (B) scale marker 5µm. Note red arrows showing short- rod undergoing division cells.

4.3.6.8. Internal morphology of *P. putida* (SV3) observed by transmission electron microscopy (TEM).

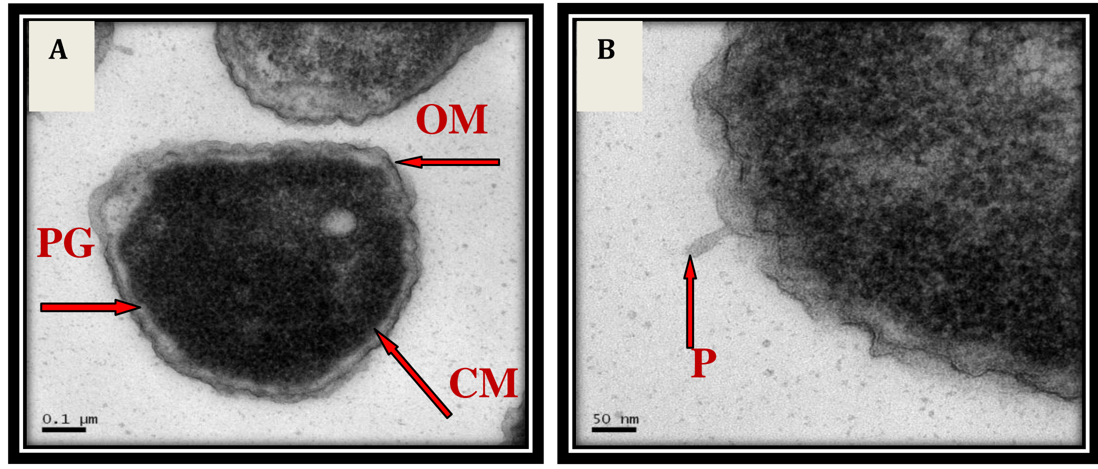


Figure 4.29: Transmission electron micrograph shows the morphology of the *Pseudomonas putida* (A) showing (PG) peptidoglycan layer (CM) cytoplasmic membrane and (OM) outer membrane. Scale marker 0.1µm. (B) showing (P) pilus or fimbria. Scale marker 50 nm.

4.4. Conclusions

The major initial aim of this Chapter was to isolate extremophilic bacteria, from weathered volcanic ash, a non-extreme environment. The volcanoes of which have not been active since 2007. Three bacteria (*Geobacillus thermoleovorans, Enterobacter mori* and *Pseudomonas putida*) were isolated as pure cultures and identified by 16S rRNA gene sequencing. Table 4.3 shows the main characteristics of this isolates and the main characteristics of *G. thermoleovorans* was as follows: a) the isolate was grown at 45°C to 70°C with 55°C being optimal in both media M9 and LB b) *G. thermoleovorans* is a thermophile since it requires a temperature of around 55°C for optimum growth and was unable to grow outside the temperatures range of 45°C-70°C. c) *G. thermoleovorans* can grow in media at neutral pH and can tolerate pH values between 5.5 and 8.0. d) *G. thermoleovorans* does not require NaCl for growth however; it was able to grow in a NaCl concentration of 1.0M NaCl. e) The effect of UV-C significantly reduced C.F.U, while UV-B decreased C.F.U with increasing length of exposure.

The second isolate *Enterobacter. mori* was able to grow on rich media as LB and minimal salts media M9. The strain was grown at 25°C to 55°C, 37°C being optimal in both media. The strain was grown at pH 4.0 to pH 10.0, with pH 7.0 being optimal in LB, Horikoshi and M9 minimal salts media. *E. mori* was shown to be capable of growing and surviving in both alkaline and acidic conditions *E. mori* does not require NaCl for growth, but can tolerate salt concentration more than 1.0M NaCl, and a NaCl concentration at 1.5M in LB medium. *E. mori* survived when exposed to UV-C radiation for 5 to 20sec and it cannot survive after 30sec. However, *E. mori* colony forming units decreased with increasing dose of UV-B radiation and it could not resist a 1 min exposure.

Pseudomonas putida was able to grow on rich media as LB and minimal salts media M9. The strain was grown at 25°C to 45°C, 25°C being optimal in both media. The strain was grown at pH 5.0 to pH 9.0, with pH 7.0 being optimal in each media. There was no observable growth under alkaline conditions (pH 10.0 and pH 12.0), or acidic conditions (pH 3.0, pH 4.0) in LB, Horikoshi and M9 media for 24 hours or 48 hours. It can be concluded therefore that *P.putida* can tolerate a salt concentration of, 1.0M NaCl. It cannot however, survive 30sec of exposure to UV-C. However, *P. putida* colony forming units decreased with an increasing dose of UV-B radiation and were killed after 1 min exposure.

CHAPTER 5

CHAPTER 5

DETERMINATION OF COMPATIBLE SOLUTES BY USING NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

5.1. Introduction

A wide variety of microorganisms produce compatible solutes in order to allow them to adapt to different extreme environments (Kurz, 2008). The accumulation of compatible solutes is an approach commonly employed by bacteria to provide cellular protection against high salt concentration (osmolarity); while other bacteria (e.g. *Bacillus subtilis*) accumulate the compatible solute, glycine betaine as an effective protection against cold and heat stress (Holtmann and Bremer, 2004; Hoffmann and Bremer, 2011). Other thermophilic bacteria (e.g. *Rhodothermus mariuns* and *Thermus thermophilus*), accumulate 2-*O*-Mannosylglycerate as a major compatible solute (Nunes *et al*., 1995). Compatible solutes have been widely studied as organic osmolytes responsible for osmotic balance, and at the same time, being compatible with the cellular metabolism (Galinski, 1993). A wide range of these solutes has been studied using nuclear magnetic resonance (NMR) and high performance liquid chromatography (HPLC).

Nuclear magnetic resonance (NMR) spectroscopy is a valuable technique for the rapid detection of compatible solutes which accumulate intracellularly in microorganisms after exposure to a range of different stresses (Nunes *et al*., 1995). Nuclear magnetic resonance spectroscopy is also a very powerful and adaptable technique for investigating biological molecules and their interactions in solution (Fenn *et al*., 2002).

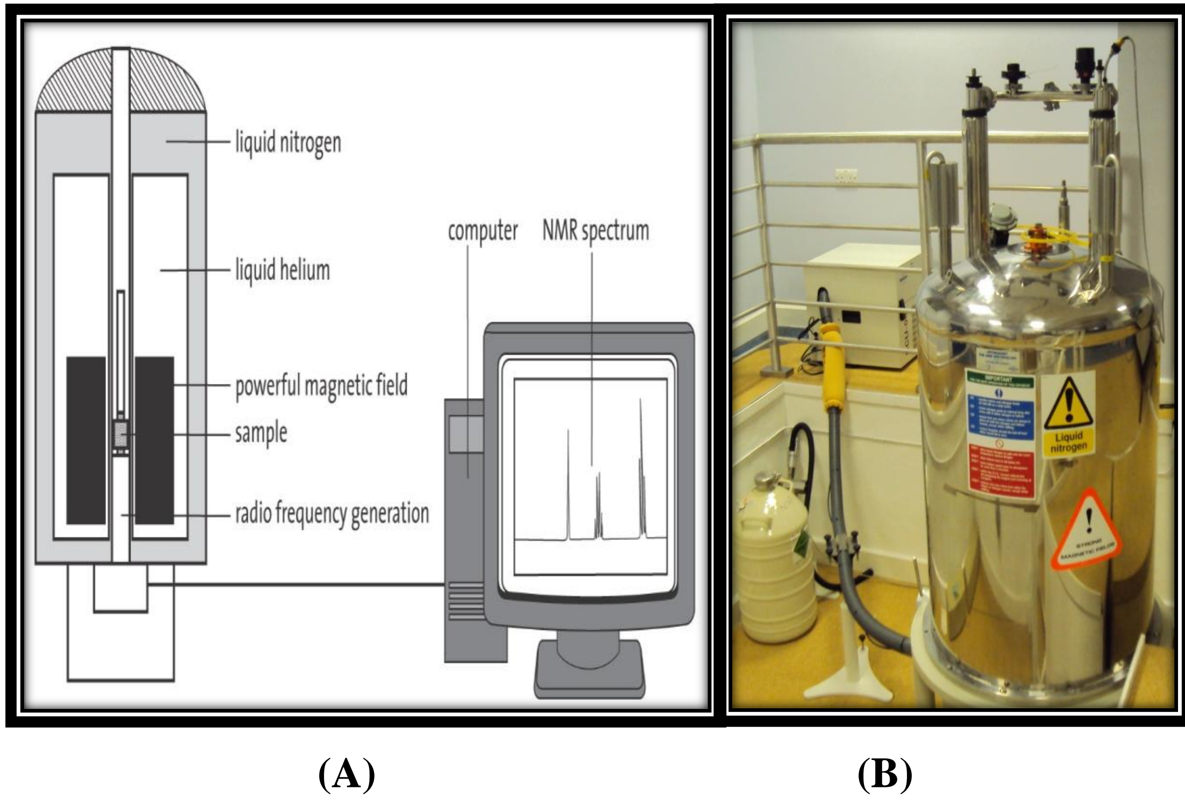
The aim of the work discussed in this Chapter was to identify the compatible solutes accumulated by three strains which been identified earlier *viz*: i) *Geobacillus*

thermoleovorans, (TV1) subjected to high temperatures stress; ii) *Enterobacter mori,* (EV2) at salinity and different external pH values; and iii) *Pseudomonas putida,* (SV3) when exposed to different salinity concentrations.

5.2. Material and Methods

5.2.1. Nuclear magnetic resonance (NMR) spectroscopy apparatus

During NMR analysis, the sample being investigated is initially placed in an extremely magnetic field, which is cooled by liquid nitrogen and helium. Pulses of radio waves passed into the sample then emit a radio wave reply and the response is then analysed electronically to produce a NMR spectrum (Fig 5.1) (Fenn *et al*., 2002).



(A) **(B)**

Figure 5.1: (A) The explanatory scheme for NMR apparatus (*Reproduced and updated from Fenn et al, 2002*) used with permissions. (B) The NMR apparatus used in this experiment

5.2.2. Sample preparation for NMR analysis

Samples (5 ml) of *Geobacillus thermoleovorans* (TV1), *Enterobacter mori* (EV2) and *Pseudomonas putida* (SV3) cultures were transferred into (15 ml) tubes, centrifuged at 5000 ×g for 10 minutes and the supernatant was discarded carefully. The resulting pellets were kept in -80°C freezer until been analysed. The pellets were thawed by re-suspending them in 1ml of MillQ water and vortexed for 1 minute at room temperature, and then been sonicated (2× 20 seconds) using Soniprep 15, SANYO at 15 amplitude microns. The samples were then centrifuged at 5000 ×g for 10 minutes, and the resultant supernatant layers were transferred into microcentrifuge tubes then transferred to -80°C for 2 hours. The samples were then dried using (Heto Power Dry PL9000) at -91.4°C condenser and 0.36 KPa pressure for 48 hours.

The final samples preparation step before NMR analysis was the addition of ^{1}H to 2D ^{1}H-^{1}H HSQC and 2D C-H C-HSQC. NMR spectra were obtained by re-dissolving the dried sample cells in 500 µl of D_2O in a microcentrifuge tube. Followed by addition of 5µl of trimethly siylpropionate (TSP) to the NMR tube, and then the dissolved samples were analysed using NMR equipment (Frings *et al*., 1993).

5.2.3. Effect of adaptation to different temperatures on compatible solutes accumulated by *Geobacillus thermoleovorans* (TV1)

The effect of adaptation to different temperatures (45°C, 55°C, 65°C and 70°C) on the growth of *G. thermoleovorans* (TV1) was investigated in two media (e.g. LB and M9). Aliqouts of actively growing cultures (e.g. 1 ml) were used to inoculate 50 ml of M9 media at 45°C, with continuous shaking for 24 hours. The OD_{600} of each culture were measured and recorded, and then another aliquot (e.g. 1ml) from each culture of the new M9 culture grown at 45°C were transferred into 50 ml of M9 media and were grown at 55°C and been incubated under the same conditions.

Again, the concentration (e.g. OD_{600}) was measured for the both bacterial cultures, and then portions of 1ml from the M9 culture grown at 55°C were transferred into 50 ml M9 media and been grown at 65°C; this was incubated under the same conditions. Finally, 1ml from the M9 cultures grown at 65°C were transferred into fresh aliquots of 50 ml M9 media and grown at 70°C and were incubated using the same conditions. The same procedure was performed for growth of the bacterial strain in LB medium. After each experiment, 5 ml of every sample were centrifuged for 10 minutes at 3000 ×g, then the supernatant was discarded and the pellets were kept in -80°C freezer until been used. NMR analysis was carried out as described earlier in (Section 5.2.2).

5.2.4. Effect of adaptation to different salinity, pH on compatible solutes accumulated by *Enterobacter mori* (EV2)

E. mori (EV2) was adapted to different concentration of NaCl (0.17, 0.50, 1.0 and 1.5 M) in M9 minimal medium and (0.17, 0.50, 1.0, 1.5 and 2.0 M) in LB medium (see Chapter 4, Section 4.2.5.10) for more detailed about adaptation to different salinity in M9 medium), and the same procedure was performed to adapt the isolate in LB medium. In addition, *E. mori* (EV2) was adapted to different pH values (e.g. pH 4.0, pH 7.0, and pH10.0) in both M9 and LB media. The same procedure to adapt to different salinity was used, as described previously to adapt to different pH values. Each sample was prepared for NMR analysis as described above.

5.2.5. Effect of adaptation to different salinity on compatible solutes accumulated by *Pseudomonas putida* (SV3)

In this experiment, the isolate of *P. putida* (SV3) was adapted to different concentrations of NaCl (e.g. 0.17, 0.50 and 1.0) in M9 minimal medium and (e.g. 0.17, 0.50, 1.0 and 1.5) in LB medium (as described in Chapter 4, Section 4.2.5.10).

Similar procedures were performed to adapt the investigated bacterial strain in LB medium. Each sample was prepared for NMR analysis as described earlier in this Chapter (Section 5.2.3).

5.3. Results and Discussion

5.3.1. NMR analysis of compatible solutes

The aim of NMR analysis was to identify the compatible solutes accumulated by three strains *G. thermoleovorans* (TV1), *E. mori* (EV2) and *P. putida* (SV3) when exposed to different stress conditions. The effect of media type used in this experiment was also investigated by using two media (e.g. M9 salt minimal medium and rich LB medium) for different stress conditions.

5.3.2. Accumulation of compatible solutes as strategies for adapting to a high temperatures stress by *G. thermoleovorans*

The spectrum of compatible solutes in *G. thermoleovorans* strain has been established with NMR techniques at different temperatures. Fig 5.2 shows the compatible solutes accumulation by *G. thermoleovorans* when the strain was grown in M9 minimal salt medium at pH 7.0 and was adapted at different temperatures (e.g. 45°C, 55°C, 65°C and 70°C). *G. thermoleovorans* in M9 medium did not accumulate any osmolyte in its cells, which might be due to presence of intracellular metal ions at high temperatures. Similarly, the compatible solutes accumulated by *G. thermoleovorans,* when the strain was grown in LB medium at pH 7.0, were adapted at different temperatures (e.g. 45°C, 55°C, 65°C and 70°C) are shown in Fig 5.3. It can also be seen that betaine was the main compatible solute at 55°C in LB medium.

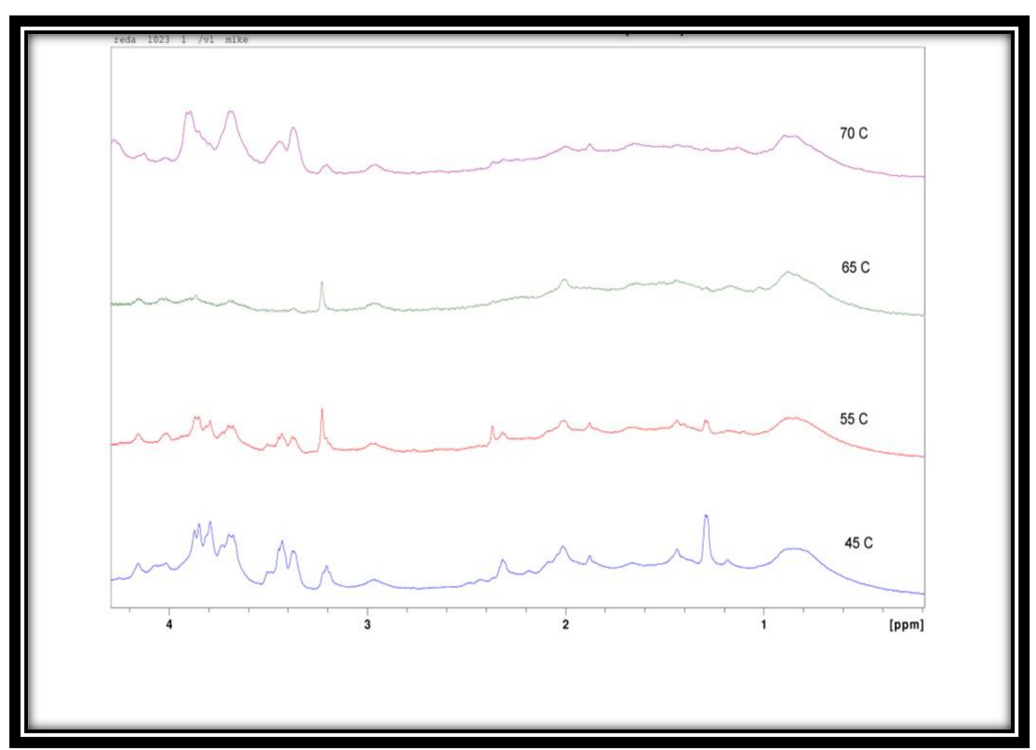


Figure 5.2: NMR ^{1}H spectra of cell extracts from *G. thermoleovorans* at 45°C, 55°C, 65°C and 70°C in M9 minimal salt medium spectra.

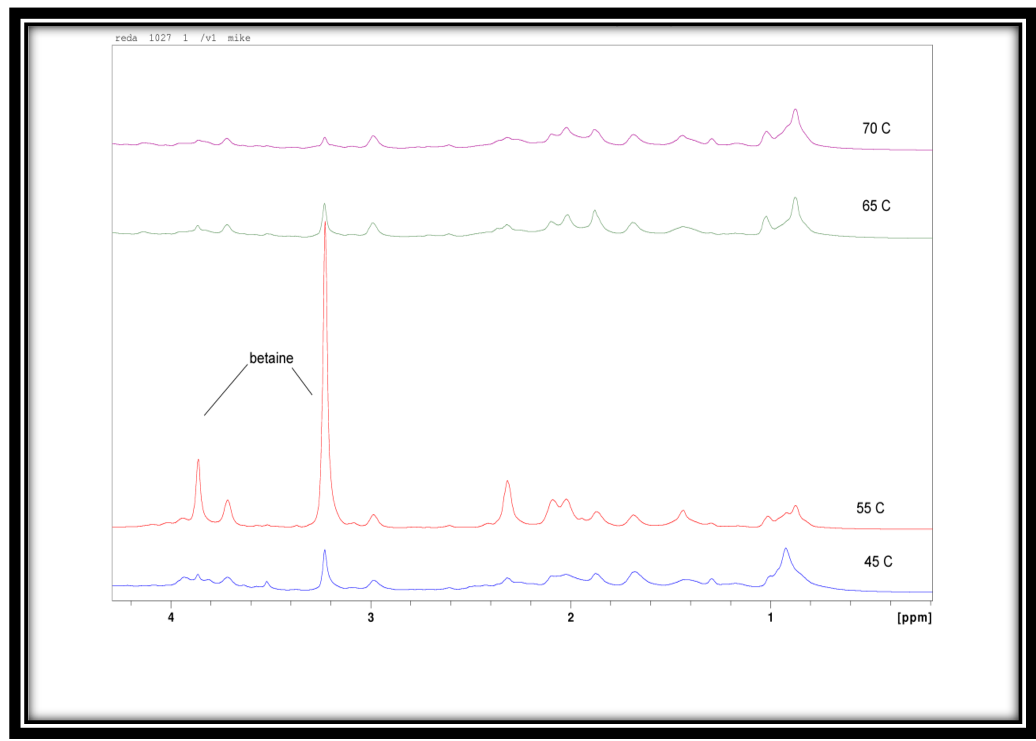


Figure 5.3: NMR ^{1}H spectra of cell extracts from *G. thermoleovorans* at 45°C, 55°C, 65°C and 70°C in LB medium spectra.

5.3.3. Accumulation of compatible solutes as a strategy for adapting to salinity stress by *E. mori*

Compatible solutes present in *E. mori* strain grown at different salinities were analysed. Fig 5.4 shows the compatible solutes accumulated by *E. mori* when the isolate was grown in M9 minimal salt medium at pH 7.0 and was adapted at different salinities (0.17, 0.5, 1.0 and 1.5 M) NaCl. From the spectra shown in this Figure, it is clear that when *E. mori,* was subjected to different salinity stresses, has shown different amounts of compatible solutes (e.g. proline) were produced. The maximum amount of proline was produced at 1.0 M; however, it diminished again at 1.5M. The spectra appeared at 0.5 and 1.5 M NaCl have numerous signals due to interference from various sugars. The most obvious change occurred was the instant increase in proline as salt concentration was increased.

Fig 5.5 shows the compatible solutes accumulation by *E. mori* when the strain was grown in LB medium at pH 7.0 to adapt different salinities (e.g. 0.17, 0.5, 1.0, 1.5 and 2.0 M NaCl). It can be clearly seen that when *E. mori* when exposed to different stresses at different NaCl values shows different amounts of compatible solutes, mainly of betaine. Betaine is the main organic solute present in the LB medium at 1.5 M NaCl, however, it was very scant at 0.17M NaCl. While double this amount was formed when it was exposed to 0.5 M NaCl, and approximately 4X at 1.0 M NaCl fold than that for 0.17M NaCl. The maximum amount of betaine was produced by the isolate at 1.5M NaCl, and was drastically diminished at 2.0M NaCl. These results are with full agreement with findings reported by Imhoff and Rodriguez-Valera (1984), which stated that various species of the family *Enterobacteriaceae* accumulate betaine as the main compatible solute under different solute stresses.

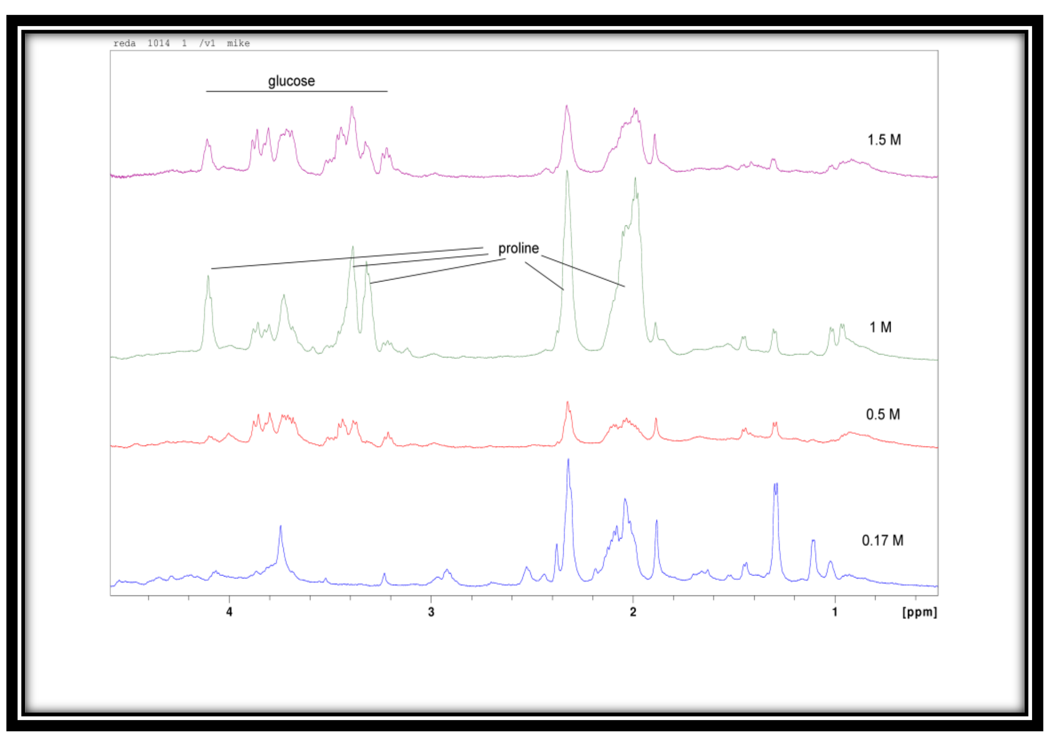


Figure 5.4: NMR ^{1}H spectra of cell extracts from *E. mori* at 0.17, 0.5, 1.0 and 1.5 NaCl (M) in M9 minimal salt medium spectra.

