Physiological and Molecular Characterisation of an Alkaliphilic *Bacillus* Isolated from a Moderately Alkaline Environment

by

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A Thesis Submitted for Degree of Doctor of Philosophy

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March, 2010

Dedication

To

My Father "Muhammed"

My Mother "Salima"

My Wife "Halima"

My daughters "Shahd and Shada"

My son "Suhaib"

For Their Love, Encouragement and Support

Abstract

Two strains of alkaliphilic, strictly aerobic, Gram-positive, non-motile bacteria were isolated. First, an alkaliphilic bacterium (optimum growth at pH 10) designated as MAK7 was isolated from a water and sediment sample obtained from a non-extreme environment (River Lathkill in the Derbyshire Peak District). Second, an alkaliphilic *Bacillus* sp. was isolated as a laboratory contaminant from a culture of MAK7 grown at pH 10. Phylogenetic analysis, based on 16S rRNA gene sequences, showed that isolate MAK7 was an unclassified strain of *Bacillus,* most closely related to a *Bacillus* isolate associated with marine sponges (99.4% sequence identity). The laboratory contaminant *Bacillus* sp. was most closely related to the species *Bacillus cereus* (100% sequence similarity). Generation times of 55 and 40 minutes were observed at external pH 10 in Horikoshi medium for alkaliphilic *Bacillus* MAK7 and *B. cereus* respectively. The internal pH of *Bacillus* MAK7 and *B. cereus* at pH 10 was 9.00 ± 0.08 and 8.76 ± 0.28 respectively. To cope with the reversed ΔpH at pH 10, the membrane potential of both strains increased significantly. However, there was a significant overall drop in the proton motive force at pH 10 to -112 mV for *Bacillus* MAK7 and -97 mV for *B. cereus.* The optimum salinity for growth was determined to be 100 mM NaCl for *Bacillus* MAK7 and 400 mM NaCl for *B. cereus*. However, neither strain showed a Na^+ or K^+ requirement for growth or optimal respiration rates, which is unusual for alkaliphilic bacteria. Increasing concentrations of NaCl were inhibitory to the respiration rate of both strains, but KCl concentrations up to 400 mM did not inhibit respiration. Enzyme activities (malate dehydrogenase, fumarase and hexokinase) in crude cell-free extracts were measured to investigate the effects of alkaline pH on metabolic pathways.

Acknowledgements

First and foremost, all thanks to the Almighty Allah who makes this work possible. There are many people who I would like to thank for their help over the past four years, I can only apologise now if I manage to forget to name them personally. I blame it on my complete brain failure.

I would like to thank my supervisors Dr. Jim Gilmour for his invaluable help, guidance, encouragement and patience during the period of this study. I would also like to thank my advisors Professor Milton Wainwright and Dr. John Rafferty for their advice.

I would also like to take this opportunity to thank Dr. Professor Anne Moir in the Department of Molecular Biology and Biotechnology in the Sheffield University, who kindly donated the plate of *Bacillus subtilis* which was used to complete one of the parts of my study.

I would like to thank my parents for their emotional and standing by me through my every step in life. I would like to say a special thank you to my wife Mrs. Halima Alhani for her encouragement and support over the years. I also would like to thank my daughters: Shahd and Shada and my son: Suhaib who offered me happiness.

I would like to thank the laboratory technicians in the Department of Molecular Biology and Biotechnology in the Sheffield University for there help and all the others in the laboratory who were always there to help guide and assist me through all my experiments.

Last and in the no way the last I would like to thank my colleagues in laboratory F21 in the Department of Molecular Biology and Biotechnology in the University of Sheffield.

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Abbreviations

a_i	Concentration inside the cell
a_o	Concentration outside the cell
ATP	Adenosine triphosphate
bp	base pair (s)
BSA	Bovine serum albumin
°C	Centigrade
CAPS	3-(cyclohexylamino)-l-propanesulfonic acid
CFE	Crude cell free
CTAB	Cetyltrimethylammonium bromide
dpm	Disintegration per minute
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
EB	Ethidium bromide
ECV	Extracellular volume
EDTA	Ethylenediaminetetraacetic acid
EMBL	European Molecular Biology Laboratory
g	Gram (s)
h	Hour (s)
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
ICV	Intracellular volume

IPTG	**Isopropyl ß-D-1-thiogalactopyranoside**
kb	**Kilobase (s)**
LB	**Luria-Bertani medium**
M	**Molar**
mg	**Milligram (s)**
min	**Minute (s)**
ml	**Millilitre (s)**
mM	**Millimole (s)**
MW	**Molecular weight**
NAD	**Nicotinamide adenine dinucleotide(oxidised form)**
NADH	**Nicotinamide adenine dinucleotide(reduced form)**
NCIMB	**National Collection of Industrial, Marine and Food Bacteria**
OAA	**Oxaloacetic acid**
OD	**Optical density**
PCR	**Polymerase chain reaction**
PEP	**Phospho-enol-pyruvate**
PMF	**Proton Motive Force**
pH_i	**Internal pH**
pH_o	**External pH**
pK	**Is equal to the pH at which a compound is half dissociated**
PV	**Pellet volume**
RNA	**Ribonucleic acid**
rRNA	**Ribosomal Ribonucleic acid**

RNase	**Ribonuclease**
rpm	**Revolutions per minutes**
SOC	**Super optimal broth with catabolite repression**
TAE	**Tris-acetate-EDTA**
TCA	**Trichloroacetic acid**
TPP^+	**Tetraphenyl-phosphonium cation**
Tris	**Tris (hydroxymethyl) methylamine**
V/V	**Volume per unit volume**
W/V	**Weight per unit volume**
X-gal	**5 -bromo - 4- chloro -3-indolyl - ß-D-galactopyranoside**
Δp	**Electrochemical proton gradient or proton motive force**
ΔpH	**Transmembrane pH gradient**
Δψ	**Transmembrane electrical potential**
µg	**Microgram**
µl	**Microlitre (s)**
%	**Percentage**

Introduction and Aims

Chapter One

1.1. Extreme Environments

Generally, moderate environments are important to support a wide range of living organisms and usually have pH near neutral, temperature between 20 and 40°C, air pressure of 1 atmosphere and adequate levels of available water, nutrients and salts (Satyanarayana *et al.,* 2005). In contrast, extreme environments can be described as having a much reduced biodiversity with most organisms present being microorganisms (Gomes and Steiner, 2004). Extreme environments pertain to conditions that would be uncomfortable for the normal functioning of humans (Satyanarayana *et al.*, 2005). The extreme conditions may be high or low pH, high or low temperature, high metal concentrations, high salinity, very low water activity, very low nutrient content, high pressure, high radiation or low oxygen tension (Albers *et al.*, 2001; Gomes and Steiner, 2004; Satyanarayana *et al.*, 2005; Redecke *et al.,* 2007).

The range of extreme environments include high temperatures between 55 to 121°C or low temperatures between – 2 to 10°C, high alkalinity environments that have pH values above 9 or high acidity environments that have pH values lower than 4 and high salinity environments containing 2 – 5 M NaCl (Hough and Danson, 1999; van den Burg, 2003; Gomes and Steiner, 2004). There are also high pressure environments that have hydrostatic pressures up to 1400 atmospheres (Satyanarayana *et al.,* 2005). Additionally, there are manmade extreme conditions including cool houses, steam heated buildings and acid mine waters (Satyanarayana *et al.,* 2005).

1.2. Extremophilic Microorganisms

Originally, extreme environments were thought to prevent the existence of life, but it is now known that extreme environments are populated by many groups of extreme microorganisms that are specifically adapted to grow and survive under extreme conditions (Horikoshi, 1991a). Unlike many microorganisms that cannot grow and survive under extreme conditions, extreme microorganisms can develop and grow optimally when one or several stress conditions are in the extreme range (Edwards 1990; Horikoshi, 1991a; Albers *et al.,* 2001).

MacElroy (1974) was one of the first to use the term extremophile to refer to an organism that can grow and thrive in extreme environmental conditions, as reported by Gomes and Steiner, (2004). Many extremophiles are found within the Domain Archaea (Woese *et al.,* 1990; Albers *et al.,* 2001), however, many Bacteria and even some Eukarya can tolerate extreme conditions (Albers *et al.*, 2001; Konings *et al.*, 2002).

Life in extreme environments has been studied intensively, focusing attention on the diversity of organisms and the molecular and regulatory mechanisms involved. Extremophiles are structurally adapted at the molecular level to resist these extreme conditions (Gomes and Steiner, 2004). Furthermore, some extremophiles are polyextremophiles that able to withstand multiple extremes, i.e. adapted to more than one type of extreme environment, e.g. the acidothermophiles *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius* that have been grown at pH 3 and 80°C (Gomes and Steiner,

2004; Irwin and Baird, 2004). Thermophilic alkalitolerant bacteria have also been isolated, such as *Anaerobranca* spp. (Engle *et al.*, 1995).