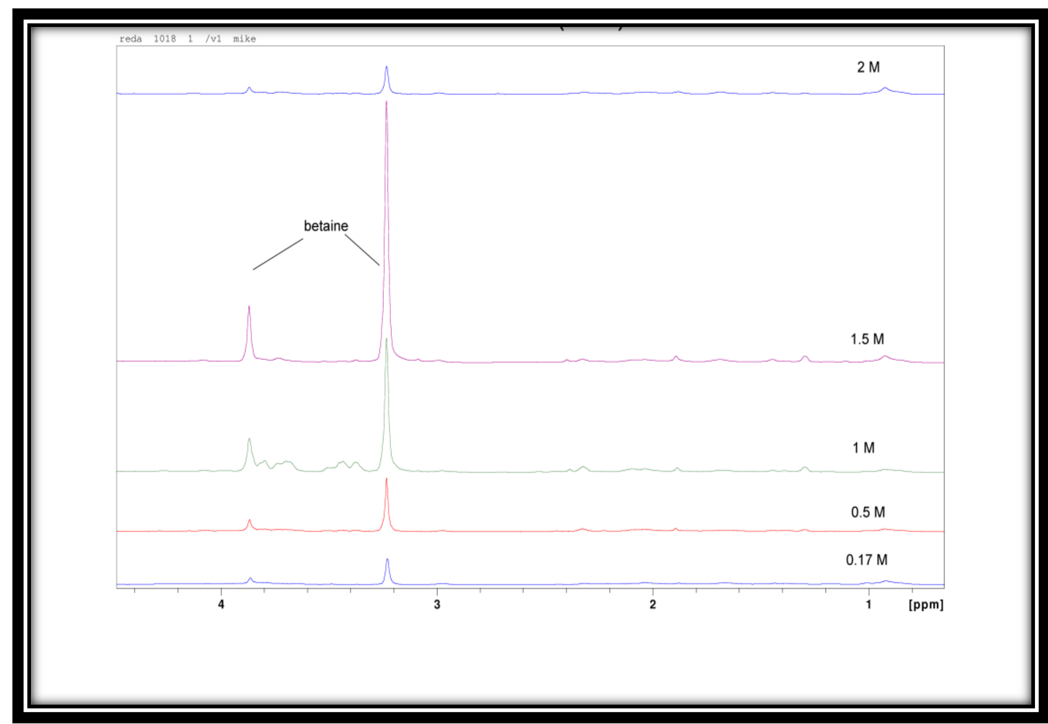


Figure 5.5: NMR ^{1}H spectra of cell extracts from *E. mori* at 0.17, 0.5, 1.0, 1.5 and 2.0 NaCl (M) in LB medium spectra.

5.3.4. Accumulation of compatible solutes as a strategy for adapting to pH stress by *E. mori*

Compatible solute accumulation in *E. mori* strain grown at different pH were analysed in this experiment. Fig 5.6 shows the compatible solutes accumulation results by *E. mori* when the strain was grown in M9 minimal salt medium and was subjected to adapt at different pH values (e.g. pH 4, pH 7.0 and pH 10.0). From the spectra shown in the diagram, it is clear that *E. mori,* subcultured in different pH values, clearly accumulaed two types of compatible solutes, i.e. glutamate and betaine. At pH 4.0, betaine was the main compatible solute accumulated, whereas, glutamate was dominant at pH 7.0.

Fig 5.7 shows the compatible solutes accumulated by *E. mori* when the strain was grown in LB medium and was adapted to different pH values (e.g. pH 4.0, pH 7.0 and pH 10.0). In the case of *E. mori* subcultured on LB medium at different pH values, the obtained results showed that betaine was the main compatible solute present; which was dominant at pH 10.0, while it was formed in lower amounts at lower pH values (e.g. pH4.0 and pH7.0).

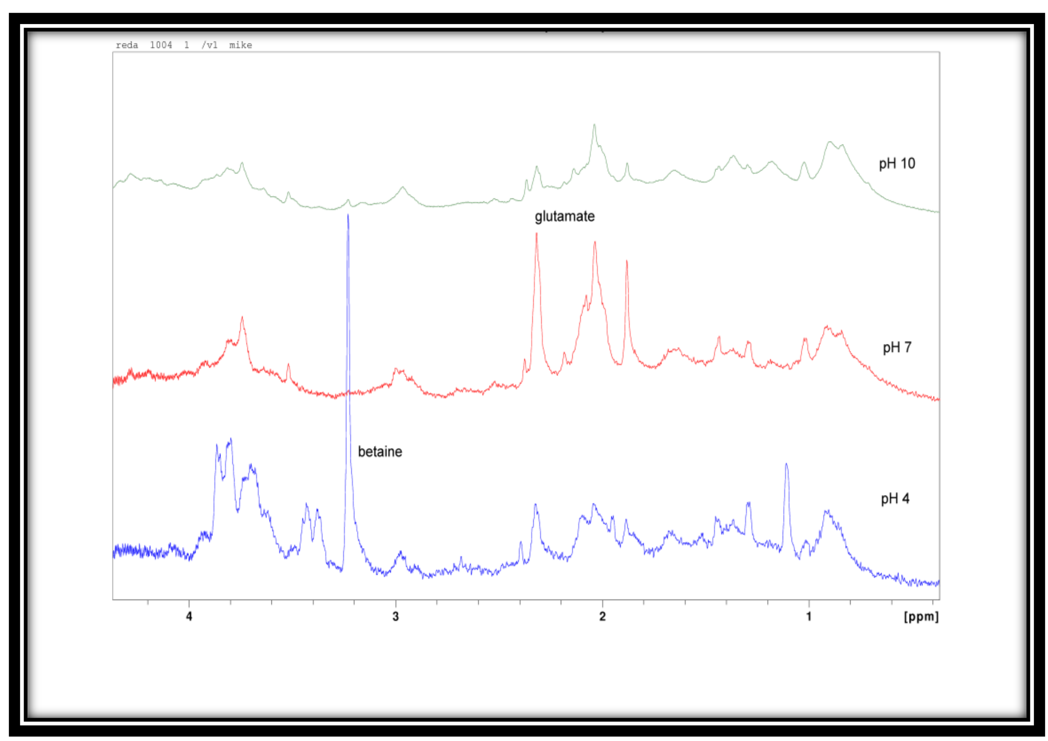


Figure 5.6: NMR ^{1}H spectra of cell extracts from *E. mori* at pH 4.0, pH 7.0 and pH10.0 in M9 minimal salt medium spectra.

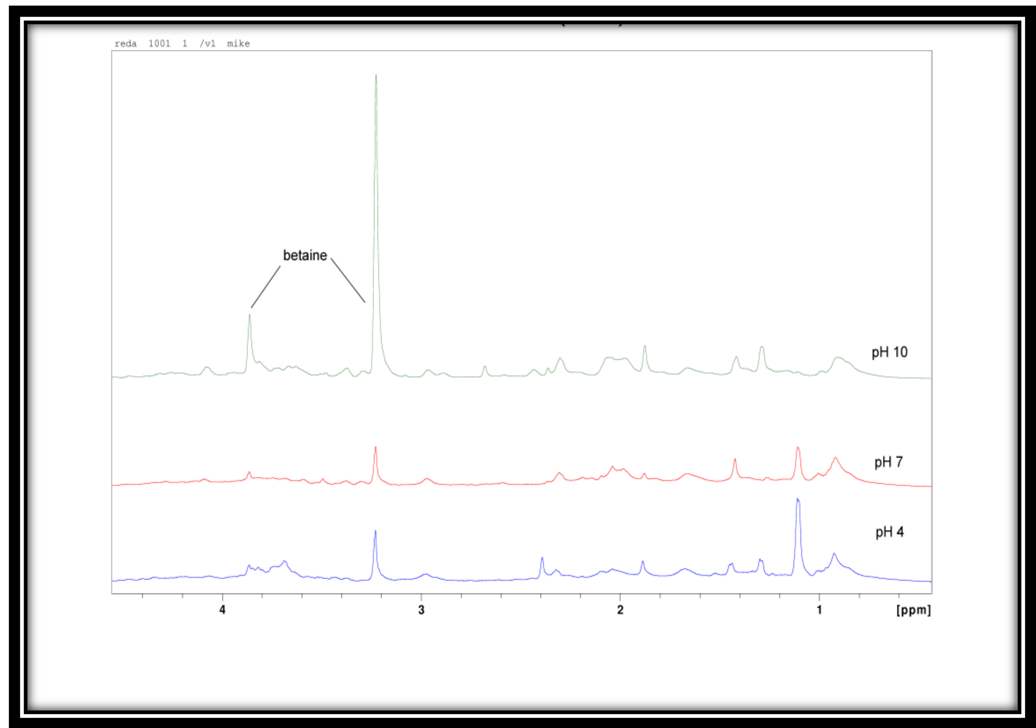


Figure 5.7: NMR ^{1}H spectra of cell extracts from *E. mori* at pH 4.0, pH 7.0 and pH10.0 in LB medium spectra.

5.3.5. Accumulation of compatible solutes as a strategy for adapting to salinity stress by *P. putida*

In this experiment, the NMR spectrum of compatible solutes occurred in *P. putida* strain has been analysed at different salinity conditions. Fig 5.8 shows the compatible solutes accumulation by *P. putida* when the strain was grown in M9 minimal salt concentration at pH 7.0, and been adapted to different salinity gradients of NaCl (e.g. 0.17M, 0.5M and 1.0 M). From the spectra shown in (Fig 5.8), it is clear that the organism accumulated proline and betaine at high salinity (e.g. 1.0 M), and that the amount of betaine produced increased as salinity increased. However, there has also been an increase in proline (in fact there has been more proline present than betaine, even though the betaine peak is bigger). This can be explained by the fact that the betaine signal which is sharper and is produced by 9 protons, compared to the proline signals which are broader and is produced from only 2 protons.

Fig 5.9 also shows the compatible solute accumulation by *P. putida* when the strain was grown in LB medium at pH 7.0; and been adapted to different salinities (e.g. 0.17M, 0.5M, 1.0M and 1.5 M NaCl). Based on the spectra shown in (Fig 5.9), it can be clearly seen that betaine was produced in most abundance at the highest salt concentration (e.g. 1.5M NaCl). Whereas, at all salinities below 1.5 M NaCl, glutamate and saccharide were confirmed in variant amounts. According to Kets *et al* (1996) study, they reported that *P. putida* been found to accumulate betaine induced by the presence of sodium chloride and sucrose. Additionally, *P. putida* accumulates Nα-acetylglutaminylglutamine (NAGGA), and also mannitol as the main compatible solute for osmoadaptation.

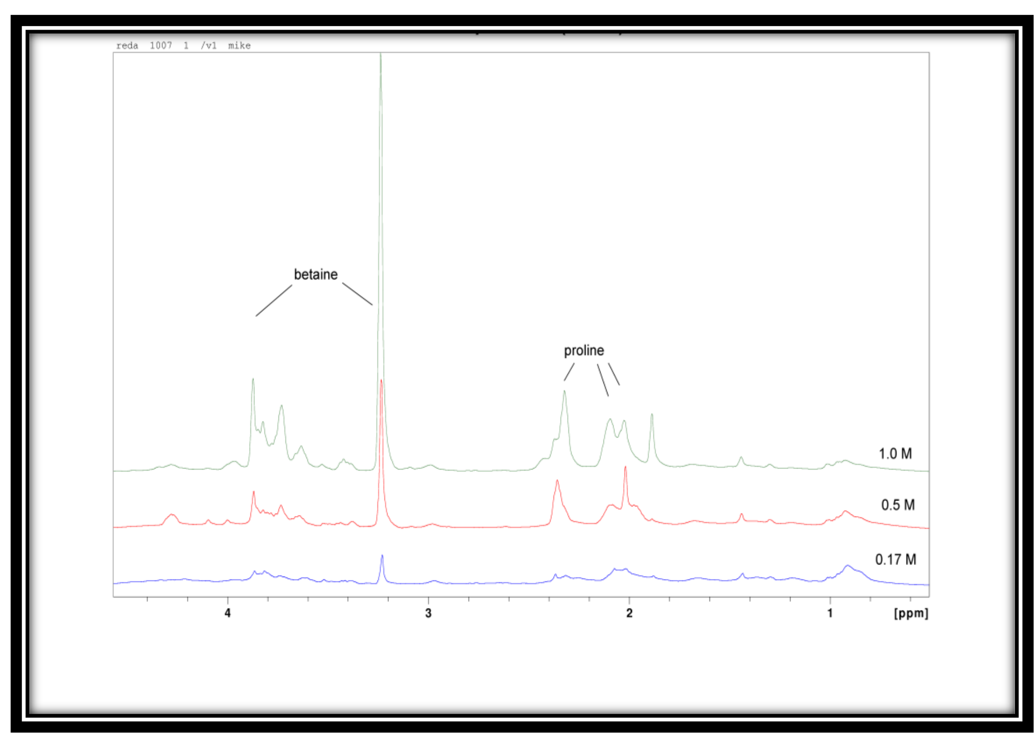


Figure 3.8: NMR ^{1}H spectra of cell extracts from *P. putida* at 0.17, 0.5 and 1.0 NaCl (M) in M9 minimal salt medium spectra.

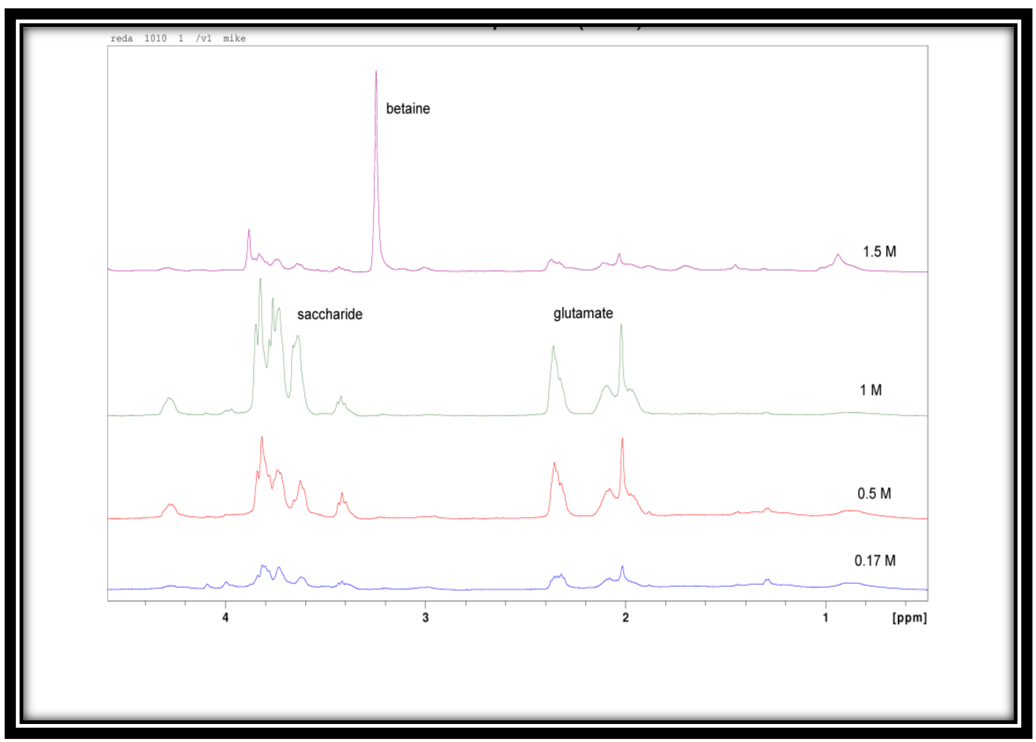


Figure 3.9: NMR ^{1}H spectra of cell extracts from *P. putida* at 0.17, 0.5, 1.0 and 1.5 NaCl (M) in LB medium spectra.

5.4. Conclusions

In the present work, the accumulation of compatible solutes for *G. thermoleovorans*, *E. mori* and *P. putida* were detected in both media, i.e. low nutrient (M9 minimal salt medium) and rich nutrient (LB) medium with different stresses. *G. thermoleovorans* was adapted to grow at different high temperatures and the main compatible solute at 55°C in LB medium was betaine. Whereas, *G. thermoleovorans* did not accumulate any compatible solute in M9, at all temperatures. *E. mori* use proline as the main compatible solute at 1.0M NaCl in M9 medium. In addition, betaine was also identified at 1.5 M NaCl in LB medium. Glutamate was dominant at pH 7.0 and betaine was the main compatible solute present at pH 4.0 in the M9 medium. Moreover, betaine was dominant at pH 10.0 in LB medium. *P. putida* use betaine as a main compatible solute and proline as an additional compatible solute at high salinity (1.0 M) in M9 medium. In LB medium *P. putida* accumulated betaine at the highest salt concentration 1.5 M NaCl. Whereas, at 0.17, 0.5, 1.0 M NaCl, glutamate and saccharide were accumulated in varying amounts.

CHAPTER 6

CHAPTER 6

DETECTION OF *MYCOPLASMA* IN DESERT SURFACE SOILS AND A RANGE OF ENVIRONMENTAL SAMPLES USING THE EZ-PCR TEST

6.1. Introduction

Mycoplasma was discovered in 1889 by a German biologist Frank. The name is originally from the Greek *myKes*, for fungus and plasma. Frank obviously thought he had isolated a fungus, although *Mycoplasma,* were originally referred to as Pleuropneumonia-Like Organisms (PPLO), because of their capability to cause contagious bovine pleuropneumonia (CBPP). *Mycoplasmas* are the smallest of free-living life forms, and are found in humans, plants, animals, insect, soil and sewage (Greenwood, *et al*., 2002). *Mycoplasma* is a genus of bacteria with special characteristics that differ from other bacteria and viruses. *Mycoplasma* is prokaryotic has the cell size between 150-250 nm, which lack cell walls and as a result, are not inhibited by most frequently used antibiotics, such as penicillin, cycloserine and other antibiotics which affect cell wall growth (Tadesse and Alem, 2006), they are also not stained by Gram's stain. *Mycoplasma* requires specific growth media and need up to three weeks for growth and become mature enough for them to be positively identified (Cassell, 1995). Colonies on agar show a characteristic ‟Fried-egg or mulberry‟ appearance. *Mycoplasma* species have cell membranes containing sterols and as a result are able to resist osmotic lyses**,** and are, as a result, more stable than other prokaryotes.

Mycoplasma contains RNA and DNA and as genome with a low molecular weight genome between 500 to 1100 bp, (G+C content 23 to 40%), a fact which considerably reduces their biosynthetic capabilities and explains why they tend to be

dependent on their hosts. They have no DNA homology match with known bacteria**,** but they are similar to some parasitic organisms (e.g. *Chlamydia* and *Rickettsia*) (Madigan *et al*., 2012). Some species are known to be human pathogens and cause pneumonia and other respiratory diseases, (e.g. as caused by *M. pneumonia*). Other species (such as *M. genitalium*) also cause pelvic inflammatory disease (Neumayr *et al*., 2003).

In the laboratory**,** cell cultures are prone to contamination by *Mycoplasma sp*, which can lead to the destruction of cell lines or changes in cell growth and metabolism. Since *Mycoplasma* species are difficult to culture and identify using the microscope alternative detection methods using the PCR reaction, have been developed (Cassell, 1995; Marois *at al*., 2002). Only a few published papers have reported the isolation and identification of *Mycoplasma* from the environment using molecular identification techniques (McAuliffe *et al*., 2006). *Mycoplasma* were found in drinking water, feathers, droppings, dust and chicken and turkey waste and on lettuce (Marois *et al*., 2000). Environments from which these organisms have been isolated include: bedding sand used in dairy units (Justice Allen *et al.*, 2010), rabbit faeces (Angulo *at al*., 1987), a swine waste disposal system (Orning *et al*., 1978) and polluted water from poultry farms (Marois *et al*., 2000); *Mycoplasma* have also been isolated from insects and fly larvae (Oduori *et al*., 2005).

The aim of the work, presented in this Chapter, was to determine whether *Mycoplasma* sp can be isolated from varies desert surface soils, rock samples and other environmental samples. In this study, environmental samples were tested in order to detect any occurrence of *Mycoplasma* using an EZ-PCR *Mycoplasma* Test Kit (Geneflow Limited, Cat No.20-700-20).

6.2. Materials and Methods

6.2.1. Sample and site description

A variety of primitive desert surface soils, rock samples and other environmental samples were studied, as follows:

1. Desert surface soils with and without lichen cover from Tabernas, Spain.
2. Volcanic ash soils with and without plant cover collected from the French Indian Ocean Island of Reunion.
3. Desert varnish from Ashikhara, Oman.
4. Rock samples from Ghar Al Hibashi caves, Saudi Arabia and rock samples from the Jarnan cave, Oman.
5. Volcanic rock samples from the French Indian Ocean Island of Reunion.
6. Agricultural loam soil samples from the Sheffield district.
7. Moss soil samples from the Sheffield district.
8. Garden soil samples from the garden of Royal Hallamshire Hospital, (main entrance) Sheffield.

6.2.2. EZ-PCR *Mycoplasma* test kit procedures

Portions (1g) of each sample were transferred to 10 ml dH_2O solutions, with continuous shaking for15 min at 250 rpm. Afterwards, Portions (1.0 ml) were then transferred to 1.5 ml microcentrifuge tubes and were centrifuged at 1000 ×g for 1min. The supernatant was transferred to a fresh tube and centrifuged at 13000×g for 10 min to pellet the *Mycoplasma*. In order to estimate the *Mycoplasma*, the supernatant was carefully discarded and the pellet was then re-suspended in 50µl of buffer solution, and mixed thoroughly using vortex mixer (the pellets were not always visible).Finally, the test samples were then heated at 95°C for 3 minutes, and immediately stored at -20°C for later use.

6.2.3. PCR amplification

For each test sample, a reaction mixture was prepared (on ice in a PCR tube). One positive and one negative control were also prepared (see Table 6.1). The tubes were then placed in a thermal cycler and the *Mycoplasma* programme was run. When the PCR amplification was finished, the PCR tubes were removed from the thermal cycler and aliquots of 4µl (5× loading day) were added to each tube. For positive control, the size of the PCR product was (270bp) and the PCR was checked. Whilst in the negative control tube, the DNA template was replaced with ddH_2O.

Table 6.1: Reaction mixture in a PCR tube for amplification *Mycoplasma* DNA

Component	**Quantity**
Sterile Milli-Q water	35.0-39.0µl
Reaction Mix	10.0 µl
Test sample Positive and Negative control	5.0 µl

The thermal cycling order for PCR amplification of *Mycoplasma* used in this experiment was as follows: initial denature at 94°C for 30 sec, following by 35 cycles of DNA denaturation at 94°C for 30 sec, then primer annealing step at 60°C for 120 sec, strand elongation at 72°C for 60 sec, final denaturation at 94°C for 30 sec, primer annealing at 60°C for 120 sec, and final elongation at 72°C for 5 min. The thermal cycling conditions used for the amplification of *Mycoplasma* are shown in Table 6.2.

Table 6.2: PCR amplification procedure for *Mycoplasma sp.*

Steps	Temperature and time	Number of cycle
Initial denaturating	94°C for 30 sec	1
Denaturating	94°C for 30 sec	35
Annealing	60°C for 120 sec	
Elongation	72°C for 1 sec	
Final denaturating	94°C for 30 sec	1
Final annealing	60°C for 120 sec	
Final elongation	75°C for 5 min	
Hold	4°C	

6.2.4. Analysis of amplified products by gel electrophoresis

Aliquots (10µl) of the amplified test samples together with positive and negative controls, were mixed with (2µl) portions of 6× loading dye, and added to the wells before being separated in a 2% agarose gel. The gel was submerged in TAE buffer 1× with ethidium bromide. In order to determine the size of fragments, 6 µl of Hyper Ladder 200 bp (Bioline, UK) was used in this experiment. Finally, the samples were subjected to electrophoresis for 45 minutes at 80V. The amplified products were finally visualised on the gel and digital images were taken using "UVitec" "Uvidoc" attached to a digital camera.

6.3. Results and Discussion

6.3.1. The occurrence of *Mycoplasma* in environmental samples

Mycoplasma DNA was detected using PCR amplification. Table 6.3 below shows the results for *Mycoplasma sp.* isolation from the various environmental samples studied here. *Mycoplasma* was not isolated from any of the desert soils samples (whether covered by lichen or not), and in any volcanic ash soil samples (whether

covered by plants or not). Likewise, no *Mycoplasma* isolates were obtained from any of the desert varnish samples, or in any type of rock samples and agricultural soils. Two positive results were however found, one for the Moss soil (*Polytrichum commune*), collected from the Sheffield district, and the other for the Garden soil, collected from the garden of Royal Hallamshire Hospital, (main entrances) Sheffield.

Table 6.3: The occurrence or absence of *Mycoplasma* in different environmental samples

Samples	**Results**
Desert surface soils with lichen cover	Negative
Desert surface soils without lichen cover	Negative
Volcanic ash soils with plant cover	Negative
Volcanic ash soils without plant cover	Negative
Desert varnish	Negative
Rock samples from Ghar Al Hibashi caves	Negative
Rock samples from Jarnan caves	Negative
Volcanic rocks	Negative
Agricultural soils	Negative
Moss soils (*Polytrichum commune*)	**Positive**
Garden soils	**Positive**

The reason for the appearance of two positives against a general trend of negative for the isolation of environmental *Mycoplasma* is not immediately obvious. The appearance of *Mycoplasma* in the Moss soils and the Garden soil samples may reflect a level of pollution from animal faeces, mostly dogs and birds, and other associated bacteria in these environments. The presence of *Mycoplasma* has previously been

reported in Hailstones samples in Sheffield, a finding which may indicate their presence in rainwater and generally in the upper atmosphere, from where they could undergo intercontinental transfer (Al'Abri, 2011). Finally, there has been a report of the isolation of *Mycoplasma* from the Nile River, presumably associated with pollution by human and animal faeces (Alshammari, 2010).

6.3.2. PCR detection methods of *Mycoplasma*

In order to detect *Mycoplasma sp.,* EZ-PCR *Mycoplasma* Test Kit was used. In this kit, primers were specifically designed which target highly conserved sequences in *Mycoplasma* DNA only, and not for bacterial or animal DNA sequences. In general, this PCR dependent method is very sensitive, specific and rapid technique compared to the conventional direct culturing procedures. This kit detects various *Mycoplasma* species (e.g. *M.capricolum, M. pirum, M.fermentans, M.salivarium, M.arthritidis, M. hyorhinis, M.bovis, M. arginini, M. pneumonia, M.pulmonis, M.hominis* and *M. pirum).* In addition, *Spiroplasma* and *Acholeplasma* are detected with high specificity and sensitivity.

This technique has a unique standard which is the rRNA gene sequences of prokaryotes (including *Mycoplasma*) is well conserved; the sequences and lengths of the spacer region in the rRNA operon (e.g. the region between 16S and 23S gene) are different from one species to another. The detection procedure utilises the PCR process with this primer set consists of two main elements:

i. Amplification of a specific and conserved 16S rRNA gene region of *Mycoplasma* using two primers, and
ii. Detection of the amplified fragment by agarose gel electrophoresis.

By using this system for DNA amplification system, DNA amplification from other sources (e.g. bacteria or tissue samples) is avoided. Therefore, not only the sensitivity, but also the specificity of detection is enhanced when using this primer Set for amplification of the gene sequence with PCR; the amplified products are then detected by using agarose gel electrophoresis Fig 6.1.

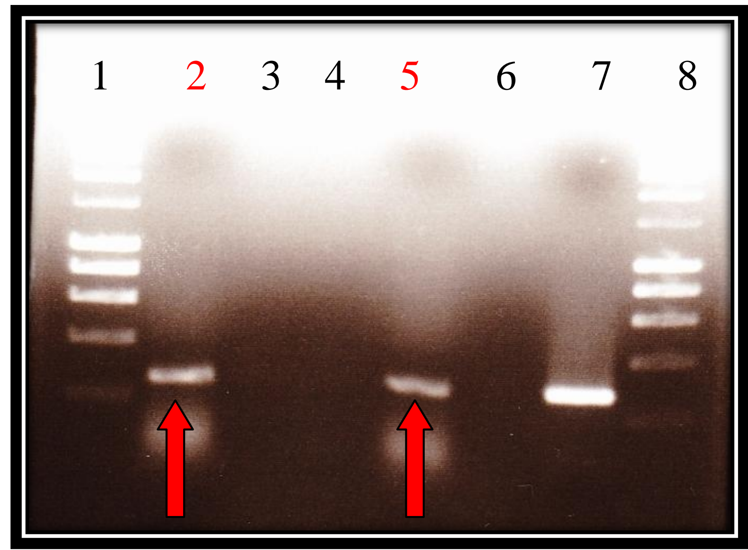


Figure 6.1: EZ-PCR detecting *Mycoplasma* analysed using electrophoresis in 2% agarose gel.
Lane 1 and 8: 1Kb hyperladder, (lane 2 and 5); positive result moss soil sample, garden soil sample respectively, (lane 3 and 4); negative result,(lane 6); negative control, and (lane 7); positive control.

The EZ-PCR-detecting *Mycoplasma* method detected DNA from both viable and non-viable *Mycoplasmas* cells. Marois *et al*., (2002) developed a reverse transcription-polymerase chain reaction (RT-PCR) assay, which was used to detect viable *Mycoplasma* in various environmental samples. It was basically developed to extracting and analysing RNA by (RT-PCR). Therefore, because of its short half-life, the detection finding suggests that the detected cells are either viable or have been viable very recently. In this study, the use of PCR *Mycoplasma* experiment has

enabled the detection of *Mycoplasma* in the environmental samples used in this experiment.

6.4. Conclusions

The aim of this work described here was to determine if *Mycoplasma* species can be isolated from various desert surface soils and a range of environmental samples. In this study, environmental samples were tested for the presence of *Mycoplasma* using an EZ-PCR *Mycoplasma* Test Kit (Geneflow Limited, Cat No.20-700-20). *Mycoplasma* sp. were isolated from a soil associated with moss (*Polytrichum commune*) and from a local garden soil, but not from any of the desert soils, volcanic ash, desert varnish, rock samples and agricultural soils.

This study clearly revealed that *Mycoplasma* was infrequently isolated from the natural environments. Although two examples of the isolation of environmental *Mycoplasma* were found, further research is clearly needed to extend this work in order to determine the distribution of these organisms in wider environmental samples, and likewise to determine how these organisms can grow without apparent hosts, or otherwise locate the hosts in such environmental samples.

CHAPTER 7

CHAPTER 7

GENERAL DISCUSSION

Soil microorganisms are essential for the cycling of nutrients and for driving above ground ecosystems. Soil microorganisms also play vital roles in the various biogeochemical cycles and are responsible for the cycling of organic compounds (Kirk *et al.*, 2004). The aim of the work described in this Thesis was to study a) microbial activity of a variety of primitive desert surface soils and in the thin biotic cover which they maintain and b) compare this microbial activity with results found for an agricultural loam soil. Bacterial population densities in the desert surface soils and fertile loam soils were also determined. Bacterial counts were found in the order: agricultural soil > desert soil with and without lichen cover > volcanic ash with and without plant cover > desert varnish and control (without varnish layers). These findings are broadly what would be expected considering the relative organic matter contents of the three soils, which is expected to be reflected in the same order as the bacterial counts.

Four microbial processes were selected for study namely: nitrification, urea hydrolysis, the oxidation of elemental sulphur to sulphate and finally, phosphate solubilisation. The highest level of microbial activity was, not surprisingly, found in agricultural soil in relation to all of the microbial processes On the other hand the lowest microbial activity was found in desert varnish. The results of the present study show that:

Nitrification: The expected outcome was seen, namely that the agricultural soil exhibited the highest rates of nitrate production, followed by the desert soil having a lichen cover, followed by the desert soil without lichen cover; the amount of nitrate

formed was similar in both volcanic ashes with and without plant cover. Lastly, no nitrification was observed in the desert varnish.

Urea hydrolysis: the agricultural soil exhibited a higher rate of ammonium production followed by the desert soil with lichen cover and the desert soil without lichen cover. The results show that the concentration of ammonium in the agricultural soils is higher than in the volcanic ash with plant cover, followed by the volcanic ash without plant cover. The levels of ammonium production found in agricultural soil are almost 3 times higher than those found in desert surface soil, whether or not they possess lichen.

Sulphur oxidation: the highest oxidation occurred in agricultural soil, then the desert soil with lichen cover, followed by the desert soil without lichen cover. The concentrations of S-oxidation found in agricultural soil are almost 4 times higher than those found in volcanic ash, whether covered by plants or not. The highest level of sulphate production was found in agricultural soil, while no oxidation occurred in the desert varnish or desert soil without varnish layer throughout the 28 day incubation period.

Phosphate solubilisation: There was a significant difference in the rate of phosphate solubilisation in agricultural and desert soils, with highest rates being found in the agricultural soil at week 1. However, no change in phosphate solubilisation occurred in the desert soils, with and without lichen cover through the entire incubation period. The levels of phosphate solubilisation found in agricultural soil are almost 4 times higher than those found in volcanic ash, whether covered or not, with vegetation. The highest levels of phosphate production was found in the agricultural soil and no phosphate release occurred in desert soil without varnish

layer throughout the 28 day incubation period, while there was a slight increase in desert varnish during 28 days incubation.

The biodiversity and isolation of diverse microorganisms from desert soil and rocks using conventional isolation techniques presents a real challenge for researchers (Fendrihan, 2010). The second approach of the study presented focused on isolating bacteria from the soil samples and applying molecular techniques to their identification, notably PCR methods and both 16S and 18S rRNA gene sequencing analysis. The majority of the isolated strains were very closely related to *Bacillus* species (e.g. 99% similarities); this was true of desert surface soils, desert varnish, volcanic rocks, and cave rock samples.

A principal initial aim of this research project was to isolate extremophilic bacteria, from non-extreme environments. Volcanic ash soil samples were collected from the French Indian Ocean Island of Reunion (near Mauritius), the volcanoes of which have not been active since 2007. Three bacteria (*Geobacillus thermoleovorans, Enterobacter mori* and *Pseudomonas putida* were isolated as pure cultures and identified by 16s rRNA gene sequencing. Standards methods were used to characterise the isolates, both morphologically and physiologically. A thermophilic *Geobacillus sp* was isolated from both geothermal and mesophilic environments. Rao *et al*. (2003) reported that *G. thermoleovorans* was isolated from a hot water spring which is located in the Waimangu volcanic valley (New Zealand). In addition, *G. thermoleovorans* has been isolated from an active volcano of Santorini at Nea Kameni Island (Greek) and the soil temperature was between 85-95°C at the location (Meintanis *et al*., 2006). Another study conducted by Romano *et al*. (2005) reported the isolation of *G. thermoleovorans* from the Pizzo sopra Ia fossa site at Stromboli Island (Eolina Island, south of Italy), the temperature of the

volcanic ash here being 75°C. The result presented here show that *G. thermoleovorans* can be isolated from mesophilic environments and the main characteristics in this study are as follows: a) *G. thermoleovorans* was able to grow on rich LB media and M9 minimal salts media. b) The isolate was grown at 45°C to 70°C with 55°C being optimal in both media. c) *G. thermoleovorans* is as thermophile since it requires a temperature of around 55°C for optimum growth and was unable to grow outside the temperatures range of 45°C-70°C. d) *G. thermoleovorans* can grow in media at neutral pH and can tolerate pH values between 5.5 and 8.0. e) *G. thermoleovorans* does not require NaCl for growth however; it was able to grow in a NaCl concentration of 1.0M NaCl. f) the effect of UV-C significantly reduced C.F.U, while UV-B decreased on C.F.U with increasing length of exposure. Most of these results agree with findings published by Zarilla and Perry (1987).

The most important reason for selecting *E. mori* for further characterisation was its ability to grow over a range of different pH values. The isolate was grown at pH 4.0 to pH 10.0, with pH 7.0 being optimal in LB, Horikoshi and M9 minimal salts media. *E. mori* was first isolated from diseased mulberry roots (China) and reported as a new species in the genus *Enterobacter* and nominated as *Enterobacter mori* (Zhu *et al.*, 2011b). The result presented here show that *E. mori* was able to grow on rich media such as LB and M9 minimal salts media, This strain was grown at 25°C to 55°C, with 37°C being optimal in both media. *Enterobacter mori* was shown to be capable of growing and surviving in both alkaline and acidic conditions *E. mori* does not require NaCl for growth, but can tolerate salt concentration more than 1.0M NaCl, and a NaCl concentration at 1.5M in LB medium. *E. mori* survived when exposed to UV-C radiation at 5 to 20sec and it cannot survive after 30sec. However,

E. mori colony forming units decreased with increasing dose of UV-B radiation and it could not resist a 1 min exposure. *Pseudomonas putida* was able to grow on rich media as LB and minimal salts media M9.The strain was grown at 25°C to 45°C, 25°C being optimal in both media. The strain was grown at pH 5.0 to pH 9.0, with pH 7.0 being optimal in each media. There was no observable growth under alkaline conditions (pH 10.0 and pH 12.0), or acidic conditions (pH 3.0, pH 4.0) in LB, Horikoshi and M9 media for 24 hours or 48 hours. It can be concluded therefore that *P. putida* can tolerate a salt concentration of 1.0M NaCl. It cannot however, survive 30sec of exposure to UV-C. However, *P. putida* colony forming units decreased with an increasing dose of UV-B radiation and were killed after 1 min exposure.

NMR analysis shows that *G. thermoleovorans* in M9 medium did not accumulate any osmolyte in its cells, a fact which might be due to presence of intracellular metal ions at high temperatures and the main compatible solute at 55°C in LB medium was betaine. *E. mori* cells accumulated proline as the main compatible solute when it was grown in M9 minimal salt medium at pH 7.0 and was adapted at different salinities (0.17, 0.5, 1.0 and 1.5 M NaCl). Betaine was shown to be the main organic solute present in the LB medium at 1.5 M NaCl and the maximum amount of betaine was produced by *E. mori* at 1.5 M NaCl, and was drastically diminished at 2.0M NaCl. Moreover, betaine was the main compatible solute accumulated at pH 4.0, while, glutamate was dominant at pH 7.0 in the M9 medium. In LB medium betaine was the main compatible solute present; which was dominant at pH 10, while it was formed in lower amounts at lower pH values (e.g. pH4 and pH7). Finally, *P. putida* in M9 medium, accumulated proline and betaine at high salinity (e.g. 1.0 M), and the amount of betaine produced increased as salinity increased. The results show that *P. putida* accumulated betaine in most abundance at the highest salt concentration (e.g.

1.5M NaCl). Whereas, at all salinities below 1.5 M NaCl, glutamate and saccharide were accumulated in varying amounts.

The final study in this Thesis was aimed at determining whether *Mycoplasma* sp can be isolated from various desert surface soils, rock samples and other environmental samples. The results show that *Mycoplasma* sp was detected in only two environment samples, one for a Moss soil, collected from the Sheffield district, and the other for a soil collected from the garden of the Royal Hallamshire Hospital, (main entrances), Sheffield.

1.7. Suggestions for future work

So far the microbial diversity studies on the cave environments in the Middle Eastern regions have not received an adequate attention and hence, very little is known about the interaction between the microbes and the cave environments, which ultimately results in little literature being available on this subject; the findings of this Thesis shows the need for further research work on these unique cave environments, as well as the microbiology of caves in general. The following areas of future work of particular importance arise from the work presented here namely to:

1. Cave environments have been considered extreme environments and as result, microbes from this environment are difficult to cultivate in the laboratory. Therefore, further work using culture-independent techniques, such as the use of denaturing gradient gel electrophoresis (DGGE) to identify novel species present in these caves is highly desirable.
2. It would be of interest to determine whether cave dwelling microbes are adapted to growth in the cave environment or are they just survive in such extreme environments.

3. Since microorganisms play a significant role in nutrient cycles, it would be interesting to study these cycles (e.g. nitrogen, sulphur, carbon, iron and phosphorus) in cave environments.
4. It is also recommended that further studies should employ scanning electron microscopy (SEM) and transmission electron microscopy (TEM), in order to determine further the morphological and physiological features of microorganisms isolated from cave environments.

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APPENDIX

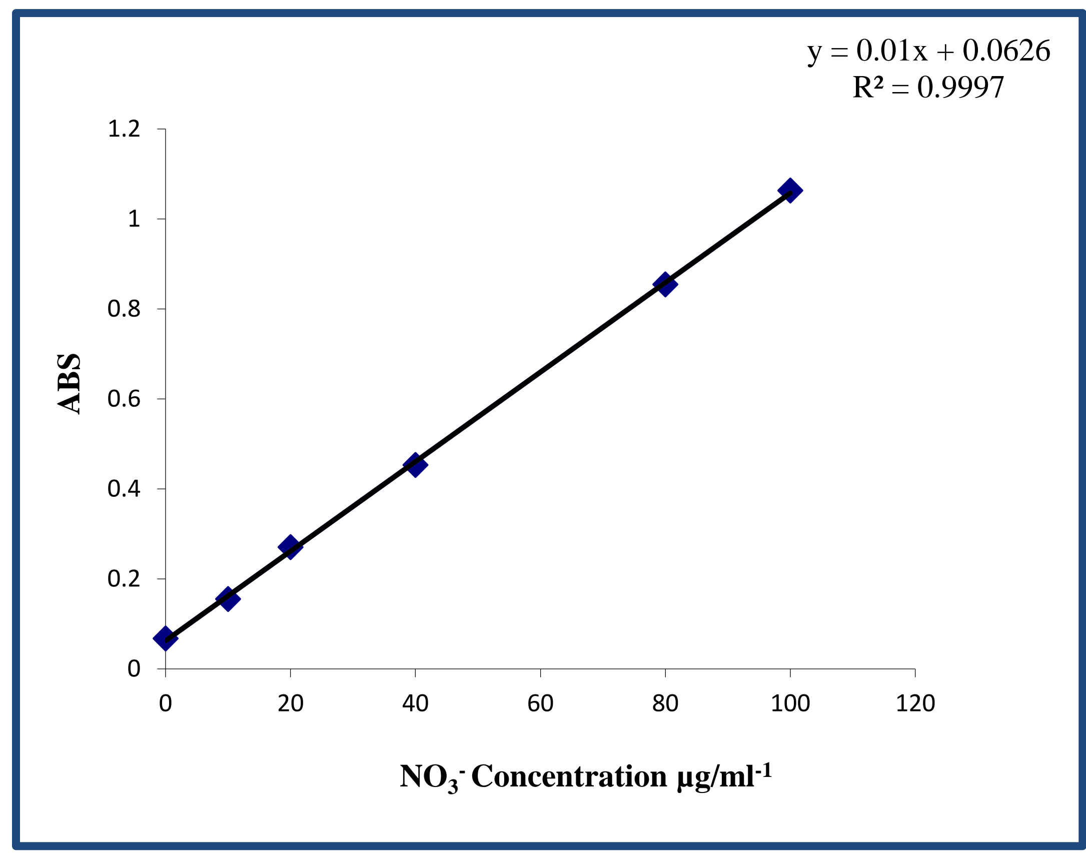
APPENDIX

Appendix A

Preparation of standard curves:

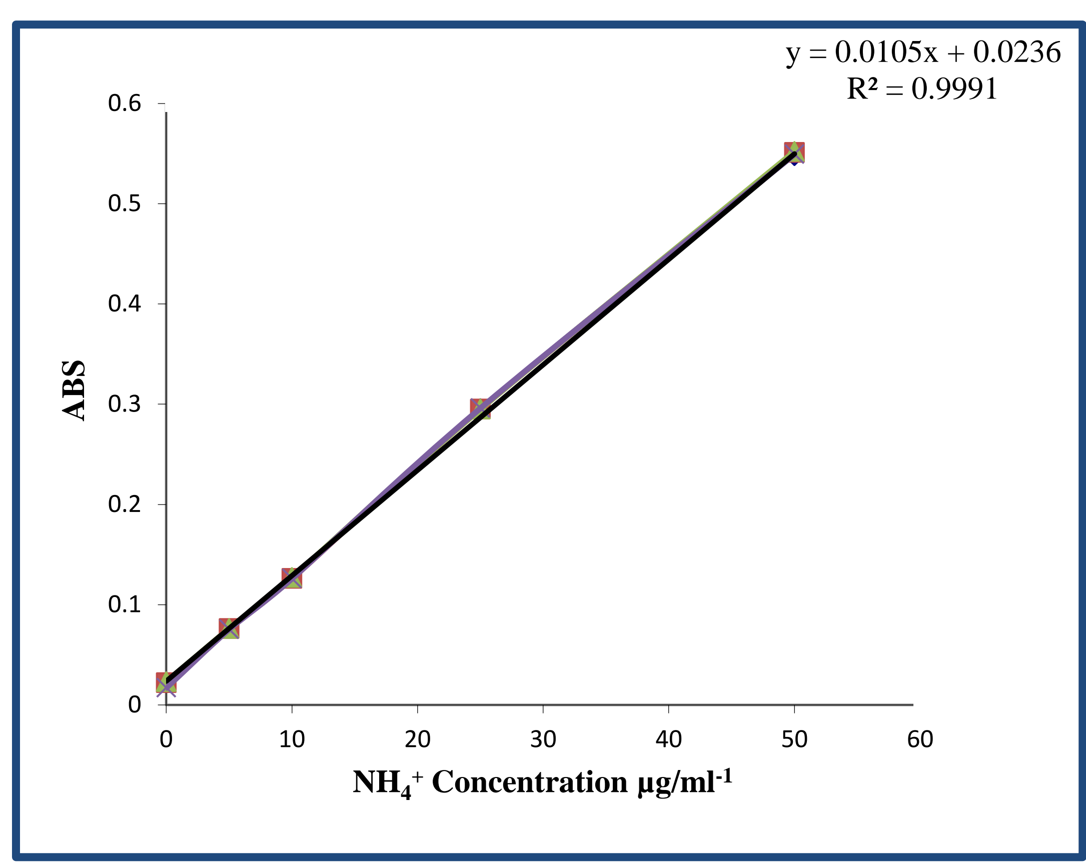
(1) Standard curve for nitrate

To produce a calibration curve for nitrate, 1.37 g of sodium nitrate ($NaNO_3$) was dissolved in 1 L dH_2O to obtain 1000µg NO_3^--N ml^{-1}. Then diluted the solution 10 times (10 ml sodium nitrate solution with 90 ml of dH_2O) equivalent 100µ g / NO_3^--N ml^{-1} This solution was diluted with dH_2O to produce solution 0,10,25,50,75, and 100 µg NO_3^--Nml^{-1}. All solution were analysed to determine the nitrate ions by using chromotropic acid method (Sims and Grant, 1971).