Additionally, the extremozymes (biocatalysts) produced by extreme microorganisms are proteins that can function under extreme conditions via their extreme stability, they suggest new opportunities for biocatalysis and biotransformations (Gomes and Steiner, 2004). Table 1.1 shows the different groups of extremophiles and the extremozymes which have been obtained from these microorganisms (Hough and Danson, 1999; van den Burg, 2003). It is clear that extremophiles may have important industrial applications in terms of the utility of their metabolic ability in extreme environments and their ability to produce unusual enzymes (Goto *et al.*, 2005; Russell, 2000).

Extremophilic microorganisms are classified according to the environments they occupy e.g. alkaliphiles, acidophiles, halophiles, thermophiles, psychrophiles and osmophiles (Edwards, 1990; Gilmour, 1990; Jennings, 1990; Horikoshi, 1991a; Ulukanli and Digrak, 2002; Gomes and Steiner, 2004). Moreover, it is interesting to note that many extremophilic bacteria are amongst the most primitive of bacteria, as suggested by their location close to the root of the universal phylogenetic tree (Figure 1.1) (Hough and Danson, 1999).

Phenotype	Environment	Typical genus (Bacteria and Archaea)	Extremozymes
Thermophilic	55 – 80°C	*Methanobacterium*, *Thermoplasma*, *Thermus**, some *Bacillus** species	Amylases, Pullulanase,Glucoamylases, Glucosidases, Cellulases, Xylanases, Chitinases, Lipases, and Esterases
Hyperthermophilic	80 –113°C	*Aquifex**, *Archaeoglobus*, *Hydrogenobacter**, *Methanothermus*, *Pyrococcus*, *Pyrodictium*, *Pyrolobus*, *Sulfolobus*, *Thermococcus*, *Thermoproteus*, *Thermotoga**	Proteases
Psychrophilic	– 2 to 10°C	*Alteromonas**, *Psychrobacter**	DNA polymerases, Dehydrogenases, Proteases, Amylases, Cellulases, Dehydrogenases and Lipases
Halophilic	2–5 M NaCl	*Haloarcula*, *Halobacterium*, *Haloferax*, *Halorubrum*	Proteases, Dehydrogenases
Acidophilic	pH<4	*Acidianus*, *Desulfurolobus*, *Sulfolobus*, *Thiobacillus**	Amylases, Glucoamylases Proteases and Cellulases
Alkaliphilic	pH>9	*Natronobacterium*, *Natronococcus*, some *Bacillus** species	Proteases and Cellulases

Table 1.1: Characteristics of different groups of extremophiles and their biocatalysts (extremozymes), which could be applicable in industrial processes, *Genus of the domain Bacteria; all others are Archaea. Modified from, Hough and Danson, (1999) and van den Burg, (2003).

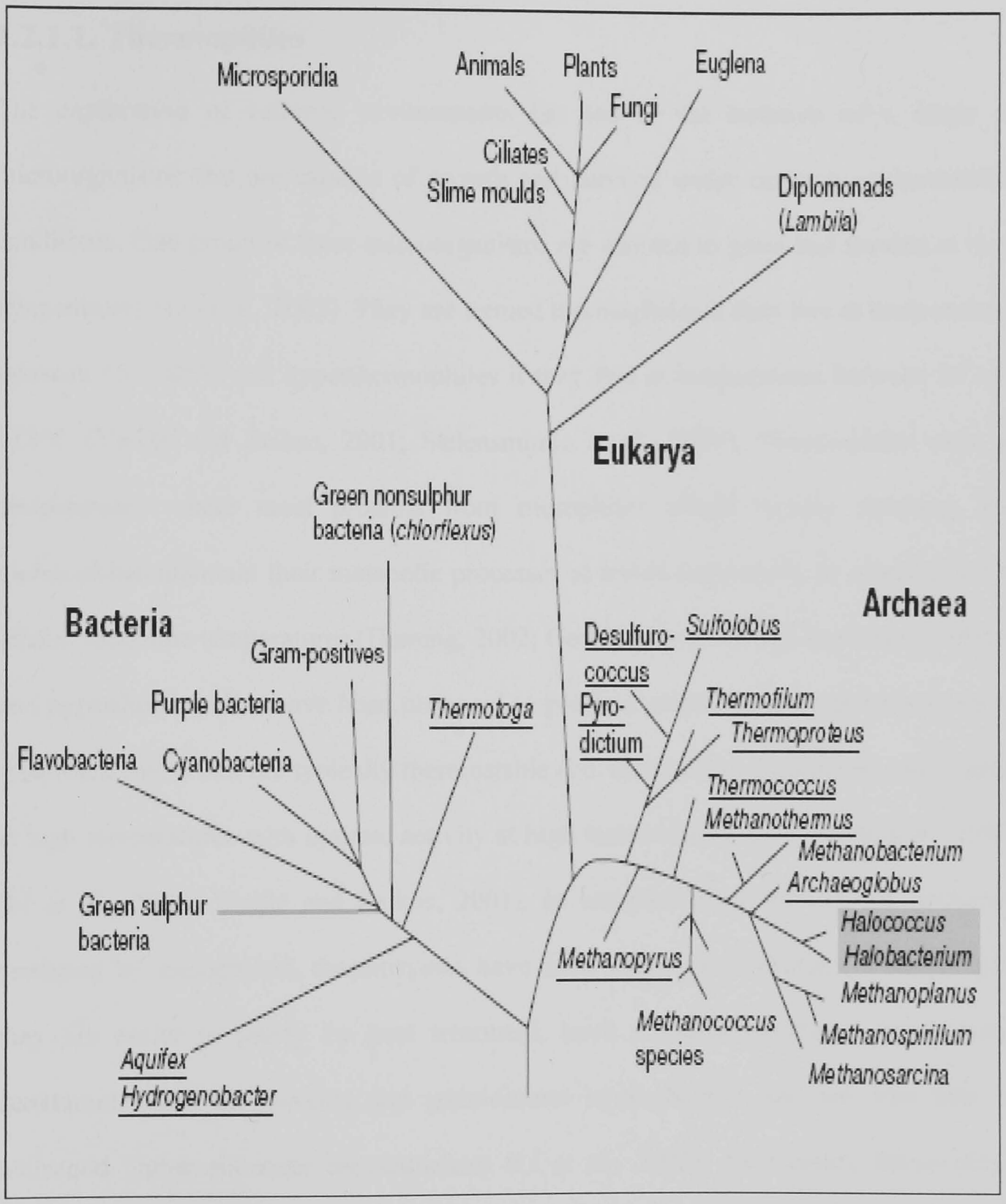


Figure1.1: The universal phylogenetic tree, constructed from rRNA sequence comparisons. Branches representing the three domains (Archaea, Bacteria, Eukarya) are indicated. Thermophilic and hyperthermophilic species are underlined and halophilic species are shaded. Taken from, Hough and Danson, (1999).

1.2.1. Extremes of Temperature and Life

1.2.1.1. Thermophiles

The exploration of extreme environments has led to the isolation of a range of microorganisms that are capable of growth and survival under extreme environmental conditions. One group of these microorganisms are adapted to grow and survive at high temperatures (Li *et al.,* 2005). They are termed thermophiles if they live at temperatures between 45 – 80°C and hyperthermophiles if they live at temperatures between 80 and 113°C (Vieille and Zeikus, 2001; Sælensminde *et al.,* 2007). Thermophiles exist at temperatures where most proteins from mesophiles would rapidly denature, but thermophiles maintain their metabolic processes at levels comparable to organisms that inhabit moderate temperatures (Deming, 2002; Georlette *et al.* 2004). Both thermophiles and hyperthermophiles have been observed to produce extremozymes (thermozymes or thermoenzymes) that are typically thermostable (i.e. resistant to irreversible inactivation at high temperatures with optimal activity at high temperatures, between 60 and 125°C) (Li *et al.,* 2005; Vieille and Zeikus, 2001). In comparison to mesozymes (enzymes produced by mesophiles), thermozymes have three major biotechnological advantages, they are easier to purify by heat treatment, have a higher resistance to chemical denaturants such as solvents and guanidinium hydrochloride and are also able to withstand higher substrate concentrations (Li *et al.,* 2005). As a result, thermophilic extremophiles have attracted most attention for their thermozymes, including proteases, lipases, cellulases, chitinases and amylases, that have been widely used in industrial applications like detergents, hydrolysis in food and feed, brewing, baking, and starch, cellulose, chitin and pectin processing (van den Burg, 2003).