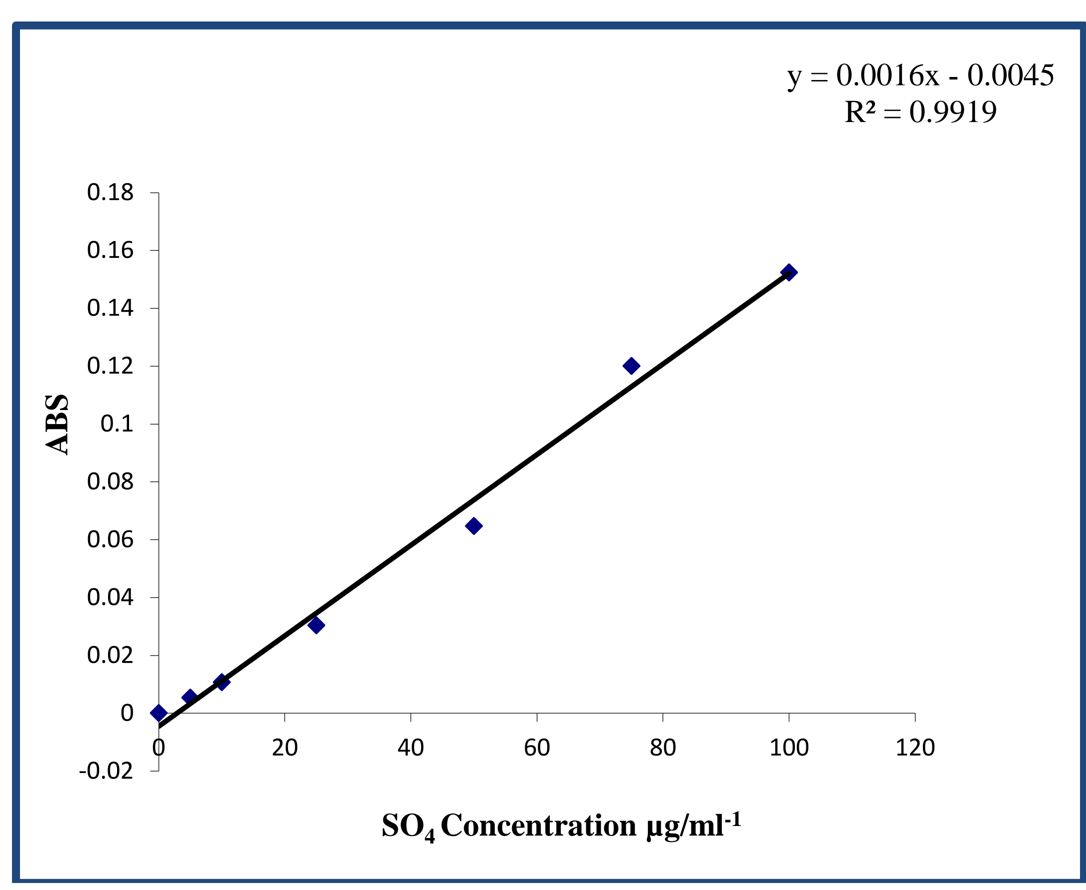
(2) Standard curve for ammonium

To produce a calibration curve for ammonium ions, 3.66 g of $(NH_4)_2SO_4$ ammonium sulphate was dissolved in 1 L dH_2O to obtain 1000µg NH_4^+-Nml^{-1}. Then diluted the solution 10 times (10 ml ammonium sulphate solution with 90 ml of dH_2O) equivalent 100µ g / NH_4^+-Nml^{-1} ·This solution was diluted with dH_2O to produce solution 0,10,25,and 50 µg NH_4^+-Nml^{-1}. All solutions were analysed to determine the ammonium ions by using indophenol blue method (Wainwright and Pugh, 1973).



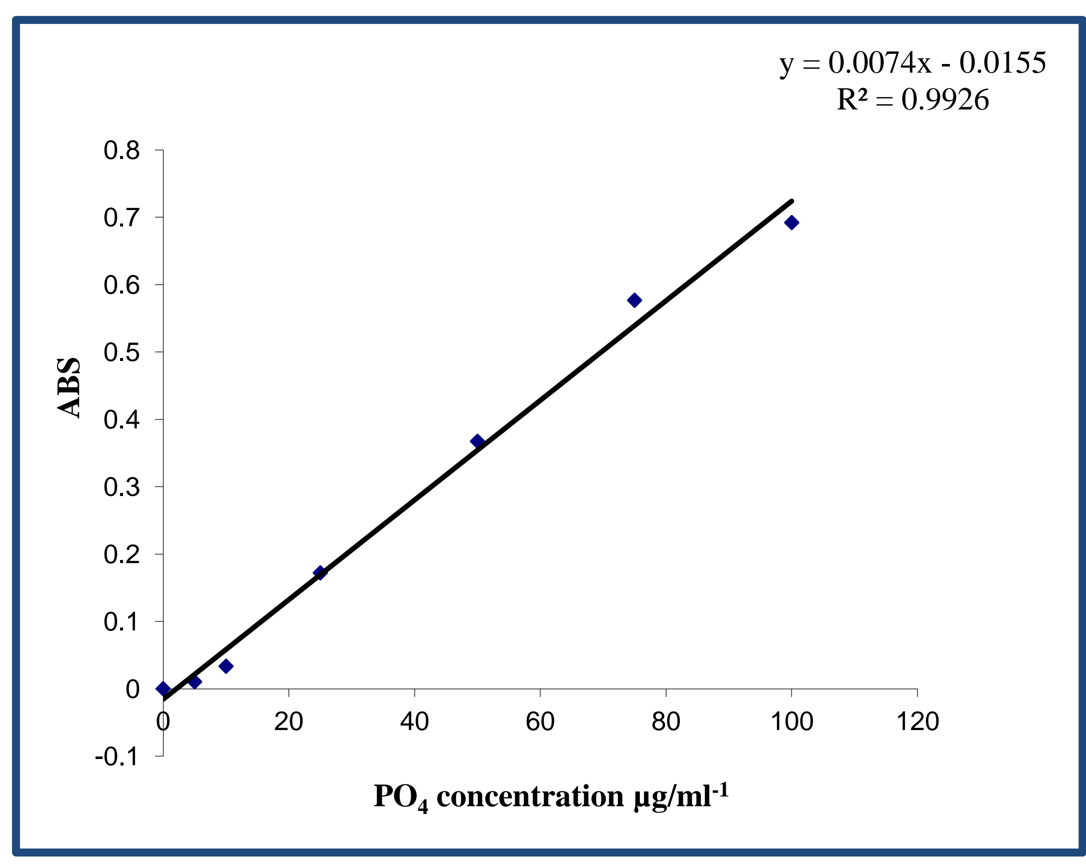
(3) Standard curve for sulphate-S

To produce a calibration curve for sulphate –S, 1.47 g of sodium sulphate ($NaSO_4$) was dissolved in 1 L dH_2O to obtain 1000µg SO_4^{2-}-Sml^{-1}. Then the solution diluted 10 times (10 ml of sodium sulphate solution with 90 ml of dH_2O) equivalent 100µ g / SO_4^{-2}-S ml^{-1}. This solution was diluted with dH_2O to produce 0,5,10,25,50,75 and 100 µg SO_4^{2-}-Sml^{-1}. All solutions were analysed to determine the sulphate-S ions by using turbidimetric analysis of sulphate-S (Hesse, 1971).



(4) Standard curve for Phosphate

To produce a calibration curve for phosphate –P, 1.48 g of sodium hydrogen phosphate (Na_2HPO_4) was dissolved in 1 L dH_2O to obtain 1000μg PO_4-Pml^{-1}. Then the solution diluted 10 times (10 ml of sodium hydrogen phosphate solution with 90 ml of dH_2O) equivalent 100μ g / PO_4-P ml^{-1}. This solution was diluted with dH_2O to produce 0,5,10,25,50,75 and 100 μg PO_4-Pml^{-1}. All solutions were analysed to determine the phosphate-P ions by using turbid-metric analysis of phosphate -P (Ajaj, 2005).



Appendix B

The phylogenetic analysis of desert surface soil, desert varnish, volcanic soil, rock samples and cave rock samples.

```
>gb|JQ317182.1|  Bacillus sp. ABR8 16S ribosomal RNA gene, partial sequence
Length=1445

 Score =  753 bits (834),  Expect = 0.0
 Identities = 421/422 (99%), Gaps = 1/422 (0%)
 Strand=Plus/Minus

Query  1     CAAAAGGTTACCCCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTG  60
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Sbjct  1422  CAAAAGGTTACCCCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTG  1363

Query  61    TACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAG  120
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Sbjct  1362  TACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAG  1303

Query  121   CTTCATGCAGGCGAGTTGCAGCCTG-AATCCGAACTGAGAATGGTTTTATGGGATTCGCT  179
             ||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  1302  CTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGAATGGTTTTATGGGATTCGCT  1243

Query  180   TAACCTCGCGGTCTTGCAGCCCTTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTC  239
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1242  TAACCTCGCGGTCTTGCAGCCCTTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTC  1183

Query  240   ATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCA  299
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1182  ATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCA  1123

Query  300   CCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGAC  359
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1122  CCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGAC  1063

Query  360   TTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCATCCTGTCC  419
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1062  TTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCATCCTGTCC  1003

Query  420   CC  421
             ||
Sbjct  1002  CC  1001
```

firmicutes | 2 leaves
Bacillus infantis strain AL10 16S ribosomal RNA gene, partial sequence
firmicutes | 3 leaves
Uncultured Bacillus sp. clone LT-10 16S ribosomal RNA gene, partial sequence
Bacillus sp. BL12 16S ribosomal RNA gene, partial sequence
029_kh_3_16S-R_EDIT
Bacillus sp. G2DM-61 16S ribosomal RNA gene, partial sequence
firmicutes | 3 leaves
Bacillus sp. SA014_1 16S ribosomal RNA gene, partial sequence
Bacillus sp. SA014_2 16S ribosomal RNA gene, partial sequence
Bacillus sp. SA014_3 16S ribosomal RNA gene, partial sequence
Bacillus sp. enrichment culture clone O5 16S ribosomal RNA gene, partial sequence
Bacillus sp. HLSB174 16S ribosomal RNA gene, partial sequence
Bacillus sp. JS-8 16S ribosomal RNA gene, partial sequence
Bacillus sp. JS-13 16S ribosomal RNA gene, partial sequence
Bacillus sp. DD2.202 16S ribosomal RNA gene, partial sequence
Bacillus sp. FS502 16S ribosomal RNA gene, partial sequence
Bacillus sp. SP2601 16S ribosomal RNA gene, partial sequence
Bacillus firmus strain RKS161- 16S ribosomal RNA (rrnE) gene, partial sequence
Bacillus sp. 64 16S ribosomal RNA gene, partial sequence
Bacillus sp. AsK15 16S ribosomal RNA gene, partial sequence
Bacillus sp. TP1MF 16S ribosomal RNA gene, partial sequence
Bacillus sp. JU1(2010) 16S ribosomal RNA gene, partial sequence
Bacillus sp. IBP-VN2 16S ribosomal RNA gene, partial sequence
Bacillus sp. M71_S33x partial 16S rRNA gene, isolate M71_S33x
Bacillus sp. ABR8 16S ribosomal RNA gene, partial sequence

firmicutes | 17 leaves
Bacillus firmus strain IAM 12464 16S ribosomal RN...
Bacillus firmus clone B563 16S ribosomal RNA gen...
Bacillus firmus 16S rRNA gene, isolate CV93a
Bacillus firmus 16S rRNA gene, isolate CV93b
Bacillus sp. ATCI02-4 16S ribosomal RNA gene, par..
Bacillus sp. BCw064 16S ribosomal RNA gene, parti.
Bacillus firmus strain 526-2 16S ribosomal RNA gen.
Bacillus sp. CU14 16S ribosomal RNA gene, partial ..
Bacillus firmus gene for 16S rRNA, partial sequence
Bacillus sp. BSi20511 16S ribosomal RNA gene, par
Bacillus sp. G2DM-32 16S ribosomal RNA gene, par.
Bacillus sp. By114(B)Ydz-dh 16S ribosomal RNA ...
Bacillus firmus strain CM21 16S ribosomal RNA gen.
Stenotrophomonas maltophilia strain ZFJ-4 16S ri...
Bacillus firmus strain ZB4 16S ribosomal RNA gene,.
Stenotrophomonas maltophilia strain BQAPs-03d 1.
Bacillus sp. W-SL-2 16S ribosomal RNA gene, parti...
Bacillus firmus strain 26-18 16S ribosomal RNA gen.
Bacillus sp. 35 16S ribosomal RNA gene, partial seq..
Bacillus sp. TSIW24 16S ribosomal RNA gene, parti.
Bacillus firmus strain UST981101-006 16S ribosoma
Bacillus sp. BR029 16S ribosomal RNA gene, partial.
Bacillus sp. BR024 16S ribosomal RNA gene, partial.
Bacillus sp. BR021 16S ribosomal RNA gene, partial.
Bacillus sp. 210_13 16S ribosomal RNA gene, parti..
Bacillus sp. 210_59 16S ribosomal RNA gene, parti..
Bacillus sp. HU29 16S ribosomal RNA (rrnE) gene, ..
Bacillus sp. C3(2009) 16S ribosomal RNA gene, par.
Uncultured bacterium clone FPURT2-B09 16S ribos..
Bacillus sp. 21F07-MB2-1 16S ribosomal RNA gen...
Bacillus firmus strain HSCS14 16S ribosomal RNA ...
Bacillus firmus strain PARZ10 16S ribosomal RNA ...
Bacillus firmus strain PARZ12 16S ribosomal RNA ...
Bacillus firmus strain KU3 16S ribosomal RNA gene,.
Bacillus sp. D-1 16S ribosomal RNA gene, partial se..
Bacillus firmus strain YD19 16S ribosomal RNA gen..
Bacillus firmus 16S rRNA gene, isolate AP15
Uncultured soil bacterium clone DLQRCAOBC104Y..
Uncultured soil bacterium clone DLQRCAOBC134Y..
firmicutes | 14 leaves

```
>ref|NR_044872.1|  Dermacoccus nishinomiyaensis 16S ribosomal RNA, partial sequence
 emb|X87757.1|  M.nishinomyaensis 16S rRNA gene
Length=1464

 Score =  911 bits (1010),  Expect = 0.0
 Identities = 509/513 (99%), Gaps = 0/513 (0%)
 Strand=Plus/Minus

Query  1     TAGGCCACCGGCTTCGGGTGTTACCGACTTTCGTGACTTGACGGGCGGTGTGTACAAGGC  60
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Query  61    CCGGGAACGTATTCACCGCAGCGTTGCTGATCTGCGATTACTAGCGACTCCGACTTCATG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1330  CCGGGAACGTATTCACCGCAGCGTTGCTGATCTGCGATTACTAGCGACTCCGACTTCATG  1271

Query  121   GGGTCGAGTTGCAGACCCCAATCCGAACTGAGACCGGTTTTTGGGATTAGCTCCACCTC  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1270  GGGTCGAGTTGCAGACCCCAATCCGAACTGAGACCGGTTTTTGGGATTAGCTCCACCTC  1211

Query  181   ACGGTTTCGCAACCCTCTGTACCGGCCATTGTAGCATGCGTGAAGCCCAAGACATAAGGG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1210  ACGGTTTCGCAACCCTCTGTACCGGCCATTGTAGCATGCGTGAAGCCCAAGACATAAGGG  1151

Query  241   GCATGATGATTTGACGTCATCCCCACCTTCCTCCGAGTTGACCCCGGCAGTCTCCCATGA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1150  GCATGATGATTTGACGTCATCCCCACCTTCCTCCGAGTTGACCCCGGCAGTCTCCCATGA  1091

Query  301   GTCCCCACCCGAAGTGCTGGCAACATGGAACGAGGGTTGCGCTCGNTGCGGGACTTAACC  360
             |||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||
Sbjct  1090  GTCCCCACCCGAAGTGCTGGCAACATGGAACGAGGGTTGCGCTCGTTGCGGGACTTAACC  1031

Query  361   CAACATCTCACGACACGAGCTGACGACAACCATGCACCANCTGTACACCAGTCCGAAGAC  420
             ||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  1030  CAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTACACCAGTCCGAAGAC  971

Query  421   GCACACATCTCTGCATGATTCCGGTGTATGTCAAGCCTTGGTAAGGTTCTNCGCGTTGNA  480
             |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||| |
Sbjct  970   GCACACATCTCTGCATGATTCCGGTGTATGTCAAGCCTTGGTAAGGTTCTTCGCGTTGCA  911

Query  481   TCGAATTAATCCGCATGCTCCGCCGCTTGTGCG  513
             |||||||||||||||||||||||||||||||||
Sbjct  910   TCGAATTAATCCGCATGCTCCGCCGCTTGTGCG  878
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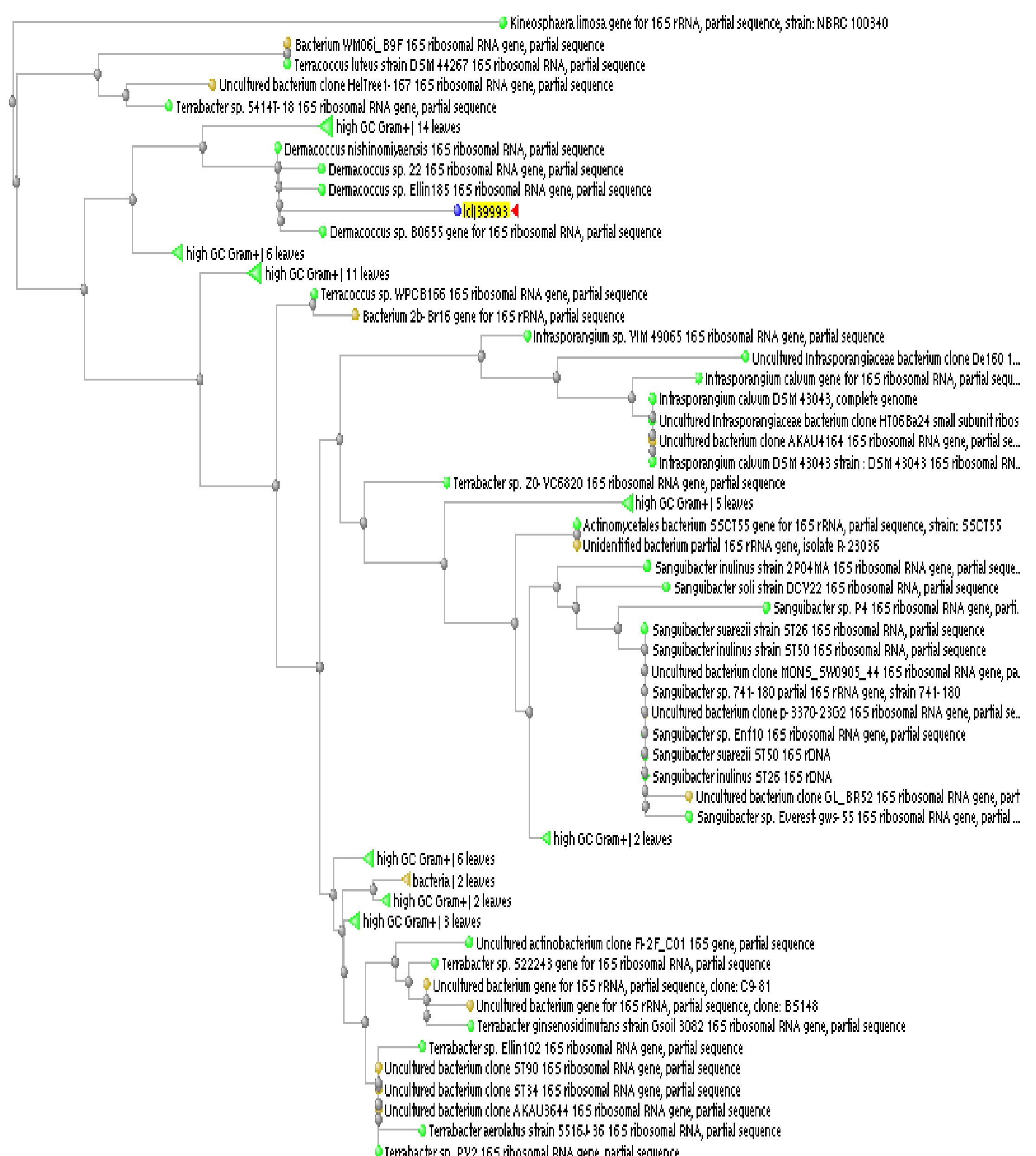

Kineosphaera limosa gene for 16S rRNA, partial sequence, strain: NBRC 100340
Bacterium WM06i_B9F 16S ribosomal RNA gene, partial sequence
Terracoccus luteus strain DSM 44267 16S ribosomal RNA, partial sequence
Uncultured bacterium clone HelTree1-167 16S ribosomal RNA gene, partial sequence
Terrabacter sp. 5414T-18 16S ribosomal RNA gene, partial sequence
high GC Gram+ | 14 leaves
Dermacoccus nishinomiyaensis 16S ribosomal RNA, partial sequence
Dermacoccus sp. 22 16S ribosomal RNA gene, partial sequence
Dermacoccus sp. Ellin185 16S ribosomal RNA gene, partial sequence
lcl|39993
Dermacoccus sp. B0655 gene for 16S ribosomal RNA, partial sequence
high GC Gram+ | 6 leaves
high GC Gram+ | 11 leaves
Terracoccus sp. WPCB166 16S ribosomal RNA gene, partial sequence
Bacterium 2b-Br16 gene for 16S rRNA, partial sequence
Intrasporangium sp. YIM 49065 16S ribosomal RNA gene, partial sequence
Uncultured Intrasporangiaceae bacterium clone De160 1...
Intrasporangium calvum gene for 16S ribosomal RNA, partial sequ...
Intrasporangium calvum DSM 43043, complete genome
Uncultured Intrasporangiaceae bacterium clone HT06Ba24 small subunit ribos
Uncultured bacterium clone AKAU4164 16S ribosomal RNA gene, partial se...
Intrasporangium calvum DSM 43043 strain : DSM 43043 16S ribosomal RN..
Terrabacter sp. Z0-YC6820 16S ribosomal RNA gene, partial sequence
high GC Gram+ | 5 leaves
Actinomycetales bacterium 55C155 gene for 16S rRNA, partial sequence, strain: 55C155
Unidentified bacterium partial 16S rRNA gene, isolate R-23036
Sanguibacter inulinus strain 2P04MA 16S ribosomal RNA gene, partial seque..
Sanguibacter soli strain DCY22 16S ribosomal RNA, partial sequence
Sanguibacter sp. P4 16S ribosomal RNA gene, parti.
Sanguibacter suarezii strain ST26 16S ribosomal RNA, partial sequence
Sanguibacter inulinus strain ST50 16S ribosomal RNA, partial sequence
Uncultured bacterium clone MON5_SW0905_44 16S ribosomal RNA gene, pa.
Sanguibacter sp. 741-180 partial 16S rRNA gene, strain 741-180
Uncultured bacterium clone p-3370-23G2 16S ribosomal RNA gene, partial se..
Sanguibacter sp. Enf10 16S ribosomal RNA gene, partial sequence
Sanguibacter suarezii ST50 16S rDNA
Sanguibacter inulinus ST26 16S rDNA
Uncultured bacterium clone GL_BR52 16S ribosomal RNA gene, part
Sanguibacter sp. Everest-gws-55 16S ribosomal RNA gene, partial ...
high GC Gram+ | 2 leaves
high GC Gram+ | 6 leaves
bacteria | 2 leaves
high GC Gram+ | 2 leaves
high GC Gram+ | 3 leaves
Uncultured actinobacterium clone FI-2F_C01 16S gene, partial sequence
Terrabacter sp. 522243 gene for 16S ribosomal RNA, partial sequence
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: C9-81
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: B5148
Terrabacter ginsenosidimutans strain Gsoil 3082 16S ribosomal RNA gene, partial sequence
Terrabacter sp. Ellin102 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone ST90 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone ST34 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone AKAU3644 16S ribosomal RNA gene, partial sequence
Terrabacter aerolatus strain 5516J-36 16S ribosomal RNA, partial sequence
Terrabacter sp. PY2 16S ribosomal RNA gene, partial sequence

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>gb|GU220477.1| Streptomyces sp. HMC5 16S ribosomal RNA gene, partial sequence
Length=1445

 Score =  230 bits (124),  Expect = 2e-57
 Identities = 136/141 (96%), Gaps = 4/141 (3%)
 Strand=Plus/Plus

Query  1    AACCACTTCGGT-GGGA-TAGTGGCGAACGTGTGAGTAACACGTGGGCAATCTGCCCTTC  58
            |||||||||||| |||| |||||||||||| |||||||||||||||||||||||||||||
Sbjct  65   AACCACTTCGGTGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTC  124

Query  59   ACTCTGGGACAAGCCCTGGAAACGGGG-CTAATACCGGATAACACCTTCTCTCTCCTGAG  117
            ||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  125  ACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATAACACCTTCTCTCTCCTGAG  184

Query  118  GGAAG-TTAAAAGCTCCGGCG  137
            ||||| |||||||||||||||
Sbjct  185  GGAAGGTTAAAAGCTCCGGCG  205
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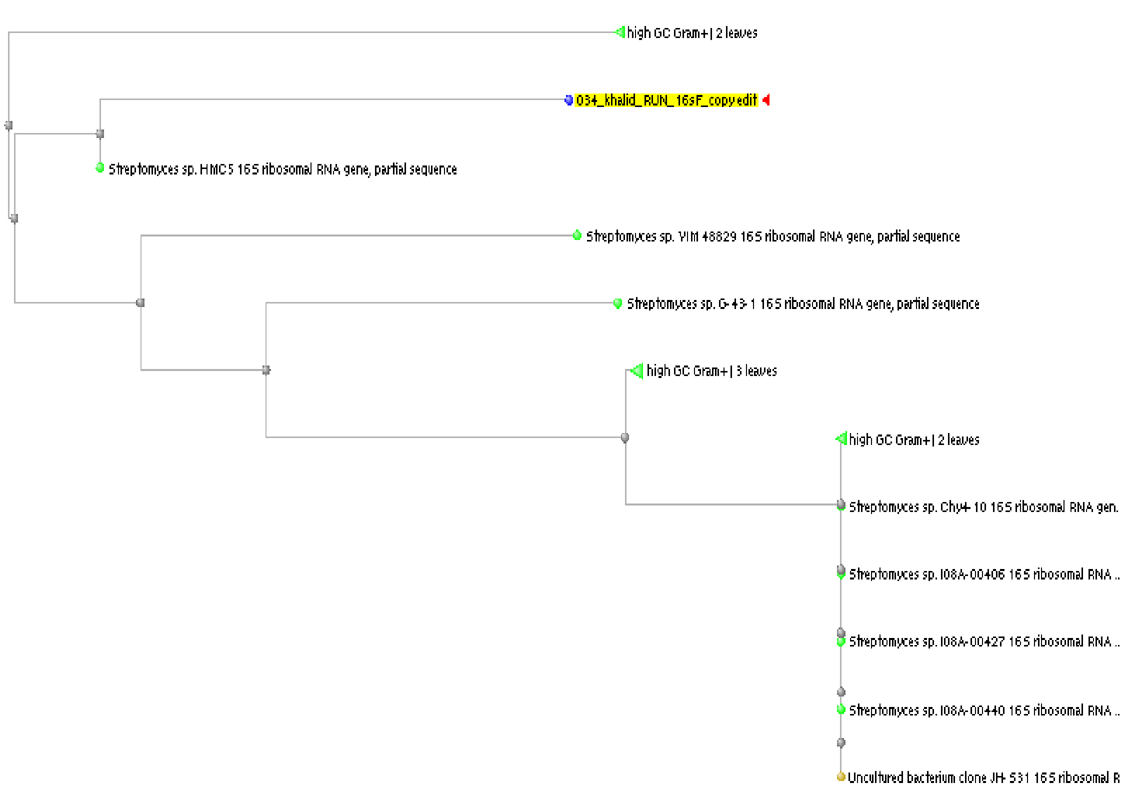