A wide diversity of prokaryotes is capable of growth at 50°C (Satyanarayana *et al.*, 2005) and thermophiles are more widespread than hyperthermophiles. Thermophiles include an extensive range of microorganisms, such as *Bacillus*, *Clostridium*, *Thiobacillus*, *Thermus* spp., actinomycetes and lactic acid bacteria and several group of archaea (Irwin and Baird, 2004). Thermophilic microorganisms have been found in natural environments such as sun-heated soils, terrestrial hot springs, composts, geothermally heated oil reservoirs and oil wells (Malhotra *et al.*, 2000). Whereas, hyperthermophiles have been isolated from hot springs and sediments around marine hydrothermal vents on the ocean floor with temperatures up to 121°C, geological evidence suggests that such microorganisms use Fe (III) as an electron acceptor (Kashefi and Lovley, 2003). These hyperthermophiles include *Pyrolobus fumarii* which is an Archaeon (Irwin and Baird, 2004).

On the basis of optimum temperature requirements, numerous species of both Bacteria and Archaea have been classified as thermophiles including *Thermus aquaticus*, *T. thermophilus*, *Thermodesulfobacterium commune*, *Sulfolobus acidocaldarius*, *Thermomicrobium roseum*, *Dictyoglomus thermophilum*, *Methanococcus vulcanicus*, *Sulfurococcus mirabilis* and *Thermotoga maritima*. Hyperthermophilic microorganisms on the other hand include, *Methanococcus jannaschii*, *Acidianus infernos*, *Archaeoglobus profundus*, *Methanopyrus kandleri*, *Pyrobaculum islandicum*, *Pyrococcus furiosus*, *Pyrodictium occultum*, *Pyrolobus fumarii*, *Thermococcus littoralis*, *Ignicoccus islandicum* and *Nannoarchaeum equitans* (Ghosh *et al.*, 2003; Satyanarayana *et al.*, 2005).

Furthermore, a few thermophilic fungi belonging to Zygomycetes such as *Rhizomucor miehei*, *R. pusillus*, Ascomycetes such as *Chaetomium thermophile* and Hyphomycetes such as *Acremonium alabamensis* have been isolated from composts, soils, nesting materials of birds, wood chips and many other sources. Additionally, some algae such as *Cyanidium caldarium* and protozoa including *Cothuria* sp., *Oxytricha falla*, *Cyclidium citrullus and Naegleria fowleri* have been reported to grow at high temperatures (Satyanarayana *et al.*, 2005).

1.2.1.2. Psychrophiles

Psychrophiles (cold-loving) are extremophilic microorganisms that are capable of growth and reproduction at low temperatures. To be classified as psychrophilic, their optimum growth temperature must be less than 15°C and they should be capable of growth at 0°C (Morita, 1975). In contrast, psychrotrophic microorganisms are more thermally adaptable and can grow at relatively low temperatures or at temperatures more suitable to mesophilic microorganisms (Irwin and Baird, 2004; Zecchinon *et al.*, 2001). Psychrophilic microorganisms are usually found in the oceans and the polar and alpine regions of the Earth and they are present in pockets of salt brine in arctic sea ice, where the temperature can drop to below –15°C (Georlette *et al.*, 2004; Irwin and Baird, 2004; Sælensminde *et al.*, 2007). Therefore, true psychrophilic microorganisms have to produce cold-adapted enzymes, which exhibit high catalytic activities at low temperature, in order to adapt to a cold habitat. Psychrophilic microorganisms have attracted attention as sources of enzymes with potential for low-temperature catalysis. In

fact, a range of cold-active enzymes have been found in psychrophiles (Gerday *et al.*, 2000).

In general, a characteristic feature of extremozymes from psychrophilic microorganisms is the correlation of high catalytic activity and low thermal stability at moderate temperatures, which can be partly explained by the increased flexibility of the molecule, compared with mesophilic and thermophilic enzymes (Bentahir *et al.,* 2000; D'Amico *et al.,* 2003). It has been supposed that increased flexibility is correlated with decreased stability and a delicate balance between stability and activity is required (Beadle and Schoicet, 2002). van den Burg (2003) reported that many extremozymes from psychrophiles could be good choices for industrial applications, because of ongoing efforts to decrease energy consumption. Evidently, there is an increasing demand for psychrophilic enzymes in detergents to develop laundry applications that could be performed at lower temperatures. Examples of extremozymes from psychrophiles that are now commercially used include proteases, amylases or lipases. Several food processing applications would also benefit from the availability of extremozymes that are active at lower temperatures (van den Burg, 2003).

Many genera have been reported to contain psychrophilic microorganisms including, *Alcaligenes*, *Alteromonas*, *Aquaspirillum*, *Arthobacter*, *Bacillus*, *Bacteroides*, *Brevibacterium*, *Gelidibacter*, *Methanococcoides*, *Methanogenium*, *Methanosarcina*, *Microbacterium*, *Micrococcus*, *Moritella*, *Octandecabacter*, *Phormidium*, *Photobacterium*, *Polaribacter*, *Polaromonas*, *Psychroserpens*, *Shewanella* and *Vibrio* (Satyanarayana *et al.,* 2005).

1.2.2. Extremes of pH and Life

1.2.2.1. Alkaliphiles (High pH)

In general, alkaliphilic microorganisms require high pH and the presence of sodium ions for their lifestyles (Horikoshi, 1999; Kitada *et al.,* 2000: Ma *et al*, 2004a). Since Vedder isolated the obligate alkaliphile, *B. alcalophilus* in 1934 many strains of alkaliphiles have been isolated from a diverse range of environments, including neutral environments. Industrial applications, especially the utilization of their enzymes to operate under alkaline conditions, have been reported by Horikoshi (1991b) and Peddie (1999). Most of these isolates were classified as belonging to the genus *Bacillus* i.e. they were aerobic alkaliphilic microorganisms, rod-shaped, Gram-positive, spore-forming, motile, and both oxidase and catalase positive (Horikoshi, 1991b; Kroll, 1990; Yumoto, 2002). Alkaliphiles will be discussed in detail in sections 1.3 to 1.10 of this chapter.

1.2.2.2. Acidophiles (Low pH)

Acidophiles thrive under conditions of low pH, these microorganisms are termed extreme acidophiles if they capable of optimal growth at less than pH 3 and moderate acidophiles if they capable of optimal growth at pH 3 – 5. Microorganisms capable of growth at low pH are largely prokaryotes, and comprise relatively few species of Bacteria and Archaea (Johnson and Hallberg, 2003 and 2008; Baker-Austin and Dopson, 2007).

Therefore, most acidophilic microorganisms have been isolated from extreme environments that present an inaccessible physical barrier which reduces the

colonization potential of other microorganisms that grow at or around neutral pH (neutralophilic). These extreme environments include both man-made and natural acidic environments that occur in the biosphere such as acid mine drainage and geothermal vents (Futterer *et al.,* 2004; Johnson and Hallberg, 2003). True acidophiles such as the archaea *Picrophilus torridus* and *P. oshimae* have been grown optimally at pH 0.7 and at 60°C (Gomes and Steiner, 2004). The biodiversity of extreme acidophiles has recently attracted considerable attention with regard to their physiology and phylogenetic affiliations. The ability to use inorganic chemicals as electron donors is common among extreme acidophiles, as ferrous iron and sulfur represent two major energy sources in many extremely acidic environments (Johnson and Hallberg, 2008). Reduction of iron and sulfur by acidophilic microorganisms is driven by their use as electron acceptors under oxygen limited conditions and in turn these reactions drive major biogeochemical processes in low pH environments. Acidophiles show significant diversity in how they assimilate carbon, some are obligate autotrophs and others are obligate heterotrophs, whereas a great number use either organic or inorganic carbon, depending on the availability of the former (Johnson and Hallberg, 2008).

However, acidic effluents associated with acid mine drainage can cause huge environmental pollution such as the contamination of drinking water. Consequently one important biotechnological application of acidophiles is to reduce this pollution, in addition to their role in metal extraction from ores (Rohwerder *et al.,* 2003; Golyshina and Timmis, 2005). Furthermore, acidophilic microorganisms can be used as a source of acid-stable enzymes with applications as lubricants and catalysts (van den Burg, 2003).

These enzymes included amylases, pullulanases, glucoamylases and glucosidases (Gomes and Steiner, 2004).

To grow at low pH, acidophiles must maintain their internal pH close to neutral (maintain a pH gradient of several pH units) across the cellular membrane while producing ATP by the influx of protons through the F_0/F_1 ATPase (Baker-Austin and Dopson, 2007). There are a variety of strategies of pH homeostasis to cope with acidic environments that involve restricting proton entry by the cytoplasmic membrane and purging of protons from the cytoplasm. The movement of protons into the cell is minimized by an intracellular net positive charge, thus the cells have a positive inside membrane potential (Satyanarayana *et al.*, 2005).