```
>gb|FJ795683.1|  Staphylococcus lentus strain 11-1 16S ribosomal RNA gene, partial
sequence
Length=902

 Score = 1200 bits (1330),  Expect = 0.0
 Identities = 676/683 (99%), Gaps = 1/683 (0%)
 Strand=Plus/Plus

Query  1    TGCAAGTCCGAGCGAACAGATGAGAAGCTTGCTTCTCTGATGTTAGCGGCGGACGGGTGA  60
            |||||||| |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16   TGCAAGTC-GAGCGAACAGATGAGAAGCTTGCTTCTCTGATGTTAGCGGCGGACGGGTGA  74

Query  61   GTAACACGTGGGTAACCTACCTATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATAC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  75   GTAACACGTGGGTAACCTACCTATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATAC  134

Query  121  CGGATAATATATTGAACCGCATGGTTCAATGTTGAAAGACGGTTTCGGCTGTCACTTATA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  135  CGGATAATATATTGAACCGCATGGTTCAATGTTGAAAGACGGTTTCGGCTGTCACTTATA  194

Query  181  GATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATACG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  195  GATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATACG  254

Query  241  TAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  255  TAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACG  314

Query  301  GGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  315  GGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTG  374

Query  361  AGTGATGAAGGTCTTAGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAATTTGTTAGTA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  375  AGTGATGAAGGTCTTAGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAATTTGTTAGTA  434

Query  421  ACTGAACAAGTCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  435  ACTGAACAAGTCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC  494

Query  481  GCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGC  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  495  GCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGC  554

Query  541  GGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCNACCGTGNAGGGTCATTGGAAACTGG  600
            |||||||||||||||||||||||||||||||||| |||||| ||||||||||||||||||
Sbjct  555  GGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGG  614

Query  601  GGAACTTGAGTGCAGAAAAGGANAGTGGAATTCCCTGTGTAGCGGTGAAATGCGCAAAGA  660
            ||||||||||||||||| |||| ||||||||||| ||||||||||||||||||||| |||
Sbjct  615  GGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGA  674

Query  661  TATGGAGGAACACCAGTGGCGAA  683
            |||||||||||||||||||||||
Sbjct  675  TATGGAGGAACACCAGTGGCGAA  697
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039_Khalid_THR__16F[1]_copy

Uncultured bacterium clone aaa51a02 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aaa43g04 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbt15d04 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbt15g02 16S ribosomal RNA gene, partial sequence
Staphylococcus lentus strain 11-1 16S ribosomal RNA gene, partial sequence
Staphylococcus lentus strain 10-3 16S ribosomal RNA gene, partial sequence
Staphylococcus lentus strain 10-1 16S ribosomal RNA gene, partial sequence
Staphylococcus lentus strain 7-5 16S ribosomal RNA gene, partial sequence
Staphylococcus lentus strain 7-3 16S ribosomal RNA gene, partial sequence
Staphylococcus lentus strain 7-4 16S ribosomal RNA gene, partial sequence
Staphylococcus lentus strain 7-1 16S ribosomal RNA gene, partial sequence
Staphylococcus lentus strain 3-1 16S ribosomal RNA gene, partial sequence
Staphylococcus lentus strain 2-2 16S ribosomal RNA gene, partial sequence
Staphylococcus lentus strain 2-1 16S ribosomal RNA gene, partial sequence
Staphylococcus lentus strain 1-1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone 1d05 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone 1f02 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone 0191 16S ribosomal RNA gene, partial sequence
Uncultured bacterium partial 16S rRNA gene, clone PIST-ADA11
Staphylococcus lentus strain CICCHLJ Q29 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28h04 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28h03 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28g11 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28g04 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28g03 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28f08 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28f05 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28f02 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28e12 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28d12 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28d11 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28d10 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28d07 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28c07 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28c06 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28c05 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28b12 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28b05 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28b01 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab28a12 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab27g12 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab27g11 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab27g08 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab27g04 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab27f02 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab27d12 16S ribosomal RNA gene, partial sequence
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Uncultured bacterium clone aab27b10 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab27b03 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab26h05 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab26h01 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab26g10 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab26g05 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab26f11 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab26f10 16S ribosomal RNA gene, partial sequence
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Uncultured bacterium clone aab26f01 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab26e09 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab26e01 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab26d08 16S ribosomal RNA gene, partial sequence
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Uncultured bacterium clone aab26b11 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab26b07 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab26b06 16S ribosomal RNA gene, partial sequence
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Uncultured bacterium clone aab26a05 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab26a01 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab25h12 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab25g11 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab25g08 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab25f07 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab25f06 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab25d10 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab25c09 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab25c08 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab25c07 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab25b04 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab25a07 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab25a03 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab40h11 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab40g02 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab40d06 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab40a05 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab40a03 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab39f04 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab39f02 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab39b12 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab39a01 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab38h04 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab38c02 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone aab38a08 16S ribosomal RNA gene, partial sequence
Staphylococcus lentus strain ATCC 29070 (= MAFF 911385) 16S ribosomal RNA, partial sequence
Uncultured bacterium clone nbt22a02 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nby556e02c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nby560e04c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nby297b03c1 16S ribosomal RNA gene, partial sequence
Staphylococcus sp. CSA7(2011) 16S ribosomal RNA gene, partial sequence

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>gb|JQ267647.1|  Bacillus amyloliquefaciens strain S76-3 16S ribosomal RNA gene,
partial sequence
Length=1500

 Score = 1040 bits (1152),  Expect = 0.0
 Identities = 576/576 (100%), Gaps = 0/576 (0%)
 Strand=Plus/Minus

Query  1     CCCACCTTCGGCGGCTGGCTCCTAAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1476  CCCACCTTCGGCGGCTGGCTCCTAAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTC  1417

Query  61    TCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGAT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1416  TCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGAT  1357

Query  121   CCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1356  CCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGA  1297

Query  181   GAACAGATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1296  GAACAGATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTG  1237

Query  241   TAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1236  TAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCC  1177

Query  301   TCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1176  TCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCA  1117

Query  361   AGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCA  420
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Sbjct  1116  AGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCA  1057

Query  421   TGCACCACCTGTCACTCTGCCCCCGAAGGGGACGTCCTATCTCTAGGATTGTCAGAGGAT  480
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Sbjct  1056  TGCACCACCTGTCACTCTGCCCCCGAAGGGGACGTCCTATCTCTAGGATTGTCAGAGGAT  997

Query  481   GTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTG  540
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Sbjct  996   GTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTG  937

Query  541   TGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTT  576
             ||||||||||||||||||||||||||||||||||||
Sbjct  936   TGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTT  901
```

firmicutes | 2 leaves

Uncultured prokaryote clone MB7-77 16S ribosomal RNA gene, partial sequence
Bacillus amyloliquefaciens strain UY1091 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain UY628 16S ribosomal RNA gene, partial sequence
Bacillus sp. enrichment culture clone WW2_90 16S ribosomal RNA gene, partial sequence
Bacillus subtilis subsp. subtilis strain KISR-1 16S ribosomal RNA gene, partial sequence
Bacillus amyloliquefaciens Y2, complete genome
Uncultured bacterium clone 12F_L2_24 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone 10F_L2_22 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone 09F_L2_21 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone 03E_L2_03 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone 01E_L2_01 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain HB-1 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain SS2 16S ribosomal RNA gene, partial sequence
Bacillus amyloliquefaciens strain P2 16S ribosomal RNA gene, partial sequence
Bacillus sp. JS, complete genome
Bacillus sp. PBCC 23 16S ribosomal RNA gene, partial sequence
Bacillus sp. PBCC 22 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain JR5-5 16S ribosomal RNA gene, partial sequence
Bacillus licheniformis strain JR2-6 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain JR2-3 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain BPD-15 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain YPP10 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain YPM8 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain U1PB1 16S ribosomal RNA gene, partial sequence
Bacillus methylotrophicus strain TPS 1 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain KPC(-8)1 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain JPM18 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain JPM1 4 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain JPB12 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain HPC20 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain FPA3 2-2 16S ribosomal RNA gene, partial sequence
Bacterium OUA-3 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain AKB13 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain DSM10 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain CE1 16S ribosomal RNA gene, partial sequence
Bacillus sp. LC03 16S ribosomal RNA gene, partial sequence
Bacillus sp. LS03 16S ribosomal RNA gene, partial sequence
Bacillus sp. LS02 16S ribosomal RNA gene, partial sequence
Bacillus amyloliquefaciens subsp. plantarum YAU B9601-Y2 complete genome
Bacillus subtilis strain CYBS-17 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain CYBS-12 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain CYBS-11 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain CYBS-8 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain CYBS-6 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain CYBS-4 16S ribosomal RNA gene, partial sequence
Lactobacillus murinus strain AU06 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain FS05 16S ribosomal RNA gene, partial sequence
Bacillus subtilis partial 16S rRNA gene, isolate GO19
Bacillus subtilis partial 16S rRNA gene, isolate SG05
Bacillus subtilis strain 55C1-1 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain 30L1-3 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain 30L1-2 16S ribosomal RNA gene, partial sequence
Bacillus subtilis subsp. subtilis strain 30P3-3 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain 30L2-2 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain 30C3-1 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain 30L2-3 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain 30AA2-4 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain 30AA2-2 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain 30N2-5 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain 30N2-4 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain 30N3-7 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain 30N3-4 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain 55N1-4 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain 30N2-9 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain W321 16S ribosomal RNA gene, partial sequence
Bacillus subtilis subsp. inaquosorum strain IS02 16S ribosomal RNA gene, partial sequence
Bacillus sp. B19(2011) 16S ribosomal RNA gene, partial sequence
Bacillus sp. B8(2011) 16S ribosomal RNA gene, partial sequence
Bacillus subtilis subsp. spizizenii gene for 16S rRNA, partial sequence, strain: NBRC 101243
Bacillus vallismortis gene for 16S rRNA, partial sequence, strain: NBRC 101236
Bacillus amyloliquefaciens gene for 16S rRNA, partial sequence, strain: NBRC 3022
Bacillus amyloliquefaciens gene for 16S rRNA, partial sequence, strain: NBRC 3025
Bacillus subtilis gene for 16S ribosomal RNA, partial sequence, strain: BMTO-A
Bacillus subtilis gene for 16S rRNA, partial sequence, strain: NBRC 13722
Bacillus subtilis gene for 16S rRNA, partial sequence, strain: NBRC 3936
Bacillus subtilis gene for 16S rRNA, partial sequence, strain: NBRC 104443
Bacillus subtilis gene for 16S rRNA, partial sequence, strain: NBRC 12112
Bacillus subtilis gene for 16S rRNA, partial sequence, strain: NBRC 12114
Bacillus subtilis gene for 16S rRNA, partial sequence, strain: NBRC 13169
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 12TCLN173
Bacillus sp. MIB5 16S ribosomal RNA gene, partial sequence
Bacillus sp. CS61 16S ribosomal RNA gene, partial sequence
Bacillus subtilis subsp. inaquosorum partial 16S rRNA gene, type strain DSM 22148T
Bacillus subtilis subsp. spizizenii TU-B-10, complete genome
Bacillus subtilis subsp. subtilis RO-NN-1, complete genome
Bacillus subtilis strain BA3_1A 16S ribosomal RNA gene, partial sequence
Bacillus subtilis subsp. subtilis partial 16S rRNA gene, strain B7B
Bacillus mojavensis strain NBSL48 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain SKE12 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain K21 16S ribosomal RNA gene, partial sequence
Bacillus sp. CE2 16S ribosomal RNA gene, partial sequence
Bacillus sp. AY-2011-RS14 partial 16S rRNA gene, isolate RS14
Bacillus subtilis strain ATF-40 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain B-762 16S ribosomal RNA gene, partial sequence
Bacillus sp. BIHB 1365 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain BN 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain IARI-CS-39 16S ribosomal RNA gene, partial sequence
030_kh_2_16S-R_EDIT_copy
Bacillus sp. TJ-C9 16S ribosomal RNA gene, partial sequence

```
> dbj|AB363733.1|  Paenibacillus lautus gene for 16S rRNA, partial sequence, strain:
NBRC 15380
Length=1480

 Score = 1043 bits (1156),  Expect = 0.0
 Identities = 579/580 (99%), Gaps = 0/580 (0%)
 Strand=Plus/Minus

Query  1     CCCCACCTTCGGCGGCTGGCTCCCTTGCGGGTTACCCCACCGACTTCGGGTGTTGTAAAC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1462  CCCCACCTTCGGCGGCTGGCTCCCTTGCGGGTTACCCCACCGACTTCGGGTGTTGTAAAC  1403

Query  61    TCTCGTGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGCGGCATGCTG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1402  TCTCGTGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGCGGCATGCTG  1343

Query  121   ATCCGCGATTACTAGCAATTCCGACTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1342  ATCCGCGATTACTAGCAATTCCGACTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACT  1283

Query  181   GAGACTGGCTTTTATAGGATTGGCTCCACCTCGCGGCTTCGCTTCCCGTTGTACCAGCCA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1282  GAGACTGGCTTTTATAGGATTGGCTCCACCTCGCGGCTTCGCTTCCCGTTGTACCAGCCA  1223

Query  241   TTGTAGTACGTGTGTAGCCCAAGTCATAAGGGGCATGATGATTTGACGTCATCCCCGCCT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1222  TTGTAGTACGTGTGTAGCCCAAGTCATAAGGGGCATGATGATTTGACGTCATCCCCGCCT  1163

Query  301   TCCTCCGGTTTGTCACCGGCAGTCATTCTAGAGTGCCCACCCGAAGTGCTGGCAACTAAA  360
             |||||||||||||||||||||||||||||||||||||||||| |||||||||||||||||
Sbjct  1162  TCCTCCGGTTTGTCACCGGCAGTCATTCTAGAGTGCCCACCCNAAGTGCTGGCAACTAAA  1103

Query  361   ATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1102  ATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACA  1043

Query  421   ACCATGCACCACCTGTCACCTCTGTCCCGAAGGCCGCCTCTATCTCTAGAGGATTCAGAG  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1042  ACCATGCACCACCTGTCACCTCTGTCCCGAAGGCCGCCTCTATCTCTAGAGGATTCAGAG  983

Query  481   GGATGTCAAGACTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATACTCCACTG  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  982   GGATGTCAAGACTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATACTCCACTG  923

Query  541   CTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAGTCTT  580
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  922   CTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAGTCTT  883
```

Paenibacillus lautus strain RSNPB21 16S ribosomal RNA gene, partial sequence
Paenibacillus sp. H25-07 partial 16S rRNA gene
Bacterium DE5 16S ribosomal RNA gene, partial sequence
Uncultured Paenibacillus sp. partial 16S rRNA gene, clone ACf23
firmicutes | 2 leaves
Uncultured Paenibacillus sp. clone BA-N-8 16S ribosomal RNA gene, partial sequence
Paenibacillus sp. XP1-2 16S ribosomal RNA gene, partial sequence
Paenibacillus sp. V4.12MC10, complete genome
Paenibacillus lautus strain SK21 16S ribosomal RNA gene, partial sequence
Paenibacillus sp. 5B45-2B 16S ribosomal RNA gene, partial sequence
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: WB18-82
firmicutes | 2 leaves
firmicutes | 3 leaves
Paenibacillus lautus strain S138 16S ribosomal RNA gene, partial sequence
028_kh_4_16S-R_EDIT
Paenibacillus sp. sc_50 16S ribosomal RNA gene, partial sequence
Paenibacillus lactis strain MB 2035 16S ribosomal RNA gene, partial sequence
Paenibacillus lactis partial 16S rRNA gene, strain IB-188D
Paenibacillus lactis strain MB 1928 16S ribosomal RNA gene, partial sequence
Uncultured bacterium partial 16S rRNA gene, clone SMG137
firmicutes | 3 leaves
Uncultured compost bacterium partial 16S rRNA gene, clone F51058
firmicutes | 2 leaves
Paenibacillus lactis strain ZYb1 16S ribosomal RNA gene, partial sequence
bacteria | 2 leaves
Uncultured bacterium clone B5592 16S ribosomal RNA gene, partial se...
Uncultured Paenibacillus sp. clone OUT6 16S ribos...
Paenibacillus sp. TR1103-3 gene for 16S rRNA, partial sequence
Paenibacillus sp. enrichment culture clone xj7 16S ribosomal RNA gene, partial .
bacteria | 4 leaves
firmicutes | 2 leaves
Uncultured bacterium partial 16S rRNA gene, clone P_2C02
firmicutes | 2 leaves
Paenibacillus sp. P538 16S ribosomal RNA gene, partial sequence
Paenibacillus sp. BM-7 16S ribosomal RNA gene, partial sequence
firmicutes | 2 leaves
Paenibacillus sp. CC-VHH111 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone SH 16S ribosomal RNA gene, partial sequence
firmicutes | 4 leaves
firmicutes | 22 leaves
firmicutes | 23 leaves

```
>gb|JF505980.1|  Exiguobacterium mexicanum strain KNUC9046 16S ribosomal RNA gene,
partial sequence
Length=1494

 Score = 1440 bits (1596),  Expect = 0.0
 Identities = 803/805 (99%), Gaps = 1/805 (0%)
 Strand=Plus/Plus

Query  1    CGTGCNTAATACATGCA-GTCGAGCGCAGGAAATCGACGGAACCCTTCGGGGGGAAGTCG  59
            ||||| ||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  25   CGTGCCTAATACATGCAAGTCGAGCGCAGGAAATCGACGGAACCCTTCGGGGGGAAGTCG  84

Query  60   ACGGAATGAGCGGCGGACGGGTGAGTAACACGTAAAGAACCTGCCCTCAGGTCTGGGATA  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  85   ACGGAATGAGCGGCGGACGGGTGAGTAACACGTAAAGAACCTGCCCTCAGGTCTGGGATA  144

Query  120  ACCACGAGAAATCGGGGCTAATACCGGATGGGTCATCGGACCGCATGGTCCGAGGATGAA  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  145  ACCACGAGAAATCGGGGCTAATACCGGATGGGTCATCGGACCGCATGGTCCGAGGATGAA  204

Query  180  AGGCGCTTCGGCGTCGCCTGGGGATGGCTTTGCGGTGCATTAGCTAGTTGGTGGGGTAAT  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  205  AGGCGCTTCGGCGTCGCCTGGGGATGGCTTTGCGGTGCATTAGCTAGTTGGTGGGGTAAT  264

Query  240  GGCCCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265  GGCCCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG  324

Query  300  AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAG  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  325  AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAG  384

Query  360  TCTGATGGAGCAACGCCGCGTGAACGATGAAGGCCTTCGGGTCGTAAAGTTCTGTTGTAA  419
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  385  TCTGATGGAGCAACGCCGCGTGAACGATGAAGGCCTTCGGGTCGTAAAGTTCTGTTGTAA  444

Query  420  GGGAAGAACAAGTGCCGCAGGCAATGGCGGCACCTTGACGGTACCTTGCGAGAAAGCCAC  479
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  445  GGGAAGAACAAGTGCCGCAGGCAATGGCGGCACCTTGACGGTACCTTGCGAGAAAGCCAC  504

Query  480  GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTAT  539
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  505  GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTAT  564

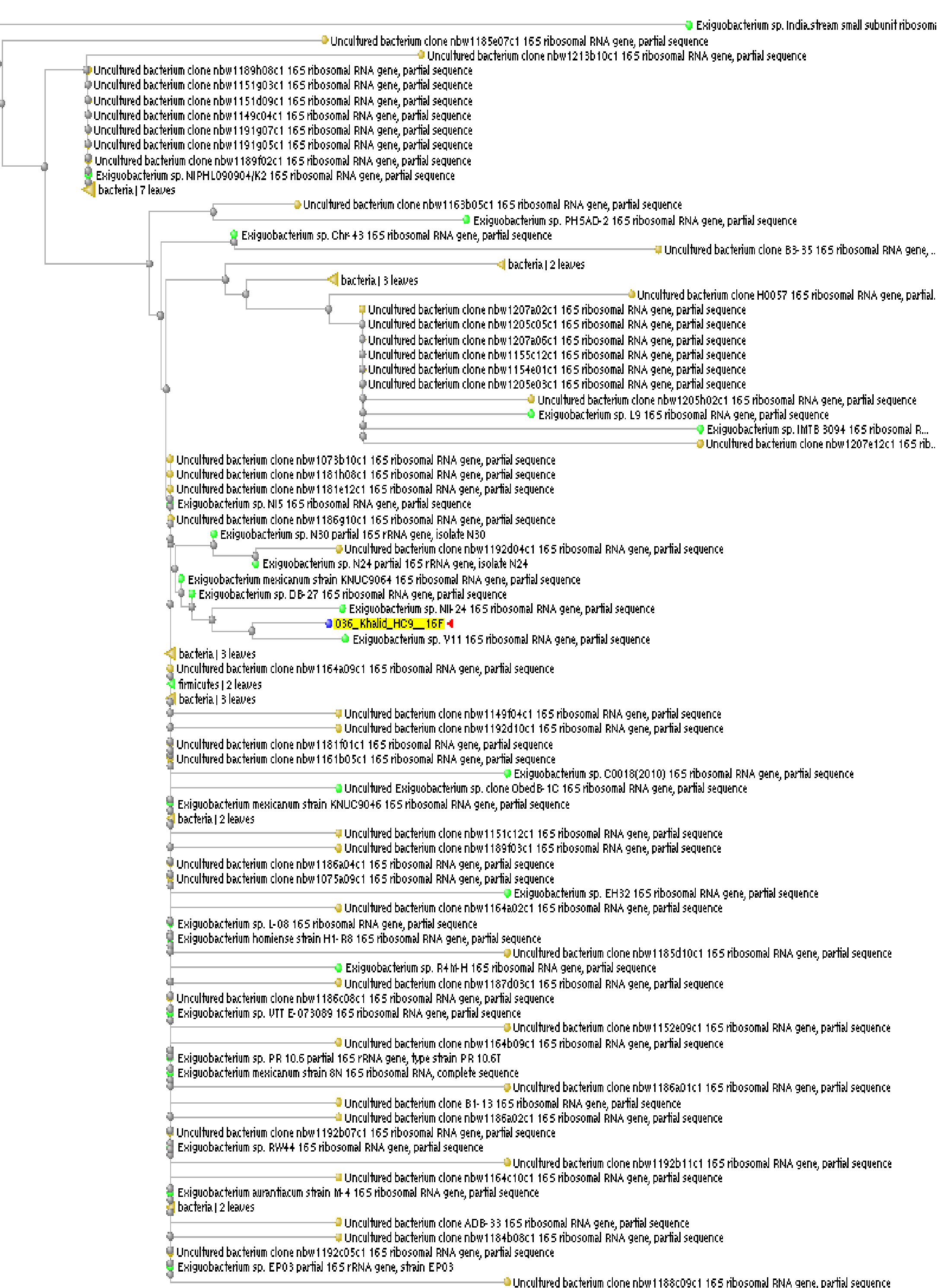
Query  540  TGGGCGTAAAGCGCGCGCAGGCGGCCTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACC  599
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  565  TGGGCGTAAAGCGCGCGCAGGCGGCCTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACC  624