1.2.3. High Salinity and Life (Halophiles)

Halophilic microorganisms are able to grow and survive in hypersaline environments by their ability to maintain osmotic balance. Halophilic microorganisms are found in all three domains of life: Archaea, Bacteria, and Eukarya (Margesin and Schinner, 2001; Madern and Zaccai, 2004). Halophilic microorganisms have two ways to adapt to saline environments. The first way is maintain an osmotic balance of cytoplasm with the external environment by accumulating high concentrations of various organic osmotic solutes e.g. ectoine or betaine. Halophilic or halotolerant microorganisms that accumulate organic compatible solutes have low intracellular salt concentrations and therefore their intracellular enzymes have no special salt tolerance. The second way is to maintain an osmotic balance of cytoplasm with the external hypersaline environment by

accumulating high concentrations of salt (KCl). Therefore, osmotic regulation requires special adaptations of the intracellular enzymes that have to function in the presence of salt (Margesin and Schinner, 2001). These adaptations include having a relatively large number of negatively charged amino acid residues on the surfaces of proteins to prevent precipitation. As a result, the solubility of halophilic enzymes is often very poor, which could limit their biotechnological applicability (Madern *et al.*, 2000).

Gilmour (1990) have observed that microorganisms requiring salt for growth can be divided into three groups; the first group is slight halophiles including many marine organisms, seawater contains about 0.5 M NaCl; second group is moderate halophiles that have optimal growth between 0.2 and 2 M NaCl; third group is extreme halophiles with optimal growth above 3 M NaCl. Furthermore, extremely halotolerant bacteria are able to grow and survive over a wide range of NaCl (0.1 to 4.5 M).

1.2.4. Other Environmental Extremes and Life

Gold (1992) described the discovery of bacteria in deep subsurface locations where they were not expected. For example, piezophilic microorganisms are able to grow and survive at depths of up to 10 km under the sea and can tolerate pressures of up to 130 MPa. Furthermore most piezophilic microorganisms require high-pressure i.e. they can not grow or grow slowly at normal atmospheric pressure. High-pressure habitats include the Marianas Trench, which is the deepest sea floor in world at about 10.9 km.

Deinococcus radiodurans has been found in extreme environments containing high levels of ionizing radiation of up to 20,000 Gy of gamma radiation. This affects cells by damaging DNA directly or producing reactive oxygen radicals that can cause mutations in DNA or strand breaks, e.g. 20,000 Gy of gamma radiation is enough to split the genome into small fragments. Many microorganisms are strictly obligate anaerobes, which do not have the ability to survive in aerobic environments (Irwin and Baird, 2004).

1.3. Definition of Alkaliphilic and Alkalitolerant Microorganisms

Normally, bacteria are widely distributed in nature and most of them grow best at around neutral pH. However, there are microorganisms that can inhabit and grow in extremely alkaline environments that can be divided into two main groups of microorganisms; alkaliphilic microorganisms and alkalitolerant microorganisms (Krulwich and Guffanti, 1989a; Yumoto, 2002). Alkaliphiles can be further divided into two main physiological groups, alkaliphiles and haloalkaliphiles. Alkaliphiles require an alkaline pH of 9 or more for their growth with optimal growth at pH 10 i.e. obligate requirement for alkaline growth conditions. Haloalkaliphilic microorganisms require both an alkaline pH of 9 and high salinity up to 5 M NaCl for their growth, which is 10 times the saline/salt content of normal ocean water (Horikoshi, 1999). An example of an alkaliphilic bacterium is *Bacillus alcalophilus*, which maintains an intracellular pH between 8.4 and 9.0 (Kroll, 1990; Krulwich, 1995; Horikoshi, 1999; Jones and Grant, 2000).

On the other hand, alkalitolerant microorganisms show good growth at pH 10 or above, but good growth also takes place at near neutral pH, they are also known as facultative

alkaliphiles (Krulwich and Guffanti, 1989a). One of the most common alkalitolerant microorganisms is *B. firmus* OF4 that grows nearly as well at pH 7.5 as at pH 10.5 in batch and continuous culture on malate containing medium (Sturr *et al.*, 1994). The effect of external pH on growth of *B. firmus* OF4 was investigated and doubling times of 54 and 38 minutes at external pH values 7.5 and 10.5 respectively were recorded. The growth rates of this strain decreased above pH 11, which correlated with an apparent decrease in the ability to tightly regulate cytoplasmic pH and with the appearance of chains of cells (Sturr *et al.*, (1994).

1.4. Diversity of Alkaliphilic Microorganisms

The study of alkaliphilic microorganisms is fairly recent, there were only 16 published scientific papers concerning alkaliphiles when Horikoshi started experiments on alkaliphilic bacteria in 1968. Nevertheless, the use of alkaliphilic microorganisms has a long history in Japan, in ancient times indigo has been naturally reduced by particular bacteria that grow under high alkaline conditions in the presence of sodium carbonate (Horikoshi, 1999). Alkaliphiles have been frequently isolated from normal neutral environments such as garden soil, although cell counts of the alkaliphilic bacteria are higher in alkaline environments. Alkaliphiles can also be isolated from acidic soil samples (Horikoshi, 1996 and 1999). The diversity of alkaliphilic bacteria is shown in Table 1.2, which is based on papers published over the last 15 years.

Microorganisms	Location	pH range for growth	pH optimum	References
Gram-positive, alkalitolerant, thermophilic bacteria	Water and soil samples from Yellowstone National Park	6.9 -10.3	8.5	(Engle *et al.*, 1995)
Aerobic, Gram-positive alkaliphilic bacterium (*Bacillus subtilis)*	Hardwood pulp	9 - 11.5	9.0	(Yang *et al.*, 1995)
A new alkaliphilic strain of *Microbacterium*	Alkaline Soda Lake in Ethiopia	9.5 - 11.5	_	(Gessesse and Gashe, 1997)
Halotolerant alkaliphilic obligate methanotrophic bacteria (*Methylobacter alcaliphilus* sp. nov.)	Moderately saline Soda Lake in Tuva (Central Asia)	7.0 -10.5	9.0 - 9.5	(Khmelenina *et al.*, 1997)
Alkaliphilic aerobic organotrophic bacteria (*Dietzia natronolimnaios* sp. nov.)	Moderately saline and alkaline East African Soda Lake	6 - 10	9.0	(Duckworth *et al.*, 1998)
Gram-positive, anaerobic alkaliphilic bacteria (*Tindallia magadii* gen. nov.)	Soda deposits in Lake Magadi, Kenya	7.5 -10.5	8.5	(Kevbrin *et. al.*, 1998)
Alkaliphilic organotrophic bacteria (*Halomonas magadii* sp. nov)	Several saline and alkaline East African soda lakes	7 - 11	9 - 10	(Duckworth *et al.*, 2000)

Table 1.2: Diversity of alkaliphilic bacteria

Microorganisms	Location	pH range for growth	pH optimum	References
Gram-positive, facultatively alkaliphilic bacterium (*Bacillus* sp. WW3-SN6)	Alkaline washwaters derived from the preparation of edible olives	7.0 - 10.5	8.0 - 9.0	(Notugias and Russell, 2000)
Alkaliphilic sulphur-oxidizing bacteria (AL2 and AL3)	Alkaline environments (Soda soil and Soda Lakes)	8.0 - 10.4	9.5 - 9.8	(Sorokin *et al.*, 2000)
Gram- negative, nonmotile bacteria (*Ancylobacter natronum* sp. nov.)	Soda Lake of the southern Transbaikal region	6.5 - 9.5	8 - 8.5	(Doronina *et al.*, 2001)
Alkaliphilic ammonia-oxidizing bacteria.	North-east Mongolian soda lakes	9.7 - 10.5	10	(Sorokin *et al.*, 2001b)
Arthrobacter ramosus (MCM B-351)	Alkaline lake of Lonar, Buldhana District of Maharashtra, India	-	10	(Nilegaonkar *et al.*, 2002)
Gram- positive, alkaliphilic bacteria (*Bacillus halmapalus*)	Soil samples in Haga, Tochigi, Japan	6.8 - 10	9	(Saeki *et al.*, 2002)
Anaerobic alkaliphilic bacteria (*Alkalibacter saccharofermentans* gen. nov.)	The cellulolytic community of alkaline lake Nizhnee Beloe (Transbaikal region of Russia)	7.2 -10.2	9.0	(Garnova *et al.*, 2004)
Akaliphilic actinomycete strain (*Nocardiopsis alkaliphile* sp. nov.)	Eastern desert of Egypt	-	9.5 - 10	(Hozzein *et al.*,2004)

Table 1.2: Diversity of alkaliphilic bacteria (Continued)

Microorganisms	Location	pH range for growth	pH optimum	References
Two strains of alkaliphilic, slightly halophilic bacteria: *Alkalimonas amylolytica* sp. nov. (N10) and *A. delamerensis* sp. nov. (1E1)	Lake Chahannor in China (N10) and Lake Elmenteita in East Africa (1E1)	-	10 -10.5	(Ma *et al.*, 2004a)
Gram- positive, aerobic akaliphilic *Bacillus* sp.	Soil and water of Lake Van (Turkey)	6.8 -10.5	9	(Berber and Yenidunya, 2005)
Gram-positive alkalitolerant bacterium,(PAT 05^T) (*Bacillus patagoniensis* sp. nov.)	The rhizosphere of the perennial shrub *Atriplex lampa* in north-eastern Patagonia, Argentina	7-10	8	(Olivera *et al.*, 2005).
Gram-negative alkalitolerant bacterium (*Chimaereicella alkaliphila* gen. nov.)	Highly alkaline groundwater in the artesian borehole feeding the spa at Cabeco de Vide in Southern	11.4	8.0	(Tiago *et al.*, 2006)
Anaerobic, Gram-negative, alkaliphilic (*Arsukibacterium ikkense* sp. nov.)	The alkaline, low-saline ikaite columns in the Ikka Fjord, SW Greenland	10.4	9.2 -10	(Schmidt *et al.*, 2007)
Obligately alkaliphilic, highly salt-tolerant natronophiles (*Natronobacillus azotifigens* gen. nov.)	Soda soils in south-western Siberia, north-eastern Mongolia and the Lybian desert	7.5 - 10.6	9.5 -10	(Sorokin *et al.*, 2008)
Facultative anaerobic, haloalkaliphilic bacteria (*Bacillus beveridgei* sp. nov.)	The shallow littoral region of the northeastern quadrant of Mono Lake	-	8.5 - 9.0	(Baesman *et al.*, 2009)

Table 1.2: Diversity of alkaliphilic bacteria (Continued)

As shown in Table1.2, there has been a rapid expansion in the numbers and types of alkaliphilic microorganisms that have been isolated from a wide range of environments (Sorokin *et al.,* 2001a). Those environments include alkaline springs, but also garden soil, which is sometimes not particularly alkaline. It is also clear that acidic soil samples and faeces are good sources of alkaliphilic microorganisms (Grant *et al.*, 1990; Horikoshi, 1991b, 1996 and 1999; Goto *et al.,* 2005).

Two strains of Gram negative, nonmotile, encapsulated cells were isolated from the soda lakes of the southern Transbaikal region. They were found to be alkalitolerant facultatively methylotrophic bacteria which grew well at pH values between 6.5 and 9.5 on methanol as the source of carbon and energy with an optimum pH value of 8.0 - 8.5 (Table 1.2, Doronina *et al.,* 2001). An alkaliphilic *Bacillu*s sp. designated as KSM-KP43 was isolated from a sample of soil in Haga, Tochigi, Japan. It grew well at pH values between 6.8 and 10 with an optimum at pH 9. The results of 16S rRNA gene sequence analysis placed this strain in a cluster with *Bacillus halmapalus*. This strain was Gram positive, strictly aerobic, motile, sporulating, and it also was able to produce an unusual serine protease (Saeki *et al.,* 2002). YIM80379^T strain was isolated from a soil sample collected from the eastern desert of Egypt and shown to have an optimum pH for growth of 9.5 – 10 and scarce or no growth at pH 7. One the basis of 16S rRNA analysis YIM80379^T was proposed to be a novel species *Nocardiopsis alkaliphila* (Table 1.2, Hozzein *et al.,* 2004). Furthermore, alkaliphilic Gram positive, endospore forming *Bacillus* spp. and non-endospore forming species of *Micrococcus, Paracoccus*, *Pseudomonas*, *Aeromonas*, *Corynebacterium* and *Actinopolyspora,* have been isolated from neutral soils (Satyanarayana *et al.,* 2005). On the other hand, *Chimaereicella*

alkaliphila, a Gram-negative bacterium, was isolated from highly alkaline groundwater at pH 11.4 (Tiago *et al.*, 2006).

Alkaliphilic microorganisms have also been isolated from a variety of environments for industrial applications (Yumoto *et al.,* 2000). Strain V1-4 belongs to the genus *Bacillus* and is able to grow in diluted Kraft black liquor at pH 11.5 and is capable of producing high levels of xylanase when cultivated in alkaline medium at pH 9 and 10. Maximal enzyme activity was obtained by cultivation in a defined alkaline medium with 2% birchwood xylan and 1% corn steep liquor at pH 9, but high enzyme production was also obtained on wheat bran (Yang *et al.*, 1995). A facultative alkaliphile designated as PAT 05^T, was isolated from the rhizosphere of the perennial shrub *Atriplex lampa* in north-eastern Patagonia, Argentina. This strain grew at pH 7 – 10 with optimum pH 8, but no growth was reported at pH 6. This strain was Gram-positive, rod-shaped, spore-forming bacterium and on the basis of 16S rRNA gene sequencing, it was identified as the type strain of *Bacillus patagoniensis* (Olivera *et al.,* 2005).

Haloalkaliphilic microorganisms have mainly been found in extremely alkaline saline environments including both soda deserts and soda lakes, examples include the Rift Valley lakes of East Africa such as Lake Magadi, the Wadi Natrun lake in Egypt and the western soda lakes of the United States (Horikoshi, 1999; Rees *et al.,* 2004; Satyanarayana *et al.,* 2005). These lakes are often coloured red due to large numbers of haloalkaliphilic Archaea such as *Natronobacterium pharaonis*, *N. gregoryi* and *Natronococcus occultus* (Figure 1.2).

Figure 1.2: Red pigmentation due to haloalkaliphilic archaea growing on Trona crusts at Lake Magadi, Kenya. Taken from, Grant (2006).

Cyanobacteria, especially *Arthrospira* (*Spirulina*) *platensis* and *Cyanospira rippkae*, and anoxygenic phototrophic bacteria of the genera *Ectothiorhodospira and* Halorhodospira are responsible for photosynthetic primary production in soda lakes (Jones *et al*, 1998; Milford *et al.,* 2000).

Haloalkaliphilic *Bacillus* strains were isolated from the water of Lake Van and the surrounding soil by Berber and Yenidunya (2005). A facultative alkaliphile designated as WW3-SN6 strain, was isolated from the alkaline wash waters derived from the preparation of edible olives. This strain grew from pH 7.0 to 10.5, with a broad optimum from pH 8.0 to 9.0 with optimum temperature between 27 and 32°C. It could grow in up to 15% (w/v) NaCl. WW3-SN6 is Gram-positive and non-motile (Ntougias and Russell, 2000).

Two strains of alkaliphilic aerobic organotrophs from a moderately saline and alkaline East African soda lake were grown at pH values between 6 and 10 with a pH optimum for growth of 9.0 with salt concentration between 0% and 10%. 16S rRNA sequencing showed that both strains are members of the monospecific genus *Dietzia maris.* However, one of the isolates differed significantly in carbon source utilisation and halotolerance to be proposed as a new species - *Dietzia natronolimnaios* (Duckworth *et al.*, 1998). Two strains of Gram-negative halotolerant alkaliphilic methanotrophic bacteria were isolated from moderately saline soda lakes in Tuva (Central Asia). These strains grow fastest at pH 9 – 9.5, but much more slowly at pH 7 and no growth occurred at pH 6.8 or less. These strains also required $NaHCO_3$ or NaCl for their growth in alkaline medium. Both strains were not capable of growing on any polycarbon substrates

tested and based on their alkaliphilic physiology, both strains were named as *Methylobacter alcaliphilus* sp. nov. (Khmelenina *et al.*, 1997).

It is clear from Table 1.2 that numerous alkaliphilic *Bacillus* strains have been isolated from different habitats and become approved species (Goto *et al.*, 2005; Olivera *et al.*, 2005; Yumoto *et al.*, 2005). They form several clusters in phylogenetic trees based on their 16S rRNA gene sequences and haloalkaliphilic *Bacillus* species form a very important part of the diversity of the *Bacillus* genus (Figure. 1.3) (Goto *et al.*, 2005).

1.5. Alkaline Environments (Habitats)

Natural alkaline environments on earth are caused by the effect of geographical, topographical and climatic conditions and include soda lakes and desert soils (Grant and Horikoshi 1992; Tiago *et al.*, 2004; Detkova and Pusheva, 2006). Alkaline environments can also be formed by industrial activities including food processing (Grant *et al.*, 1990; Jones *et al.*, 1994) and industrial-derived waste waters (Ulukanli and Digrak, 2002). Alkalinity of environments may also be caused by biological activity, such as ammonification or sulphate reduction (Horikoshi, 1991a). Grant *et al.*, (1990) observed that two types of alkaline environments exist on Earth. The first type is high calcium (Ca^{2+}) environments that are typically represented by groundwater, bearing high levels of CaOH. The second type are low Ca^{2+} environments that are usually dominated by the presence of sodium carbonate (Na_2CO_3) and have low levels of magnesium (Mg^{2+}) ions, such as soda lakes and deserts. These environments are the most stable, naturally occurring highly alkaline environments found worldwide, where pH values of 10 and above are common (Ma *et al.*, 2004a; Tiago *et al.*, 2004).