Query  600  GGGGAGGGCCATTGGAAACTGGGAGGCTTGAGTATAGGAGAGAAGAGTGGAATTCCACGT  659
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  625  GGGGAGGGCCATTGGAAACTGGGAGGCTTGAGTATAGGAGAGAAGAGTGGAATTCCACGT  684

Query  660  GTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGCC  719
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  685  GTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGCC  744

Query  720  TATAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT  779
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  745  TATAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT  804

Query  780  CCACGCCGTAAACGATGAGTGCTAG  804
            |||||||||||||||||||||||||
Sbjct  805  CCACGCCGTAAACGATGAGTGCTAG  829
```



Exiguobacterium sp. India.stream small subunit ribosomi
Uncultured bacterium clone nbw1185e07c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1213b10c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1189h08c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1151g03c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1151d09c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1149c04c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1191g07c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1191g05c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1189f02c1 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. NIPHL090904/K2 16S ribosomal RNA gene, partial sequence
bacteria | 7 leaves
Uncultured bacterium clone nbw1163b05c1 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. PH5AD-2 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. Chr-43 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone B3-35 16S ribosomal RNA gene, ..
bacteria | 2 leaves
bacteria | 3 leaves
Uncultured bacterium clone H0057 16S ribosomal RNA gene, partial.
Uncultured bacterium clone nbw1207a02c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1205c05c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1207a06c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1155c12c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1154e01c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1205e03c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1205h02c1 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. L9 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. IMTB 3094 16S ribosomal R...
Uncultured bacterium clone nbw1207e12c1 16S rib..
Uncultured bacterium clone nbw1073b10c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1181h08c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1181e12c1 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. NI5 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1186g10c1 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. N30 partial 16S rRNA gene, isolate N30
Uncultured bacterium clone nbw1192d04c1 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. N24 partial 16S rRNA gene, isolate N24
Exiguobacterium mexicanum strain KNUC9064 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. DB-27 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. NH-24 16S ribosomal RNA gene, partial sequence
036_Khalid_HC9__16F
Exiguobacterium sp. Y11 16S ribosomal RNA gene, partial sequence
bacteria | 3 leaves
Uncultured bacterium clone nbw1164a09c1 16S ribosomal RNA gene, partial sequence
firmicutes | 2 leaves
bacteria | 3 leaves
Uncultured bacterium clone nbw1149f04c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1192d10c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1181f01c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1161b05c1 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. C0018(2010) 16S ribosomal RNA gene, partial sequence
Uncultured Exiguobacterium sp. clone ObedB-1C 16S ribosomal RNA gene, partial sequence
Exiguobacterium mexicanum strain KNUC9046 16S ribosomal RNA gene, partial sequence
bacteria | 2 leaves
Uncultured bacterium clone nbw1151c12c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1189f03c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1186a04c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1075a09c1 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. EH32 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1164a02c1 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. L-08 16S ribosomal RNA gene, partial sequence
Exiguobacterium homiense strain H1-R8 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1185d10c1 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. R4M-H 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1187d03c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1186c08c1 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. VIT E-073089 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1152e09c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1164b09c1 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. PR 10.6 partial 16S rRNA gene, type strain PR 10.6T
Exiguobacterium mexicanum strain 8N 16S ribosomal RNA, complete sequence
Uncultured bacterium clone nbw1186a01c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone B1-13 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1186a02c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1192b07c1 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. RW44 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1192b11c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1164c10c1 16S ribosomal RNA gene, partial sequence
Exiguobacterium aurantiacum strain M-4 16S ribosomal RNA gene, partial sequence
bacteria | 2 leaves
Uncultured bacterium clone ADB-33 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1184b08c1 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone nbw1192c05c1 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. EP03 partial 16S rRNA gene, strain EP03
Uncultured bacterium clone nbw1188c09c1 16S ribosomal RNA gene, partial sequence

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> gb|HQ123468.1|  Bacillus mojavensis strain KK2 16S ribosomal RNA gene, partial
sequence
Length=1408

 Score = 1319 bits (1462),  Expect = 0.0
 Identities = 740/745 (99%), Gaps = 1/745 (0%)
 Strand=Plus/Plus

Query  1    AAGCGGACAGATGGGAGCTTGCTNCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTG  60
            ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  1    AAGCGGACAGATGGGAGCTTGCT-CCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTG  59

Query  61   GGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  60   GGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTG  119

Query  121  TTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  120  TTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCG  179

Query  181  CGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  180  CGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCT  239

Query  241  GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  240  GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA  299

Query  301  GTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  300  GTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAG  359

Query  361  GTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGNATAGGGCGGT  420
            ||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  360  GTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGT  419

Query  421  ACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  420  ACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATA  479

Query  481  CGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTCCTTA  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  480  CGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTCCTTA  539

Query  541  AGTCTGATGTGAAAGCCCCCGGCTCACCCGGGGAGGGTCATTGGAAACTGGGGAACTTGA  600
            |||||||||||||||||||||||||| |||||||||||||||||||||||||||||||||
Sbjct  540  AGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGA  599

Query  601  GTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGA  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  600  GTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGA  659

Query  661  ACACCAGTGGCGAANGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGG  720
            |||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  660  ACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGG  719

Query  721  GAGCGAACAGGATTAAATACCCTGG  745
            ||||||||||||||| |||||||||
Sbjct  720  GAGCGAACAGGATTAGATACCCTGG  744
```

025_Khalid_HC33__16F

Bacillus mojavensis strain KK2 16S ribosomal RNA gene, partial sequence
Bacillus mojavensis strain IFO15718 16S ribosomal RNA, partial sequence
Bacillus subtilis strain CICC10034 16S ribosomal RNA gene, partial sequence
Bacillus mojavensis strain BCRC 17124 16S ribosomal RNA gene, partial sequence
Bacillus mojavensis strain A21 16S ribosomal RNA gene, partial sequence
Bacillus mojavensis isolate 6 16S ribosomal RNA gene, partial sequence
Bacillus sp. M71_D80 partial 16S rRNA gene, isolate M71_D80
Bacillus mojavensis strain 5512Ba-19 16S ribosomal RNA gene, partial sequence
Bacillus mojavensis strain Hswx10 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain 8-2 16S ribosomal RNA gene, partial sequence
Bacillus sp. M71_S52 partial 16S rRNA gene, isolate M71_S52
Bacillus sp. B27(2009) 16S ribosomal RNA gene, partial sequence
Bacillus sp. GCNB6 16S ribosomal RNA gene, partial sequence
Bacillus sp. CICCHLJ Q35 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain PCL1605 16S ribosomal RNA gene, partial sequence
Bacillus sp. GMTB-C1-2 16S ribosomal RNA gene, complete sequence
Bacillus sp. P209 gene for 16S rRNA, partial sequence
Bacillus mojavensis strain ifo 15718 16S ribosomal RNA, partial sequence
Bacillus subtilis gene for 16S rRNA, partial sequence, strain: NBRC 13722
Bacillus malacitensis strain MD4 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain WQ-17 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain WQ-12 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain WQ-10 16S ribosomal RNA gene, partial sequence
Bacterium B572 16S ribosomal RNA gene, partial sequence
firmicutes | 17 leaves
firmicutes | 8 leaves
firmicutes | 29 leaves
firmicutes | 7 leaves
Bacterium FJAT-13827 16S ribosomal RNA gene, partial sequence
firmicutes | 6 leaves
firmicutes | 8 leaves

```
>gb|JQ271812.1|  Bacillus muralis strain 6N02 16S ribosomal RNA gene, partial
sequence
Length=1055

 Score =  931 bits (1032),  Expect = 0.0
 Identities = 531/537 (99%), Gaps = 3/537 (1%)
 Strand=Plus/Minus

Query  1     GAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1029  GAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTA  970

Query  61    CAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  969   CAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCT  910

Query  121   TCATGCAGGCGAGTTGCAGCCTGCA-TCCGAACTGAGAATGGCTTTATGGGATTCGCTTA  179
             ||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  909   TCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGAATGGCTTTATGGGATTCGCTTA  850

Query  180   CCTTCGCAGGTTTGCAGCCCTTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCAT  239
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  849   CCTTCGCAGGTTTGCAGCCCTTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCAT  790

Query  240   AAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACC  299
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  789   AAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACC  730

Query  300   TTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTT  359
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  729   TTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTT  670

Query  360   AACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGTCCCC  419
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  669   AACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGTCCCC  610

Query  420   CGAAGGNAAAAGCCCTATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAGGTTCTT  479
             ||||||  |||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  609   CGAAGGGGAAAGCCCTATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTT  550

Query  480   CGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGGTGCGGGCCCCCGTCAAT  536
             ||||||||||||||||||||||||||||||||||||| ||||||| |||||||||||
Sbjct  549   CGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTT-GTGCGGG-CCCCCGTCAAT  495
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017_khalid_Red_16sF_copy

Bacillus sp. IMCd03 partial 16S rRNA gene, strain IMCd03
Bacillus simplex strain DHXJ10 16S ribosomal RNA gene, partial sequence
Bacillus sp. MJBC16 16S ribosomal RNA gene, partial sequence
Bacterium T56 16S ribosomal RNA gene, partial sequence
Bacillus simplex strain CE27 16S ribosomal RNA gene, partial sequence
Bacillus simplex strain Dr18 16S ribosomal RNA gene, partial sequence
Bacillus muralis strain cp5 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone TX2_2L18 16S ribosomal RNA gene, partial sequence
Uncultured Bacillus sp. clone CB8 16S ribosomal RNA gene, partial sequence
Bacillus simplex strain P51 16S ribosomal RNA gene, partial sequence
Bacillus thuringiensis strain AIMST KBT9-X 16S ribosomal RNA gene, partial sequence
Uncultured Brevibacterium sp. clone T-11 16S ribosomal RNA gene, partial sequence
firmicutes | 51 leaves
Bacillus simplex strain PRL3 16S ribosomal RNA gene, partial sequence
Bacillus sp. PRL4 16S ribosomal RNA gene, partial sequence
Bacillus sp. O-NR1 16S ribosomal RNA gene, partial sequence
Bacillus sp. L03(2011) 16S ribosomal RNA gene, partial sequence
Bacillus sp. R19(2011) 16S ribosomal RNA gene, partial sequence
Bacillus sp. R39(2011) 16S ribosomal RNA gene, partial sequence
Firmicutes bacterium R24 16S ribosomal RNA gene, partial sequence
Bacillus simplex strain Bi19 16S ribosomal RNA gene, partial sequence
Bacillus simplex strain CBG_LBI6 16S ribosomal RNA gene, partial sequence
Bacillus simplex strain CBG_LBI13II 16S ribosomal RNA gene, partial sequence
Bacillus simplex strain CBG_LBI20 16S ribosomal RNA gene, partial sequence
Bacillus simplex strain CBG_LBI24 16S ribosomal RNA gene, partial sequence
Bacillus simplex strain CBG_LBIQ 16S ribosomal RNA gene, partial sequence
Bacterium enrichment culture clone F56X_A11_T3-_A11 16S ribosomal RNA gene, partial sequence
Bacterium enrichment culture clone F56X_A7_T3-_A07 16S ribosomal RNA gene, partial sequence
Bacterium enrichment culture clone F56X_B11_T3-_B11 16S ribosomal RNA gene, partial sequence
Bacillus sp. LPPA 1494 partial 16S rRNA gene, strain LPPA 1494
Uncultured Bacillus sp. clone T2F21d33 16S ribosomal RNA gene, partial sequence
Bacillus sp. z7cer 16S ribosomal RNA gene, partial sequence
Uncultured bacterium isolate 1112869353162 16S ribosomal RNA gene, partial sequence
firmicutes | 17 leaves

```
>gb|JN856456.1|  Bacillus subtilis subsp. inaquosorum strain IS02 16S ribosomal
RNA gene, partial sequence
Length=1444

 Score = 1040 bits (1152),  Expect = 0.0
 Identities = 576/576 (100%), Gaps = 0/576 (0%)
 Strand=Plus/Minus

Query  1     CCCACCTTCGGCGGCTGGCTCCTAAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1427  CCCACCTTCGGCGGCTGGCTCCTAAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTC  1368

Query  61    TCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGAT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1367  TCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGAT  1308

Query  121   CCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1307  CCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGA  1248

Query  181   GAACAGATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1247  GAACAGATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTG  1188

Query  241   TAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1187  TAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCC  1128

Query  301   TCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1127  TCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCA  1068

Query  361   AGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1067  AGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCA  1008

Query  421   TGCACCACCTGTCACTCTGCCCCCGAAGGGGACGTCCTATCTCTAGGATTGTCAGAGGAT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1007  TGCACCACCTGTCACTCTGCCCCCGAAGGGGACGTCCTATCTCTAGGATTGTCAGAGGAT  948

Query  481   GTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTG  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  947   GTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTG  888

Query  541   TGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTT  576
             ||||||||||||||||||||||||||||||||||||
Sbjct  887   TGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTT  852
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firmicutes | 2 leaves

Bacillus subtilis strain NEB 3 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain NEB 44 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain CSY388 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain CSY191 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain HD-1 16S ribosomal RNA gene, partial sequence
Bacillus sp. WM1001 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain TP-Snow-C17 16S ribosomal RNA gene, partial sequence
Bacillus amyloliquefaciens strain AcT-13 16S ribosomal RNA gene, partial sequence
Bacillus subtilis subsp. subtilis gene for 16S rRNA, partial sequence, strain: JCM 10629
Bacillus subtilis strain P10 16S ribosomal RNA gene, partial sequence
Bacillus amyloliquefaciens strain P2 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain P4 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain P6 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain P53 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain P79 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain ZWQ-2 16S ribosomal RNA gene, partial sequence
Bacillus subtilis partial 16S rRNA gene, strain CH1
Bacillus sp. SS1 partial 16S rRNA gene, strain SS1
Bacillus subtilis strain A9 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain KIBGE-IB17 16S ribosomal RNA gene, partial sequence
Bacillus subtilis BSn5, complete genome
Bacillus subtilis strain Amp1 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain KJB06-35 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain AVT-KSU309 16S ribosomal RNA gene, partial sequence
Bacillus mojavensis strain PDR-KSU303 16S ribosomal RNA gene, partial sequence
Bacillus axarquiensis strain PDR-KSU302 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain PDR-KSU304 16S ribosomal RNA gene, partial sequence
Bacillus amyloliquefaciens strain SUM-KSU302 16S ribosomal RNA gene, partial sequence
Bacillus axarquiensis strain SUM-KSU303 16S ribosomal RNA gene, partial sequence
Bacillus subtilis subsp. spizizenii strain SUM-KSU305 16S ribosomal RNA gene, partial sequence
Bacillus subtilis subsp. spizizenii strain WSE-KSU303 16S ribosomal RNA gene, partial sequence
Bacillus subtilis subsp. subtilis strain WSE-KSU304 16S ribosomal RNA gene, partial sequence
Bacillus mojavensis strain WSE-KSU305 16S ribosomal RNA gene, partial sequence
Bacillus subtilis subsp. subtilis strain WSR-KSU310 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain NBM48 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain Baws1 16S ribosomal RNA gene, partial sequence
Bacillus sp. 3417BRRJ 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain NBY44 16S ribosomal RNA gene, partial sequence
Bacillus sp. MW1001 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain MB5 NIOT 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain CLW-BA1-5 16S ribosomal RNA gene, partial sequence
Bacillus sp. enrichment culture clone C-11 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain CG24 16S ribosomal RNA gene, partial sequence
Bacillus sp. GC-1 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain MUSc-1 16S ribosomal RNA gene, partial sequence
Bacillus sp. 3614BRRJ 16S ribosomal RNA gene, partial sequence
Bacillus sp. 5001BRRJ 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain GYPB04 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain TUL322 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain BPRIST007 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain BPRIST008 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain BPRIST009 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain BT18 16S ribosomal RNA gene, partial sequence
Bacillus amyloliquefaciens strain Hy7 16S ribosomal RNA gene, partial sequence
Bacillus sp. N14(2011) 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain A45 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain U42E 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain U122A 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain U154B 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain U170B 16S ribosomal RNA gene, partial sequence
Bacillus sp. B2-4 16S ribosomal RNA gene, partial sequence
Bacillus sp. CE2 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain K21 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain SKE12 16S ribosomal RNA gene, partial sequence
Bacillus mojavensis strain NBSL48 16S ribosomal RNA gene, partial sequence
Bacillus subtilis subsp. subtilis partial 16S rRNA gene, strain B7B
Bacillus subtilis strain BA3_1A 16S ribosomal RNA gene, partial sequence
Bacillus subtilis subsp. subtilis RO-NN-1, complete genome
Bacillus subtilis subsp. spizizenii TU-B-10, complete genome
Bacillus subtilis subsp. inaquosorum partial 16S rRNA gene, type strain DSM 22148T
Bacillus sp. CS61 16S ribosomal RNA gene, partial sequence
Bacillus sp. MIB5 16S ribosomal RNA gene, partial sequence
Bacillus subtilis subsp. inaquosorum gene for 16S rRNA, partial sequence, strain: 14-1
Bacillus subtilis strain EN3 16S ribosomal RNA gene, partial sequence
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 12TCLN173
Bacillus subtilis gene for 16S rRNA, partial sequence, strain: NBRC 13169
Bacillus subtilis gene for 16S rRNA, partial sequence, strain: NBRC 12114
Bacillus subtilis gene for 16S rRNA, partial sequence, strain: NBRC 12112
Bacillus subtilis gene for 16S rRNA, partial sequence, strain: NBRC 104443
Bacillus subtilis gene for 16S rRNA, partial sequence, strain: NBRC 3936
Bacillus subtilis gene for 16S rRNA, partial sequence, strain: NBRC 13722
Bacillus subtilis gene for 16S ribosomal RNA, partial sequence, strain: BMTO-A
Bacillus amyloliquefaciens gene for 16S rRNA, partial sequence, strain: NBRC 3025
Bacillus amyloliquefaciens gene for 16S rRNA, partial sequence, strain: NBRC 3022
Bacillus vallismortis gene for 16S rRNA, partial sequence, strain: NBRC 101236
Bacillus subtilis subsp. spizizenii gene for 16S rRNA, partial sequence, strain: NBRC 101243
Bacillus sp. B8(2011) 16S ribosomal RNA gene, partial sequence
Bacillus sp. B19(2011) 16S ribosomal RNA gene, partial sequence
Bacillus amyloliquefaciens CAU-B946 complete genome
Bacillus subtilis gene for 16S ribosomal RNA, partial sequence, strain: 318
Bacillus sp. MH-I6 16S ribosomal RNA gene, partial sequence
Bacillus sp. EL31410 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain MZ-16 16S ribosomal RNA gene, partial sequence
Bacillus subtilis strain HS-116 16S ribosomal RNA gene, partial sequence
Bacillus sp. 13843 16S ribosomal RNA gene, partial sequence
Bacillus sp. 13900 16S ribosomal RNA gene, partial sequence
Bacillus amyloliquefaciens strain 576-3 16S ribosomal RNA gene, partial sequence
Bacillus subtilis subsp. inaquosorum strain I502 16S ribosomal RNA gene, partial sequence
030_kh_2_16S-R_EDIT

```
>gb|JN644531.1|  Exiguobacterium indicum strain BR18_1A 16S ribosomal RNA gene,
partial sequence
Length=1548

 Score = 1425 bits (1580),  Expect = 0.0
 Identities = 795/798 (99%), Gaps = 0/798 (0%)
 Strand=Plus/Minus

Query  1     CTACCCCACCTTCGACGGCTGGCTCCTTGCGGTTACCTCACCGGCTTCGGGTGTTGCAAA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1486  CTACCCCACCTTCGACGGCTGGCTCCTTGCGGTTACCTCACCGGCTTCGGGTGTTGCAAA  1427

Query  61    CTCTCGTGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGCAGTATGCT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1426  CTCTCGTGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGCAGTATGCT  1367

Query  121   GACCTGCGATTACTAGCGATTCCGACTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAAC  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1366  GACCTGCGATTACTAGCGATTCCGACTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAAC  1307

Query  181   TGGGAACGGCTTTATGGGATTGGCTCCACCTCGCGGTCTCGCTGCCCTTTGTACCGTCCA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1306  TGGGAACGGCTTTATGGGATTGGCTCCACCTCGCGGTCTCGCTGCCCTTTGTACCGTCCA  1247

Query  241   TTGTAGCACGTGTGTAGCCCAACTCATAAGGGGCATGATGATTTGACGTCATCCCCACCT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1246  TTGTAGCACGTGTGTAGCCCAACTCATAAGGGGCATGATGATTTGACGTCATCCCCACCT  1187

Query  301   TCCTCCGGTTTGTCACCGGCAGTCTCCCTAGAGTGCCCAACTGAATGCTGGCAACTAAGG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1186  TCCTCCGGTTTGTCACCGGCAGTCTCCCTAGAGTGCCCAACTGAATGCTGGCAACTAAGG  1127

Query  361   ATAGGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1126  ATAGGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAA  1067

Query  421   CCATGCACCACCTGTCACCATTGTCCCCGAAGGGAAAACTTGATCTCTCAAGCGGTCAAT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1066  CCATGCACCACCTGTCACCATTGTCCCCGAAGGGAAAACTTGATCTCTCAAGCGGTCAAT  1007

Query  481   GGGATGTCAAGAGTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1006  GGGATGTCAAGAGTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACC  947

Query  541   GCTTGTGCGGGTCCCGGTCAATTCATTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGGC  600
             ||||||||||||||| |||||||| |||||||||||||||||||||||||||||||||||
Sbjct  946   GCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGGC  887

Query  601   GGAGTGCTTAATGCGTTAGCTTCAGCACTGAAGGGCGGAAACCCCCCAACACCTAGCACT  660
             ||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  886   GGAGTGCTTAATGCGTTAGCTTCAGCACTGAGGGGCGGAAACCCCCCAACACCTAGCACT  827

Query  661   CATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCG  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  826   CATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCG  767

Query  721   CCTCAGCGTCAGTTACAGACCAAAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCT  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  766   CCTCAGCGTCAGTTACAGACCAAAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCT  707

Query  781   ACGCATTTCACCGCTACA  798
             ||||||||||||||||||
Sbjct  706   ACGCATTTCACCGCTACA  689
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Uncultured bacterium clone CZ139 16S ribosomal RNA gene, partial sequence
Uncultured organism clone ELU0014-T176-S-NIPCRAMgANa_000298 small subunit ribosomal RNA gene, partial sequence
Exiguobacterium acetylicum strain LT5 16S ribosomal RNA gene, partial sequence
Exiguobacterium acetylicum 16S ribosomal RNA gene, partial sequence
Exiguobacterium acetylicum strain DSM 20416 16S ribosomal RNA, partial sequence
Uncultured bacterium clone CZ212 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. DST7 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone CZ14 16S ribosomal RNA gene, partial sequence
firmicutes | 2 leaves
Bacterium A4(2009) 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. NNN611 16S ribosomal RNA gene, partial sequence
bacteria | 2 leaves
firmicutes | 2 leaves
Uncultured bacterium clone aab55a12 16S ribosomal
firmicutes | 2 leaves
Exiguobacterium sp. 3-18(2010) 16S ribosomal RNA gene, partial sequence
Bacterium 1-gw3-10 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. 3-6(2010) 16S ribosomal RNA gene, partial sequence
Exiguobacterium oxidotolerans isolate AE3 16S ribosomal RNA gene, complete sequence
Exiguobacterium sp. ARCTIC-P28 16S ribosomal RNA gene, partial sequence
firmicutes | 12 leaves
Exiguobacterium sp. B01 gene for 16S ribosomal RNA, partial sequence
Bacterium REGD5 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. 392-28 small subunit ribosomal RNA (rrn) gene, partial sequence
Exiguobacterium sp. 4_O_52 16S ribosomal RNA gene, partial sequence
Pseudomonas sp. EMB 1 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. FB165 16S ribosomal RNA gene, partial sequence
Exiguobacterium undae small subunit ribosomal RNA (rrn) gene, partial sequence
Exiguobacterium oxidotolerans strain T-2-2 16S ribosomal RNA, partial sequence
firmicutes | 4 leaves
firmicutes | 2 leaves
Uncultured bacterium clone 14-77 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone MONS_IW0905_60 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. A19 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. Sh3 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone MONS_IW0905_58 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. F5272 small subunit ribosomal RNA gene,..
firmicutes | 2 leaves
firmicutes | 3 leaves
Uncultured bacterium clone KSC2-41 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. D25(2010) 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone AH11 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. WPCB005 16S ribosomal RNA gene, partial sequence
firmicutes | 8 leaves
firmicutes | 4 leaves
firmicutes | 4 leaves
Uncultured bacterium clone Niu3 16S ribosomal RNA gene, partial sequence
Bacterium D2(2009) 16S ribosomal RNA gene, partial sequence
Bacillaceae bacterium NR184 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. BM-1 16S ribosomal RNA gene, partial sequence
Exiguobacterium indicum strain BR18_1A 16S ribosomal RNA gene, partial sequence
firmicutes | 3 leaves
Exiguobacterium indicum strain TSWCW22 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. L8 16S ribosomal RNA gene, partial sequence
Exiguobacterium sp. 2009I11 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone 1-5F 16S ribosomal RNA gene, partial sequence
firmicutes | 3 leaves
Glacial ice bacterium SB12K-2-2 16S ribosomal RNA gene, partial sequence
Exiguobacterium acetylicum strain QD-3 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone 2-3A 16S ribosomal RNA gene, partial sequence
017_Khalid_HC1__16R
Exiguobacterium indicum strain : HHS 31 16S ribosomal RNA, partial sequence

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>gb|HM133938.1|  Bacillus cereus strain BOCs315 16S ribosomal RNA gene, partial
sequence
Length=414

 Score =  544 bits (602),  Expect = 2e-151
 Identities = 304/306 (99%), Gaps = 0/306 (0%)
 Strand=Plus/Minus

Query  1    CAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCT  60
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  408  CAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCT  349

Query  61   TCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAACGGTTTTATGAGATTAGCTCC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  348  TCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAACGGTTTTATGAGATTAGCTCC  289

Query  121  ACCTCGCGGTCTTGCAGCTCTTTGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  288  ACCTCGCGGTCTTGCAGCTCTTTGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCAT  229

Query  181  AAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCNCCGGCAGTCACC  240
            |||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||
Sbjct  228  AAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACC  169

Query  241  TTAGAGTGCCCAACTAAATGATGGCAACTAAAATCAAGGGTTGCGCTCGTTGCGGGACTT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  168  TTAGAGTGCCCAACTAAATGATGGCAACTAAAATCAAGGGTTGCGCTCGTTGCGGGACTT  109

Query  301  AACCCA  306
            ||||||
Sbjct  108  AACCCA  103
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028_Khalid_HC7__16R
firmicutes | 4 leaves
Bacillus cereus isolate HKS 2-1 16S ribosomal RNA gene, partial sequence
Uncultured Bacillus sp. clone EGU378 16S ribosomal RNA gene, partial sequence
firmicutes | 13 leaves
Bacillus thuringiensis strain 61436 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain BFE 5384 16S ribosomal RNA gene, partial sequence
Bacillus thuringiensis strain CKB19 16S ribosomal RNA, partial sequence
Bacillus thuringiensis strain AP-CMST 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain BPH33 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain BE-1C 16S ribosomal RNA gene, partial sequence
Bacillus sp. TN106 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain BGCs311 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain BGCs313 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain BOCs315 16S ribosomal RNA gene, partial sequence
Bacillus thuringiensis strain XA-01 16S ribosomal RNA, partial sequence
Bacillus cereus strain Cr-50 16S ribosomal RNA gene, partial sequence
Bacillus anthracis strain IARI-L-24 16S ribosomal RNA gene, partial sequence
Bacillus sp. C1-18 16S ribosomal RNA gene, partial sequence
Bacillus sp. B3-5 16S ribosomal RNA gene, partial sequence
Bacillus sp. bC35(2011) 16S ribosomal RNA gene, partial sequence
Bacillus sp. bB3(2011) 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain CIFRI V-TSB18 16S ribosomal RNA gene, partial sequence
Bacillus sp. DB-1 16S ribosomal RNA gene, partial sequence
Bacillus sp. KU7 gene for 16S rRNA, partial sequence
Bacillus thuringiensis serovar finitimus strain ALL-01 16S ribosomal RNA gene, par
Bacillus cereus strain HN 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain WN-ly313 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain MBG33 16S ribosomal RNA gene, partial sequence
Bacillus sp. 3551BRRJ 16S ribosomal RNA gene, partial sequence
Bacillus sp. 3550BRRJ 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain MBG34 16S ribosomal RNA gene, partial sequence
Bacillus sp. T31(2011) 16S ribosomal RNA gene, partial sequence
Bacillus sp. LM24(2011) 16S ribosomal RNA gene, partial sequence
Bacillus thuringiensis serovar finitimus YBT-020, complete genome
Bacillus cereus strain RV.B1.90 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain Jinshan2010 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain DZ4 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain 5133 16S ribosomal RNA gene, partial sequence
Bacillus sp. GZT 16S ribosomal RNA gene and 16S-23S ribosomal RNA intergenic
Uncultured bacterium clone yf2clone154 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone yf2clone92 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone yf2clone47 16S ribosomal RNA gene, partial sequence
firmicutes | 6 leaves
Bacillus cereus gene for 16S rRNA, partial sequence, specimen_voucher: GTC:0289
Bacillus cereus gene for 16S rRNA, partial sequence, specimen_voucher: GTC:0289
Bacillus cereus gene for 16S rRNA, partial sequence, specimen_voucher: GTC:0291
Uncultured Bacillus sp. clone FAC185 16S ribosomal RNA gene, partial sequence
Bacillus sp. enrichment culture clone M2 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain PHAs008 16S ribosomal RNA gene, partial sequence
Bacillus sp. cp64 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone 112 16S ribosomal RNA gene, partial sequence
Bacillus thuringiensis strain NBIGP 24 16S ribosomal RNA gene, partial sequence
Bacillus sp. MC-BAC-7 16S ribosomal RNA gene, partial sequence
Bacillus sp. MC-BAC-2 16S ribosomal RNA gene, partial sequence
Bacillus sp. MC-BAC-4 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone UU2f2_79-T7 16S ribosomal RNA gene, partial sequenc
Bacillus sp. BB2_1A 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain MSU AS 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain AcdSP3 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain AcdSP4 16S ribosomal RNA gene, partial sequence
Bacillus sp. EN1 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain HT21 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain M6 16S ribosomal RNA gene, partial sequence
Bacillus cereus strain FA 16S ribosomal RNA gene, partial sequence
Bacillus sp. R-26209 partial 16S rRNA gene, strain R-26209
Bacillus sp. R-25385 partial 16S rRNA gene, strain R-25385
Bacillus cereus NC7401 genomic DNA, complete genome
Bacillus cereus gene for 16S rRNA, partial sequence, strain: NBRC 103938
Bacillus thuringiensis serovar monterrey gene for 16S rRNA, partial sequence
Bacillus cereus F837/76, complete genome
Uncultured bacterium clone Mo14 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone MoP14 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone MoP27 16S ribosomal RNA gene, partial sequence
firmicutes | 7 leaves