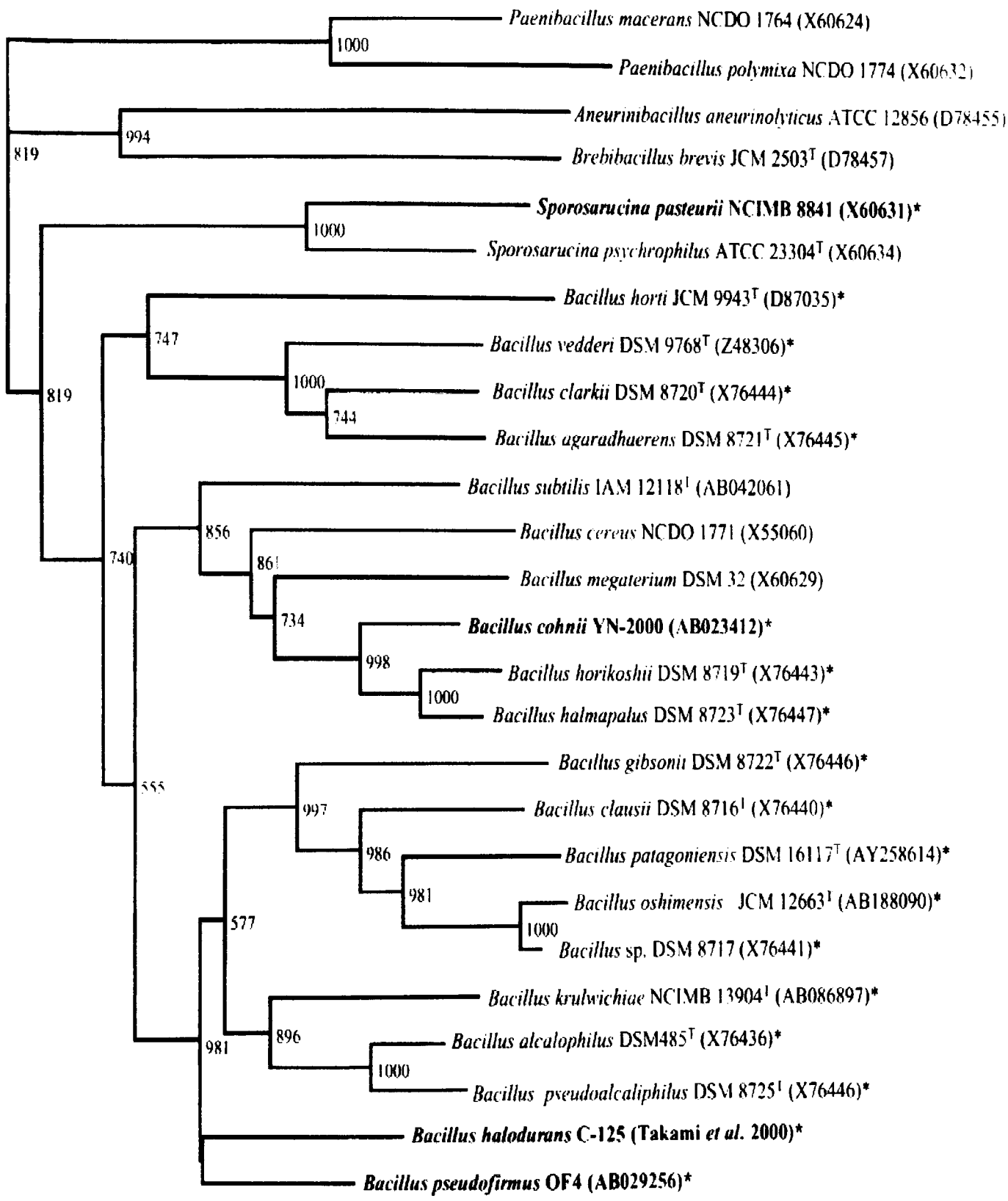


Figure 1.3: Phylogenetic tree derived from 16S rRNA gene sequence data of alkaliphilic and neutralophilic *Bacillus* spp. and related strains. The phylogenetic positions of *B. cohnii* YN-2000, *B. pseudofirmus* OF4, *B. halodurans* C-125, *Sporosarucina pasteurii* are in bold. Asterisks indicate that the strain is an alkaliphile. Numbers indicate bootstrap values greater than 500. Bar: 0.01 K_{nuc}. Taken from, Goto *et al.*, (2005).

Although, such sites are widely distributed throughout the world as a result of their inaccessibility, few such lakes have been explored from the microbiological point of view (Grant *et al.*, 1990). However, the best studied soda lakes are those of the East Africa Rift valley which have been investigated since the early 1930s (Grant *et al.*, 1990; Jones *et al.*, 1998), wherein detailed limnological and microbiological investigations have been carried out. Microbiological studies of central Asian soda lakes have also been well documented (Zhilina and Zavarzin, 1994) and a phylogentically diverse set of alkaliphiles has been identified (Ma *et al.*, 2004b).

Some soda lakes may contain both high concentrations of sodium carbonate and high concentration of other salts, especially sodium chloride, leading to the formation of alkaline saline lakes. This means that the haloalkaliphilic microorganisms that inhabit these environments must be adapted to both high pH and high salinity as well as to stresses that are secondary to these major ones, such as low water activity and scarcity of some micronutrients (Grant *et al.*, 1990; Jones *et al*, 1998; Grant, 2004; Rees *et al.*, 2004; Tiago *et al.*, 2004). The salinity range of soda lakes in the Rift Valley is from 5% (w/v) to saturation (33%) with pH values from 8.5 to above 11.5 (Rees *et al.*, 2004). Lake Chahannor, a soda lake in the Mongolia autonomous region of China, has been reported to have pH values from 9.5 to 10.2 with up to 5 M Na^+, high concentrations of both Cl^- and CO_3 , very low levels of Mg^{2+} and also undetectable amounts of Ca^{2+} (Ma *et al.*, 2004a; Wei *et al.*, 2007).

1.6. Background on Bioenergetics

Mitchell (1961) described the chemiosmotic theory which explains the key role that membranes play in the generation of metabolic energy in the form of ATP (Albers *et al.*, 2001). Electron transport in the cytoplasmic membrane of bacteria induces a difference in electrochemical potential of H^+ across the membrane (proton motive force, PMF, Δp), which is the sum of both membrane potential (transmembrane electrical potential) and transmembrane pH gradient, and is required for production of ATP by H^+-ATPase. The proton motive force is calculated using following equation:

$$\text{PMF or } \Delta p = \Delta\Psi - Z\,\Delta\,\text{pH}$$

$$\Delta\text{pH} = \text{pH}_i - \text{pH}_o$$

$$Z = 2.3\ RT/F = 58.5 \cong 59\ \text{mV}$$

$\Delta\Psi$ (mV) is membrane potential (electrical potential of membrane is outside positive and inside negative), while ΔpH is transmembrane pH gradient (outside acidic in neutrophiles and outside alkaline in alkaliphiles), pH_i is internal pH, pHo is pH outside, R is gas constant, T is absolute temperature and F is the Faraday constant (Albers *et al.*, 2001;Yumoto, 2002; Olsson *et al.*, 2003; Goto *et al.*, 2005).

The respiratory chain pumps out H^+ from the inside to the outside of the membrane associated with the flow of electrons from NADH to O_2. ATPase produces ATP by translocating H^+ from the outside to the inside of the cell membrane of bacteria. Moreover, the cell membrane of bacteria possesses a number of transporter systems for

obtaining substrates from the outside of the membrane, which work by using the electrochemical potential of H^+ across the cell membrane (Δp) as their driving force.

1.7. Mechanisms of Cytoplasmic pH Regulation in Alkaliphilic Bacteria

Protons are known to be involved in almost every physiological and biochemical reaction. For this reason, it is necessary for every living cell to regulate its cytoplasmic pH around an optimal and compatible pH for cellular activities and stability of most proteins that support growth (Padan *et al.*, 2005). Therefore, bacteria that can grow at the extremes of external pH values (below pH 4 for acidophiles and above pH 9 for alkaliphiles) must be able to maintain their cytoplasmic pH (intracellular pH) within a fairly narrow range around pH 7 (Booth, 1985; Cook, 2000; Yumoto, 2002). In general acidophiles show internal pH values in the range of 6 – 7, neutrophlies have intracellular pH values between 7.5 – 8, and alkaliphiles exhibit cytoplasmic pH values between pH 8.2 – 9 (Kashket, 1981; Brink and Konings, 1982; Hackstadt, 1983; Guffanti *et al.*, 1984). Alkaliphilic microorganisms require cellular adaptations to avoid alkalization of their cytoplasmic pH, in order to keep their intracellular functions, such as DNA replication processes and protein synthesis, working properly. The importance of maintaining the cytoplasmic pH at near-neutral pH values could be observed as decreasing growth rates of alkaliphiles correlating with increasing cytoplasmic pH (Sturr *et al.*, 1994). Furthermore, alkaliphilic microorganisms have structures outside the cell membrane such as cell wall, outer membrane and flagella that are in direct contact with the external pH and thus must be able to function at high pH values (Kroll, 1990). The mechanisms that alkaliphiles use to regulate their internal pH are described below and are summarised in Figure 1.4.