```
>gb|JQ237672.1|  Bacillus sp. Hswx95 16S ribosomal RNA gene, partial sequence
Length=1453

 Score = 1269 bits (1406),  Expect = 0.0
 Identities = 709/713 (99%), Gaps = 0/713 (0%)
 Strand=Plus/Plus

Query  1    AGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  40   AGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTG  99

Query  61   CCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGTTTGAACCG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  100  CCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGTTTGAACCG  159

Query  121  CATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  160  CATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATT  219

Query  181  AGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  220  AGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTG  279

Query  241  ATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAAT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  280  ATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAAT  339

Query  301  CTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  340  CTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGA  399

Query  361  TCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGNATAGGGCGGTACCTTGACG  420
            |||||||||||||||||||||||||||||||||||||||| |||||||||||||||||||
Sbjct  400  TCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACG  459

Query  421  GTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  460  GTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG  519

Query  481  CAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTCCTTAAGTCTGATG  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  520  CAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTCCTTAAGTCTGATG  579

Query  541  TGAAAGCCCCCGGCTCACCCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAG  600
            ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  580  TGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAG  639

Query  601  AGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTATAGATGTGGAGGAACACCAGTG  660
            |||||||||||||||||||||||||||||||||||||| |||||||||||||||||||||
Sbjct  640  AGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTG  699

Query  661  GCGAAGGGGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGA  713
            ||||||| |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  700  GCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGA  752
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026_Khalid_HC3__16F

firmicutes | 30 leaves

Bacillus sp. WUST NAP-5 16S ribosomal RNA gene, partial sequence

Bacillus sp. M71_S52 partial 16S rRNA gene, isolate M71_S52

Bacillus mojavensis strain BCRC 17531 16S ribosomal RNA gene, partial sequence

Bacillus sp. GMTB-C1-2 16S ribosomal RNA gene, complete sequence

firmicutes | 12 leaves

Bacterium BS72 16S ribosomal RNA gene, partial sequence

Uncultured Bacillus sp. clone P2 16S ribosomal RNA gene, partial sequence

Bacillus tequilensis strain KM30 16S ribosomal RNA gene, partial sequence

Bacillus tequilensis strain km18 16S ribosomal RNA gene, partial sequence

Bacillus tequilensis strain km16 16S ribosomal RNA gene, partial sequence

Bacillus sp. KR-8104 16S ribosomal RNA gene, partial sequence

Bacillus tequilensis strain BPRIST017 16S ribosomal RNA gene, partial sequence

[Brevibacterium] halotolerans strain S4Ba-1 16S ribosomal RNA gene, partial sequence