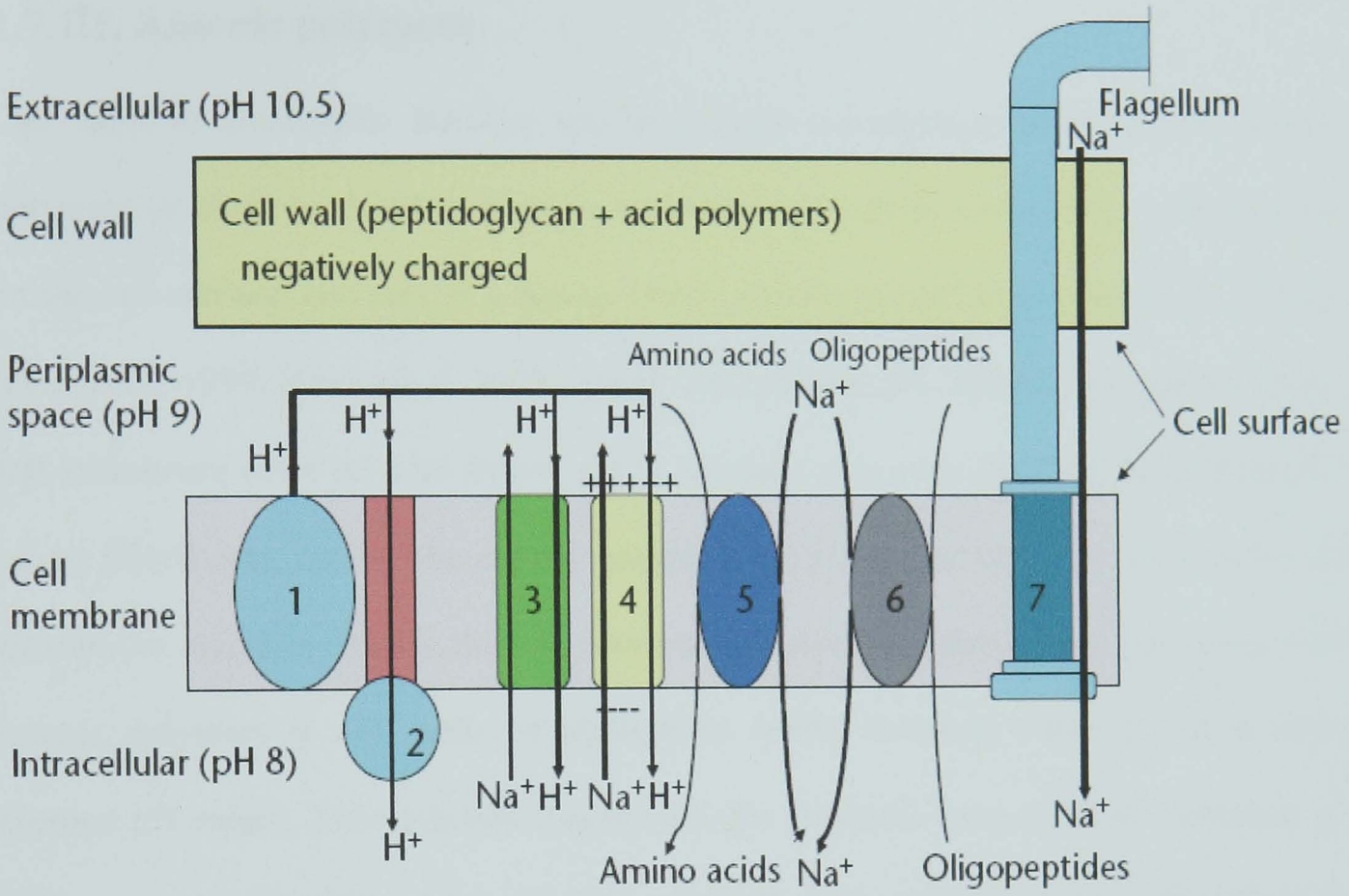


Figure 1.4: Schematic representation of cytoplasmic pH regulation. Taken from, Horikoshi (2008).

Where:

1. Respiratory chain

2. F_1 F_0-ATPase

3. ΔpH-dependent Na^+/H^+ antiporter

4. $\Delta\psi$- dependent Na^+/H^+ antiporter

5. Amino acids/ Na^+sympoter

6. Oligopepetides/ Na^+sympoter

7. Flagellar motor (mot)

1.7.1. Passive Mechanisms for Cytoplasmic pH Regulation in Alkaliphiles

1.7.1.1. Anionic polymers

Cell walls of alkaliphilic *Bacillus* species include a peptidoglycan layer with acidic polymers (teichoic acid and teichuronic acid) which serves as a negatively charged area on the cell surface, and acts as a barrier which reduces the pH value at the cell surface (Horikoshi, 1999; Aono *et al.* 1999; Tsujii, 2002; Horikoshi, 2008). The surface of the cell membrane must be kept below pH 9, because it is very unstable at alkaline pH values (Horikoshi, 2008). The anionic polymers are present in higher concentrations in alkaliphilic *Bacillus* species than in neutrophlilic *Bacillus* species and the amount of anionic polymers in cell walls of alkaliphilic strains increases when grown at high external pH values. This indicates that the anionic polymers serve a passive function in pH homeostasis and Figure 1.5 shows a schematic representation of the cell wall of a Gram-positive bacterium (Tsujii, 2002).

Evidently, the anionic polymer layer works by fixing anions to the polymer chains and consequently, positive ions are then bound in the aqueous part of the layer till equilibrium point is achieved.

1.7.1.2. Membrane fatty acids

Independent analyses confirmed that the fatty acid composition of the cell membrane plays a key role in protecting the cell from alkaline environments and moreover, both Gram-positive and Gram-negative alkaliphilic bacteria have been shown to possess very similar whole-cell fatty acid profiles (Hicks and Krulwich, 1995; Banciu *et al.*, 2005).