Bacillus sp. Hofans2 16S ribosomal RNA gene, partial sequence

Bacillus sp. GCNB5 16S ribosomal RNA gene, partial sequence

Bacillus sp. M71_D80 partial 16S rRNA gene, isolate M71_D80

Bacillus axarquiensis strain LNXM78 16S ribosomal RNA gene, partial sequence

Bacillus sp. TT407 gene for 16S rRNA, partial sequence

Bacillus mojavensis strain cT170 16S ribosomal RNA gene, partial sequence

Bacterium FJAT-13827 16S ribosomal RNA gene, partial sequence

firmicutes | 3 leaves

Bacillus axarquiensis strain BK52-132 16S ribosomal RNA gene, partial sequence

Bacillus sp. ABCQ623 16S ribosomal RNA gene, partial sequence

firmicutes | 17 leaves

firmicutes | 16 leaves

Bacillus subtilis strain L-13 16S ribosomal RNA gene, partial sequence

```
>gb|JN650255.1|  Uncultured Paenibacillus sp. clone SCTB032 16S ribosomal RNA
gene, partial sequence
Length=1478

 Score = 1072 bits (1188),  Expect = 0.0
 Identities = 597/599 (99%), Gaps = 0/599 (0%)
 Strand=Plus/Minus

Query  1     TACCCCACCTTCGGCGGCTGGCTCCCGTGAGGGTTACCCCACCGACTTCGGGTGTTGTAA  60
             |||||||||||||||||||||||||| || ||||||||||||||||||||||||||||||
Sbjct  1465  TACCCCACCTTCGGCGGCTGGCTCCCTTGCGGGTTACCCCACCGACTTCGGGTGTTGTAA  1406

Query  61    ACTCTCGTGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGCGGCATGC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1405  ACTCTCGTGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGCGGCATGC  1346

Query  121   TGATCCGCGATTACTAGCAATTCCGACTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1345  TGATCCGCGATTACTAGCAATTCCGACTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAA  1286

Query  181   CTGAGACTGGCTTTTATAGGATTGGCTCCACCTCGCGGCTTCGCTTCCCGTTGTACCAGC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1285  CTGAGACTGGCTTTTATAGGATTGGCTCCACCTCGCGGCTTCGCTTCCCGTTGTACCAGC  1226

Query  241   CATTGTAGTACGTGTGTAGCCCAAGTCATAAGGGGCATGATGATTTGACGTCATCCCCGC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1225  CATTGTAGTACGTGTGTAGCCCAAGTCATAAGGGGCATGATGATTTGACGTCATCCCCGC  1166

Query  301   CTTCCTCCGGTTTGTCACCGGCAGTCATTCTAGAGTGCCCACCATCATGTGCTGGCAACT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1165  CTTCCTCCGGTTTGTCACCGGCAGTCATTCTAGAGTGCCCACCATCATGTGCTGGCAACT  1106

Query  361   AAAATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACG  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1105  AAAATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACG  1046

Query  421   ACAACCATGCACCACCTGTCACCTCTGTCCCGAAGGCCGCCTCTATCTCTAGAGGATTCA  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1045  ACAACCATGCACCACCTGTCACCTCTGTCCCGAAGGCCGCCTCTATCTCTAGAGGATTCA  986

Query  481   GAGGGATGTCAAGACTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATACTCCA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  985   GAGGGATGTCAAGACTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATACTCCA  926

Query  541   CTGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCA  599
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  925   CTGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCA  867
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Paenibacillus sp. FSL_H7-792 16S ribosomal RNA gene, partial sequence
bacteria | 4 leaves
Paenibacillus sp. enrichment culture clone xj7 16S ribosomal RNA gene, partial sequence
bacteria | 5 leaves
Paenibacillus sp. TR1103-3 gene for 16S rRNA, partial sequence
Uncultured bacterium clone zs03043 16S ribosomal RNA gene, partial sequence
Cohnella sp. PA228 partial 16S rRNA gene, isolate PA228
Uncultured bacterium clone p6m03ok 16S ribosomal RNA gene, partial sequence
firmicutes | 9 leaves
Paenibacillus lactis strain MB 2035 16S ribosomal RNA gene, partial sequence
Paenibacillus lactis strain MB 1928 16S ribosomal R
Paenibacillus lactis partial 16S rRNA gene, strain IB-188D
firmicutes | 3 leaves
Uncultured compost bacterium partial 16S rRNA gene, clone F51058
firmicutes | 2 leaves
bacteria | 2 leaves
firmicutes | 12 leaves
Paenibacillus sp. KUDC3609 16S ribosomal RNA gene, partial sequence
Paenibacillus lautus strain A1-14 16S ribosomal RNA gene, partial sequence
027_kh_5_16S-R
Paenibacillus lautus strain D519 16S ribosomal RNA gene, partial sequence
firmicutes | 7 leaves
Paenibacillus lautus strain 5138 16S ribosomal RNA gene, partial sequence
firmicutes | 22 leaves
firmicutes | 3 leaves
firmicutes | 2 leaves
Paenibacillus sp. XP1-2 16S ribosomal RNA gene, partial sequence
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: WB18-82
firmicutes | 3 leaves
firmicutes | 2 leaves
Paenibacillus sp. H25-07 partial 16S rRNA gene
Uncultured Paenibacillus sp. clone BA-N-8 16S ribosomal RNA gene, partial sequence
Uncultured Paenibacillus sp. partial 16S rRNA gene, clone ACf23
firmicutes | 2 leaves
Bacterium DE5 16S ribosomal RNA gene, partial sequence
Paenibacillus lautus strain SK21 16S ribosomal RNA gene, partial sequence

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>gb|JQ082506.1| Penicillium corylophilum isolate TBG1-14 internal transcribed
spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal
transcribed spacer 2, complete sequence; and 28S ribosomal
RNA gene, partial sequence
Length=556

 Score =  280 bits (310),  Expect = 2e-72
 Identities = 186/200 (93%), Gaps = 6/200 (3%)
 Strand=Plus/Plus

Query  1    TCCCACCCATGTTTATTGTACCTTGTTGCTTCGGCGGGCCCGCCTCACGGCCGCCGGGGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  28   TCCCACCCATGTTTATTGTACCTTGTTGCTTCGGCGGGCCCGCCTCACGGCCGCCGGGGG  87

Query  61   -CTTCTGCC-TCTGTCC-GCGCCGGCG--AGACACCATTGAAC-CTGTCTGAAGATTGCA  114
             |||||||| |||| || ||||| ||  |||||||||||||| ||||||||||||||||
Sbjct  88   GCTTCTGCCCTCTGGCCCGCGCCCGCCGAAGACACCATTGAACACTGTCTGAAGATTGCA  147

Query  115  GTCTGAGCAATTAGCTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGCGAT  174
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||   ||
Sbjct  148  GTCTGAGCAATTAGCTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCAT  207

Query  175  CGAGCATGAACGCAGCGAAA  194
            |||   | |||||||||||||
Sbjct  208  CGATGAAGAACGCAGCGAAA  227
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040_sa_No.1_ITS1
Penicillium sp. 3 WL-2011 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, c
fungi | 3 leaves
Penicillium fagi 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S rib...
Uncultured ascomycete ITS region including 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene, clone BF-OTU238
Penicillium oxalicum strain PO10 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence
Penicillium corylophilum internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Penicillium corylophilum strain FRR 802 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete
Penicillium corylophilum isolate V8 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal R.
Penicillium sp. Vega347 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence
Penicillium corylophilum isolate CV304 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosom
Penicillium sp. CMV-2009e isolate CV15 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribos..
Penicillium sp. CMV-2009e isolate CV51 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribos..
Uncultured fungus genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, clone: DSF_43_B097
Uncultured Penicillium clone R14c25 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete s...
Penicillium sp. POPeuph22 genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene, isolate POPeuph22
Penicillium corylophilum genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, strain 0310ARD9L_1
Penicillium corylophilum isolate ACT1-2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S riboso..
Uncultured fungus genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, clone: SC_B2_5_3_3
Uncultured fungus genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, clone: SC_B3_4_3_1
Penicillium sp. MAB-2010i strain CID 265 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, compl..
Penicillium corylophilum strain DHMJ01 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete
Penicillium corylophilum strain DHMJ07 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete
Penicillium corylophilum strain CBS 330.79 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S rib.
Penicillium corylophilum isolate TBG1-17 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribos..
Penicillium corylophilum strain CBS 231.38 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S rib.
Penicillium corylophilum strain DHMJ03 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete
Penicillium corylophilum strain DAOM 221130 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial s...
Uncultured fungus genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, clone: SC_B3_5_3_4
Uncultured fungus genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, clone: SC_B3_2_3_1
Penicillium corylophilum isolate TBG1-8 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S riboso..
Penicillium corylophilum strain ATHUM 5095 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, com
Uncultured Ascomycota genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, clone BF-OTU624
Penicillium corylophilum genomic DNA containing ITS1, 5.8S rRNA gene and ITS2, isolate MI-F10
Penicillium corylophilum strain G9 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequ..
Uncultured fungus isolate UPSC_E6_54 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequenc
Penicillium sp. CMV-2009e isolate CV18 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribos..
Penicillium corylophilum isolate CV298 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosom
Fungal sp. AB39 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ri...
ascomycetes | 4 leaves
Penicillium corylophilum isolate TBG1-14 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribos..
Penicillium corylophilum strain DHMJ09 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete
Penicillium corylophilum strain DHMJ06 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete
Penicillium sp. MS-2011-F13 genomic DNA containing ITS1, 5.8S rRNA gene and ITS2, strain F13
Ascomycota sp. ARIZ OCAsh2-7 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed ...
Uncultured fungus genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, clone: SC_B2_5_3_6
Uncultured fungus genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, clone: SC_B2_5_3_1
Penicillium corylophilum strain ATHUM 5102 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, com
Penicillium sp. BF037 genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene, isolate BF037
Penicillium corylophilum genomic DNA containing ITS1, 5.8S rRNA gene and ITS2, isolate MI-F1
Penicillium corylophilum strain CBS 330.79 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, comp
Uncultured fungus isolate UPSC_B11_23 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial seque..
ascomycetes | 7 leaves
Penicillium sp. F10 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S r

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>gb|HM172832.1| Mucor hiemalis culture-collection FSU<DEU>:9871 internal
transcribed spacer 1, partial sequence; 5.8S ribosomal RNA
gene and internal transcribed spacer 2, complete sequence;
and 28S ribosomal RNA gene, partial sequence
Length=564

 Score =  610 bits (676),  Expect = 2e-171
 Identities = 350/358 (98%), Gaps = 0/358 (0%)
 Strand=Plus/Minus

Query  1    TAATACTAGAGCATTCCTTTATATTaaaaaaaTGTTCAGGCAGAAAGAACAATGGTTCAG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  491  TAATACTAGAGCATTCCTTTATATTAAAAAAATGTTCAGGCAGAAAGAACAATAGTTCAG  432

Query  61   GCCTAATAAGTTTAAAGAATTCAAGCAAGTCGAAATTCTCAGTTCCATTCACAACAAAAT  120
            |||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||
Sbjct  431  GCCTAATAAGTTTAAAGAATTCAAACAAGTCGAAATTCTCAGTTCCATTCACAACAAAAT  372

Query  121  TATGAATGTGGGGTGTTTTTGATACTGAAACAGGCGTGCTCTATGGAATACCATTGAGCG  180
            ||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  371  TATGAATGTGGGGTGTTTTTGATACTGAAACAGGCGTGCTCAATGGAATACCATTGAGCG  312

Query  181  CTAGTTGCGTTCAAAGACTCGATGATTCACTGAATATGCAATTCACACTAGTTATCGCAC  240
            | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  311  CAAGTTGCGTTCAAAGACTCGATGATTCACTGAATATGCAATTCACACTAGTTATCGCAC  252

Query  241  TTTGCTACGTTCTTCATCGATGCGAGAACCCAGAGATCCGTTGTTAAAAGTTGTTTTATA  300
            |||||||||||||||||||||||||||||| |||||||||||||||||||||||||||||
Sbjct  251  TTTGCTACGTTCTTCATCGATGCGAGAACCAAGAGATCCGTTGTTAAAAGTTGTTTTATA  192

Query  301  AGTTTTTTACGCTCATGTTACAATAATAATACTGAATTCTTTTGGTaaaaaaaTAATA  358
            ||||||||||||| ||||||||||||||||||||||||||||||||||| || |||||
Sbjct  191  AGTTTTTTACGCTTATGTTACAATAATAATACTGAATTCTTTTGGTAAATAATTAATA  134
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047_sa_No.4_ITs4

Fungal sp. ZB6 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial seq..

Mucor hiemalis f. hiemalis strain FSU 6179 clone 1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 2...

Mucor hiemalis strain FSU6530 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA ..

fungi | 10 leaves

Mucor hiemalis 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CBS 201.65

Mucor hiemalis culture-collection FSU<DEU>:9903 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, pa

Mucor hiemalis culture-collection FSU<DEU>:9902 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 2...

Mucor hiemalis culture-collection FSU<DEU>:9871 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, pa

Mucor sp. BEA-2010 isolate B3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA .

Mucor hiemalis culture-collection FSU<DEU>:10222 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA..

Mucor hiemalis strain 2511A internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

Mucor sp. BEA-2010 isolate B2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ri..

Mucor hiemalis culture-collection FSU<DEU>:10221 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

fungi | 32 leaves

Mucor sp. FJ09 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S r

fungi | 6 leaves

Leaf litter ascomycete strain its186 internal transcribed spacer 1, partial seq..

fungi | 4 leaves

fungi | 4 leaves

fungi | 30 leaves

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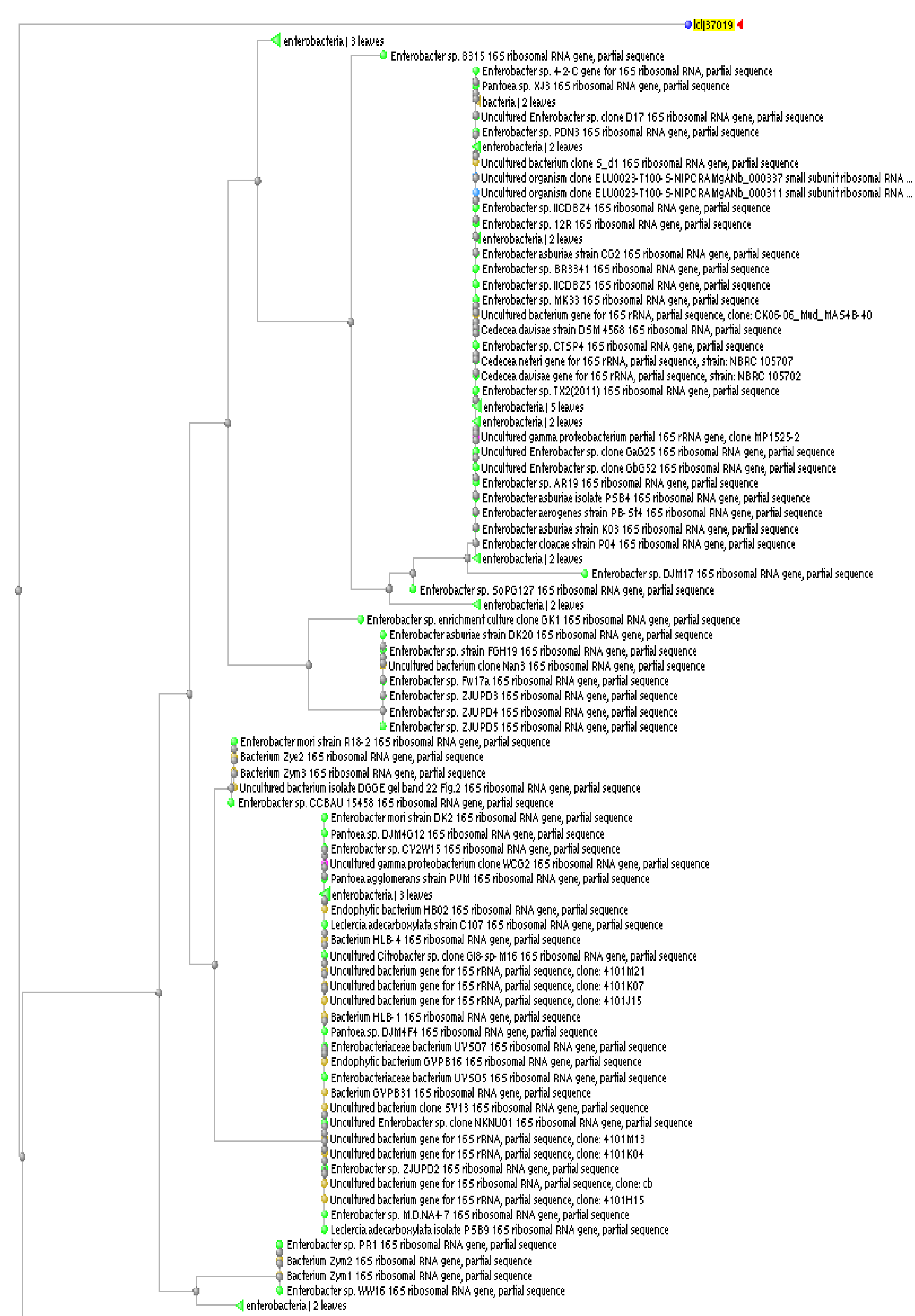
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enterobacteria | 3 leaves
Enterobacter sp. 8315 16S ribosomal RNA gene, partial sequence
Enterobacter sp. 4-2-C gene for 16S ribosomal RNA, partial sequence
Pantoea sp. XJ3 16S ribosomal RNA gene, partial sequence
bacteria | 2 leaves
Uncultured Enterobacter sp. clone D17 16S ribosomal RNA gene, partial sequence
Enterobacter sp. PDN3 16S ribosomal RNA gene, partial sequence
enterobacteria | 2 leaves
Uncultured bacterium clone 5_d1 16S ribosomal RNA gene, partial sequence
Uncultured organism clone ELU0023-T100-S-NIPCRAMgANb_000337 small subunit ribosomal RNA ...
Uncultured organism clone ELU0023-T100-S-NIPCRAMgANb_000311 small subunit ribosomal RNA ...
Enterobacter sp. IICDBZ4 16S ribosomal RNA gene, partial sequence
Enterobacter sp. 12R 16S ribosomal RNA gene, partial sequence
enterobacteria | 2 leaves
Enterobacter asburiae strain CG2 16S ribosomal RNA gene, partial sequence
Enterobacter sp. BR3341 16S ribosomal RNA gene, partial sequence
Enterobacter sp. IICDBZ5 16S ribosomal RNA gene, partial sequence
Enterobacter sp. MK33 16S ribosomal RNA gene, partial sequence
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: CK06-06_Mud_MAS4B-40
Cedecea davisae strain DSM 4568 16S ribosomal RNA gene, partial sequence
Enterobacter sp. CTSP4 16S ribosomal RNA gene, partial sequence
Cedecea neteri gene for 16S rRNA, partial sequence, strain: NBRC 105707
Cedecea davisae gene for 16S rRNA, partial sequence, strain: NBRC 105702
Enterobacter sp. TX2(2011) 16S ribosomal RNA gene, partial sequence
enterobacteria | 5 leaves
enterobacteria | 2 leaves
Uncultured gamma proteobacterium partial 16S rRNA gene, clone MP1525-2
Uncultured Enterobacter sp. clone GaG25 16S ribosomal RNA gene, partial sequence
Uncultured Enterobacter sp. clone GbG52 16S ribosomal RNA gene, partial sequence
Enterobacter sp. AR19 16S ribosomal RNA gene, partial sequence
Enterobacter asburiae isolate PSB4 16S ribosomal RNA gene, partial sequence
Enterobacter aerogenes strain PB-5f4 16S ribosomal RNA gene, partial sequence
Enterobacter asburiae strain K03 16S ribosomal RNA gene, partial sequence
Enterobacter cloacae strain P04 16S ribosomal RNA gene, partial sequence
enterobacteria | 2 leaves
Enterobacter sp. DJM17 16S ribosomal RNA gene, partial sequence
Enterobacter sp. 5oPG127 16S ribosomal RNA gene, partial sequence
enterobacteria | 2 leaves
Enterobacter sp. enrichment culture clone GK1 16S ribosomal RNA gene, partial sequence
Enterobacter asburiae strain DK20 16S ribosomal RNA gene, partial sequence
Enterobacter sp. strain FGH19 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone Nan3 16S ribosomal RNA gene, partial sequence
Enterobacter sp. Fw17a 16S ribosomal RNA gene, partial sequence
Enterobacter sp. ZJUPD3 16S ribosomal RNA gene, partial sequence
Enterobacter sp. ZJUPD4 16S ribosomal RNA gene, partial sequence
Enterobacter sp. ZJUPD5 16S ribosomal RNA gene, partial sequence
Enterobacter mori strain R18-2 16S ribosomal RNA gene, partial sequence
Bacterium Zye2 16S ribosomal RNA gene, partial sequence
Bacterium Zym3 16S ribosomal RNA gene, partial sequence
Uncultured bacterium isolate DGGE gel band 22 fig.2 16S ribosomal RNA gene, partial sequence
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Enterobacter mori strain DK2 16S ribosomal RNA gene, partial sequence
Pantoea sp. DJM4G12 16S ribosomal RNA gene, partial sequence
Enterobacter sp. CY2W15 16S ribosomal RNA gene, partial sequence
Uncultured gamma proteobacterium clone WCG2 16S ribosomal RNA gene, partial sequence
Pantoea agglomerans strain PUM 16S ribosomal RNA gene, partial sequence
enterobacteria | 3 leaves
Endophytic bacterium HB02 16S ribosomal RNA gene, partial sequence
Leclercia adecarboxylata strain C107 16S ribosomal RNA gene, partial sequence
Bacterium HLB-4 16S ribosomal RNA gene, partial sequence
Uncultured Citrobacter sp. clone GI8-sp-M16 16S ribosomal RNA gene, partial sequence
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 4101M21
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 4101K07
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 4101J15
Bacterium HLB-1 16S ribosomal RNA gene, partial sequence
Pantoea sp. DJM4F4 16S ribosomal RNA gene, partial sequence
Enterobacteriaceae bacterium UY507 16S ribosomal RNA gene, partial sequence
Endophytic bacterium GYPB16 16S ribosomal RNA gene, partial sequence
Enterobacteriaceae bacterium UY505 16S ribosomal RNA gene, partial sequence
Bacterium GYPB31 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone SY13 16S ribosomal RNA gene, partial sequence
Uncultured Enterobacter sp. clone NKNU01 16S ribosomal RNA gene, partial sequence
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 4101M13
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 4101K04
Enterobacter sp. ZJUPD2 16S ribosomal RNA gene, partial sequence
Uncultured bacterium gene for 16S ribosomal RNA, partial sequence, clone: cb
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 4101H15
Enterobacter sp. M.D.NA4-7 16S ribosomal RNA gene, partial sequence
Leclercia adecarboxylata isolate PSB9 16S ribosomal RNA gene, partial sequence
Enterobacter sp. PR1 16S ribosomal RNA gene, partial sequence
Bacterium Zym2 16S ribosomal RNA gene, partial sequence
Bacterium Zym1 16S ribosomal RNA gene, partial sequence
Enterobacter sp. WW16 16S ribosomal RNA gene, partial sequence
enterobacteria | 2 leaves
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: ...

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 Strand=Plus/Minus

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Sbjct  1065  ACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCAGAGTTCCCGAAGGCACCAATC  1006

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Sbjct  945   TAAACCACATGCTCCACCGCTTGTGCGGCC  916
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lcl|21191

Pseudomonas putida strain JGP46 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone N-121 16S ribosomal RNA gene, partial sequence
g-proteobacteria | 19 leaves
g-proteobacteria | 3 leaves
g-proteobacteria | 3 leaves
Uncultured soil bacterium clone DLQDFD6C3 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone Medium_B15 16S ribosomal RNA gene, partial sequence
Uncultured Pseudomonas sp. partial 16S rRNA gene, isolate ELC0606, clone A0601E01
Pseudomonas sp. AMAA5357 16S ribosomal RNA gene, partial sequence
g-proteobacteria | 3 leaves
g-proteobacteria | 6 leaves
Pseudomonas cremoricolorata gene for 16S rRNA, partial sequence, strain: NBRC 16634
g-proteobacteria | 10 leaves
Uncultured bacterium clone 1H3C_34 16S ribosomal RNA gene, partial sequence
Pseudomonas sp. BR1(2011) 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone yf6clone158 16S ribosomal RNA gene, partial sequence
Pseudomonas putida strain GRIRKNM2011 16S ribosomal RNA gene, partial sequence
Uncultured soil bacterium clone DLQDFD6C31 16S ribosomal RNA gene, partial sequence
Pseudomonas putida gene for 16S rRNA, partial sequence, strain: NBRC 14671
g-proteobacteria | 4 leaves
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: 12TCLN057
Uncultured Pseudomonas sp. clone 1461978 16S ribosomal RNA gene, partial sequence
g-proteobacteria | 2 leaves
Pseudomonas putida gene for 16S rRNA, partial sequence, strain: NBRC 12996
g-proteobacteria | 4 leaves
Bacterium enrichment culture clone F10 16S ribosomal RNA gene, partial sequence
Pseudomonas putida strain A3 16S ribosomal RNA gene, partial sequence
Pseudomonas putida strain GPSD-19 16S ribosomal RNA gene, partial sequence
Pseudomonas sp. CM2(2011) 16S ribosomal RNA gene, partial sequence
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Uncultured bacterium clone yf6clone21 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone yf3clone27 16S ribosomal RNA gene, partial sequence
Pseudomonas putida strain BJ10 16S ribosomal RNA gene, partial sequence
Pseudomonas sp. AMAA5330 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone yf4clone131 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone yf3clone75 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone yf2clone79 16S ribosomal RNA gene, partial sequence
Pseudomonas putida strain M56 16S ribosomal RNA gene, partial sequence
Bacterium enrichment culture clone F12 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone yf6clone67 16S ribosomal RNA gene, partial sequence
Pseudomonas putida strain M55RFD8 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone N-101 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone N-67 16S ribosomal RNA gene, partial sequence
Pseudomonas putida strain ATCC 17390 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone N-56 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone N-21 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone N-07 16S ribosomal RNA gene, partial sequence
Uncultured Pseudomonas sp. clone GI3-5-3-F11 16S ribosomal RNA gene, partial sequence
Pseudomonas cremoricolorata strain IAM 1541 16S ribosomal RNA, partial sequence
g-proteobacteria | 2 leaves
Pseudomonas putida 16S ribosomal RNA gene, partial sequence
Uncultured gamma proteobacterium partial 16S rRNA gene, clone 515A-MN7

Appendix C

Manufacturer information of UV lamps

EL Series Ultraviolet Hand Lamps User's Guide

Introduction

The EL Series of Ultraviolet Lamps offer a uniform and intense source of ultraviolet light (radiation). The lamps emit one of three UV wavelengths or white light in four watt, six watt and eight watt configurations for exposure/illumination of materials. All EL Series Lamp models can be used with the universal J138 Lamp Stand. The four watt models can be used with C-10E Cabinet for viewing materials in a darkroom environment. Eight watt lamps can be used with the C-65 Cabinet.

Important Safety Information

Caution: Shortwave and midrange UV radiation will cause damage to unprotected eyes and skin. Before operating any unit, be sure all personnel in the area are properly protected. UV Blocking Eyewear should be worn as well. UVP has a complete line of UV Blocking Eyewear: Spectacles, Goggles and Faceshield designed for this purpose.

Operation

Plug power cord into a properly grounded electrical outlet. The proper voltage of the lamp is found on the product information label.

A rocker switch is conveniently located on top of the unit to turn the lamp on or off. For models with multiple wavelengths, the rocker switch accommodates for the selection of wavelength or to shut the unit off.

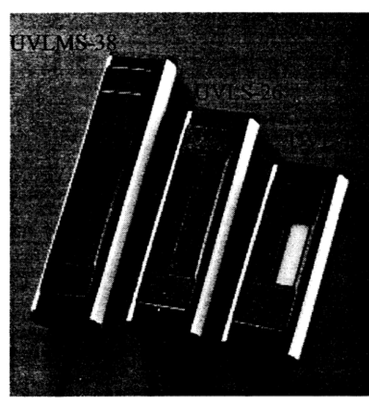
Each lamp comes with two UV tubes or one UV and one white light tubes; the UVLSM-38 is equpped with three tubes. Models with two tubes of the same wavelength operate with both tubes on at same time. Other models operate with only a single tube at one time.

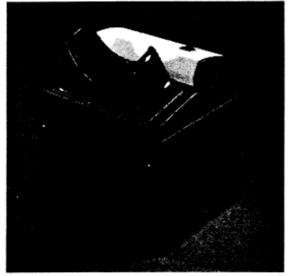
Specifications

Physical dimensions for four, six and eight watt lamps:

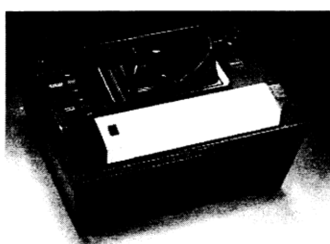
Four watt lamps:	9.8"L x 2.5"H x 3.8"W (249 x 64 x 97 mm)
Six watt lamps:	11.9"L x 2.5"H x 3.8"W (302 x 64 x 97 mm)
Eight watt lamps:	14.9"L x 2.5"H x 3.8"W (376 x 64 x 97 mm)

ULTRA-VIOLET PRODUCTS

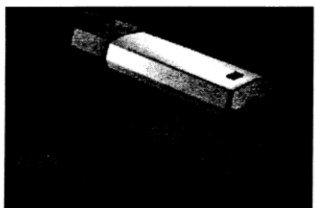




C-10E Cabinet with
four watt lamp



C-65 Cabinet with
eight watt lamp



Universal stand for all
EL Series Lamps

Four Watt Lamp Specifications

Model	Wavelength	Part Number	Nominal Volts/Hz/Amp
UVS-14	254nm/White Light	95-0266-01 95-0266-02 95-0266-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVL-14	365nm/White Light	95-0264-01 95-0264-02 95-0264-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVS-24	254nm	95-0269-01 95-0269-02 95-0269-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVL-24	365nm	95-0267-01 95-0267-02 95-0267-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVLS-24	365nm/254nm	95-0271-01 95-0271-02 95-0271-03	115/60/0.16 230/50/0.16 100/50-60/0.16

Six Watt Lamp Specifications

Model	Wavelength	Part Number	Nominal Volts/Hz/Amp
UVS-16	254nm/White Light	95-0274-01 95-0274-02 95-0274-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVL-16	365nm/White Light	95-0272-01 95-0272-02 95-0272-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVM-16	302nm/White Light	95-0273-01 95-0273-02 95-0273-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVS-26	254nm	95-0277-01 95-0277-02 95-0277-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVL-26	365nm	95-0275-01 95-0275-02 95-0275-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVM-26	302nm	95-0276-01 95-0276-02 95-0276-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVLS-26	365nm/254nm	95-0279-01 95-0279-02 95-0279-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVLM-26	365nm/302nm	95-0278-01 95-0278-02 95-0278-03	115/60/0.16 230/50/0.16 100/50-60/0.16

Eight Watt Lamp Specifications

Model	Wavelength	Part Number	Nominal Volts/Hz/Amp
UVS-18	254nm/White Light	95-0200-01 95-0200-02 95-0200-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVL-18	365nm/White Light	95-0198-01 95-0198-02 95-0198-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVM-18	302nm/White Light	95-0199-01 95-0199-02 95-0199-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVS-28	254nm	95-0249-01 95-0249-02 95-0249-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVL-28	365nm	95-0248-01 95-0248-02 95-0248-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVM-28	302nm	95-0250-01 95-0250-02 95-0250-03	115/60/0.32 230/50/0.32 100/50-60/0.32
UVLS-28	365nm/254nm	95-0201-01 95-0201-02 95-0201-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVLM-28	365nm/302nm	95-0251-01 95-0251-02 95-0251-03	115/60/0.16 230/50/0.16 100/50-60/0.16
UVLMS-38	365/302/254nm	95-0252-01 95-0252-02 95-0252-03	115/60/0.16 230/50/0.16 100/50-60/0.16

Replacement Tubes and Switches

Replacement Part	Part Number
Switch, On/Off	53-0134-01
Switch, On/Off/On	53-0135-01
Tube, 4 watt, 365nm	34-0005-01
Tube, 4 watt, 254nm	34-0066-01
Tube, 4 watt, white light	34-0003-01
Tube, 6 watt, 365nm	34-0034-01
Tube, 6 watt, 302nm	34-0044-01
Tube, 6 watt, 254nm	34-0015-01
Tube, 6 watt, white light	34-0063-01
Tube, 8 watt, 365nm	34-0006-01
Tube, 8 watt, 302nm	34-0042-01
Tube, 8 watt, 254nm	34-0007-01
Tube, 8 watt, white light	34-0056-01

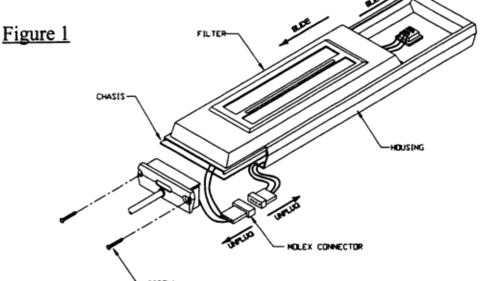
Accessories

Accessories	Part Number
J138 Lamp Stand	18-0063-01
C-65 Cabinet	95-0257-01
C-10E Cabinet	95-0072-08
Spectacles	98-0002-01
Goggles	98-0002-02
Faceshield	98-0002-04

Changing the Replacement Tubes

Always disconnect the lamp from the electrical power source prior to replacing tubes. Remove the two Phillips Head Screws located in the End Cap. Grasp the extruded housing and slide the chassis and filter out together away from the switch. Disconnect the interior Molex Connector (see Figure 1) and slide chassis and filter out of the housing completely. Once out of the extrusion, the chassis and filter will come apart. Carefully grasp the tube ends and twist one quarter turn until it works free (see Figure 2). Insert the new tube, giving it a twist to lock in place. Reassemble the lamp. Be careful not to pinch wires during assembly.

Figure 1



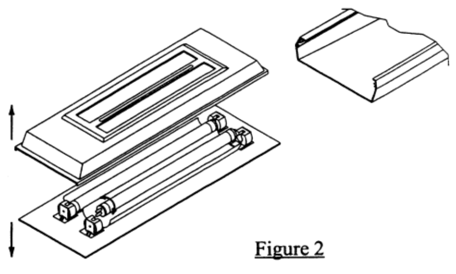


Figure 2

Cleaning the EL Series Lamps

The painted surfaces and filter areas of the lamp should cleaned with a damp sponge or cloth towel and mild soap. Never use abrasive cleaners, solvent based cleaners or scouring pads.

ALWAYS DISCONECT THE LAMP FROM THE ELECTRICAL POWER PRIOR TO CLEANING.

Maintenance/Repair/Technical Assistance

UVP offers technical support for all of its products. If you have any questions about the product's use, operation or repair, call or fax UVP Customer Service at the following offices:

In the US: Tel: (909)946-3197 or toll free (800)452-6788; Fax (909)946-3597; E-Mail uvp@uvp.com
Europe/UK: Tel: +44(0)1223-420022; Fax: +44(0)1223-420561; E-Mail: uvp@dial.pipex.com

A **Returned Goods Authorization (RGA)** number must be obtained from UVP Customer Service before returning any products.

Warranty

UVP, Inc. warrants its EL Series Lamps to be free of defects in materials and workmanship for a period of one (1) year from the date of purchase. Tubes and filters are warranted for a period of 90 days. If equipment failure or malfunction occurs durng the warranty period, UVP shall examine the inoperative equipment and have the option of repairing or replacing any part(s) which, in the judgement of UVP, were originally defective or became so under conditions of normal usage and service.

No warranty shall apply to this instrument, or part thereof, that has been subject to accident negligence, alteration, abuse or misuse by the end user. Moreover, UVP makes no warranties whatsoever with respect to parts not supplied by UVP or that have been installed, used and/or serviced other than in strict compliance with the instruments appearing in this manual.

In no event shall UVP be responsible to the end user for any incidental or consequential damges, whether foreseeable or not, including but not limited to property damage, inability to use equipment, lost business, lost profits, or inconvenience arising out of or connected with the use of instruments produced by UVP. Nor is UVP liable or responsible for any personal injuries occurring as a result of the use, installation and/or servicing of equipment.

This warranty does not supersede any statutory rights that may be available in certain countries.

Internet: http://www.uvp.com

Corporate Headquarters: UVP, Inc.
2066 W. 11th Street, Upland, CA 91786 USA
Tel: (909)946-3197 or toll free in US/Canada (800)452-6788
Fax: (909)946-3597 E-Mail: uvp@uvp.com

European Operations: Ultra-Violet Products Limited
Unit 1, Trinity hall Farm Estate, Nuffield Road, Cambridge CB4 1TG UK
Tel: +44(0)1223-420022 Fax:+44(0)1223-420561
E-mail: uvpuk@uvp.com

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