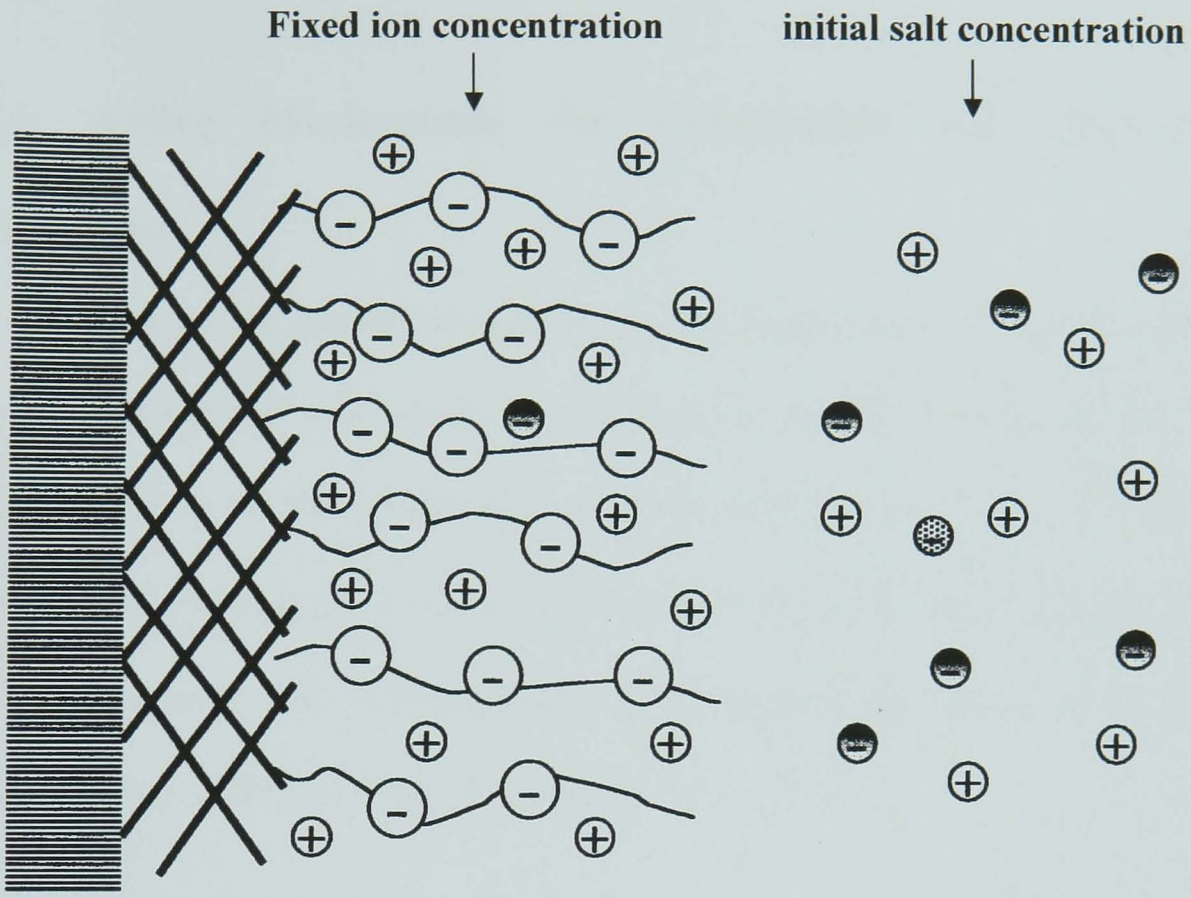


Figure 1.5: Structural model for the cell wall of Gram-positive bacteria shows plasma membrane (A) and many anionic polymer chains are situated in a brush-like shape on the surface of peptidoglycan layer (B). Added salt in the bulk aqueous phase enters partly into the anionic polymer layer (C) and equilibration is attained. The equilibrated salt concentration inside the polymer layer is much smaller than that in bulk aqueous phase because of the very high concentration of cations (sodium ions) in the polymer layer. Taken from, Tsujii (2002).

Growth in alkaline conditions favours the production of saturated or mono-unsaturated fatty acids containing either 16 or 18 carbons (Ma *et al*, 2004a).

1.7.2. Active Mechanisms for Cytoplasmic pH Regulation in Alkaliphiles

The most important active mechanism in pH homeostasis is the function of cell membrane associated with proteins that are able to catalyze an inward proton transport. Cell membranes of alkaliphiles play a crucial physiological role in maintaining pH homoeostasis by using Na^+/ H^+ antiporter system ($\Delta\psi$ dependent and ΔpH dependent), K^+/ H^+ antiporter and ATPase-driven H^+ expulsion (Horikoshi, 1999 and 2008; Krulwich *et al.*, 1997 and 1998; Wutipraditkul *et al.*, 2005).

1.7.2.1. Na^+/ H^+ antiporter

The first report of a bacterial Na^+/H^+ antiporter was in 1972 by Harold and Papineau in *Streptococcus faecalis*, and then West and Mitchell (1974) observed that the addition of Na^+ to an anaerobic cell suspension of *E. coli* showed proton extrusion. Beck and Rosen (1979) reported direct evidence of Na^+/ H^+ antiporter activity when they demonstrated the obligate coupling between Na^+ and H^+ movements, i.e. Na^+-dependent translocation of H^+ and H^+-dependent translocation of Na^+. It is important to clarify the nature and characteristics of cation/proton antiporters and Na^+/ H^+ antiporter in particular, because they are known to play a variety of important physiological roles, including pH homeostasis, regulation of cell volume, and generation of a Na^+ motive force, in both

prokaryotes and eukaryotes under alkaline conditions (Krulwich *et al.,* 1994; Brett *et al.,* 2005; Padan *et al.,* 2005). The Na^+/ H^+ antiporter does not function alone in pH homeostasis, but it was shown to maintain the cytoplasmic pH of *Bacillus firmus* at 2- 2.3 units below external pH (Krulwich *et al.*, 1998).

The Na^+/ H^+ antiporter that catalyzes net proton accumulation in the cytoplasm is powered by the Δp created by respiratory electron transport and/or by the proton-translocating F_1F_0-ATPase localized in the cytoplasmic membrane (Figure 1.4). Alkaliphilic bacteria require an effective way of cycling Na^+ back into the cell to keep the antiporter functioning and two mechanisms that are important are Na^+/solute symporters and Na^+-channels (Ito *et al.*, 2004). The Na^+/solute symporters play important roles in both pH homeostasis, by providing a route for Na^+ re-entry to keep a Na^+/ H^+ antiporter operating, and in nutrient uptake (Krulwich and Guffanti, 1989b; Ito *et al.*, 2004). Na^+-channels have non-pH related primary functions and are only important for pH homeostasis-related Na^+-uptake efficiency when Na^+-concentrations are low (Ito *et al.*, 2004).

1.7.2.2. ATPase and respiratory chain components

Respiration is remarkably effective in aerobic alkaliphilic bacteria at providing energy for regular cellular functions such as biosynthesis of molecules and motility and also for pH homeostasis at high pH. However, the proton accumulation process in the cytoplasm of alkaliphilic bacteria at high external pH values leads to a reversed ΔpH which

dramatically reduces the Δp available for ATP synthesis (Krulwich *et al.*, 1998). A much larger ΔΨ is generated to offset the reversed ΔpH, but the overall Δp is still very low. Therefore, it would make sense for alkaliphilic bacteria to possess a Na^+-coupled F_1F_0-ATPase, as found in a number of marine bacteria (Krulwich *et al.*, 1998), but numerous studies confirmed that only H^+-coupled F_1F_0-ATPases are present in alkaliphilic bacteria (Hicks and Krulwich, 1990; Hoffmann and Dimroth, 1991). Therefore, alkaliphilic bacteria require other adaptations in order to maintain efficient ATP synthesis. One of these adaptations is rapid recycling of protons to reach the ATPase directly rather than going into the bulk phase in the periplasm or cell wall (Krulwich *et al.*, 1998). An additional adaptation is to raise levels of cytochromes in the cell membrane and to use cytochromes with a lower redox potential (Hicks and Krulwich, 1995).

1.8. Industrial Applications of Alkaliphiles

Modern biotechnology has a steadily increasing requirement for new biocatalysts (Elend *et al.*, 2006). Therefore, extremophilic microorganisms that are adapted to survive in ecological niches such as at high temperatures, extremes of pH, high salt concentrations and high pressure have attracted considerable attention as a source of novel enzymes for industrial applications due to their unusual physiology and ecology (Niehaus *et al.*, 1999). These microorganisms produce unique biocatalysts (extremozymes) that function under extreme conditions comparable to those prevailing in various biotechnological applications (Margesin and Schinner, 2001; Rawlings, 2002). For example, microorganisms that can grow and survive at extreme pH values produce extremozymes that are useful for many industrial applications under either highly alkaline or highly

acidic reaction conditions. Both alkaliphilic and acidophilic microorganisms maintain their internal pH near neutrality and therefore the intracellular enzymes of these microorganisms do not need to adapt to extreme pH conditions (van den Burg, 2003; Ulukanli and Digrak, 2002). However extracellular enzymes of alkaliphiles and acidophiles do have to function at high or low pH respectively (van den Burg, 2003).

Alkaliphilic *Bacillus* strains are particularly significant for their unique enzymes that are stable over a wide range of pH values such as cellulases, proteases, lipases and amylases, these enzymes have attracted several applications and many such enzymes have already been commercialized. (Horikoshi, 1999; Takami and Horikoshi, 2000 Ulukanli and Digrak, 2002) One major application is in the modern biological detergent industry using alkalitolerant enzymes that have usually been obtained from alkaliphilic or alkalitolerant bacteria (Horikoshi, 1991b). The current proportion of total world enzyme production destined for the laundry detergent market comfortably exceeds 30 % (Horikoshi 1996; Grant, *et al.*, 1990) and there are also possible applications in pharmaceutical, food and waste treatment industries, (Grant *et al.*, 1990; Horikoshi, 1991b amd 1996: Zhilina and Zavarzin, 1994; Enomoto and Koyama, 1999; Saeki *et al.*, 2007). Furthermore, alkaliphilic microorganisms may be useful in these industrial processes which are not performed at alkaline pH e.g. antibiotic production (Grant *et al.*, 1990; Kroll, 1990; Ulukanli and Digrak, 2002).

1.8.1. Alkaline Proteases

Microbial proteases have received a great deal of attention in a variety of applications due to their stability at high temperature and pH (Gupta *et al.*, 2002; Joo *et al.*, 2002;

Kocabiyik and Erdem, 2002). Most of these proteases find applications in the food industry, in the brewing, baking, meat tenderization process, dairy industry and peptide synthesis, for infant formula preparations (Genckal and Tarib, 2006; Kocabıyık and Erdem, 2002; Ibrahim *et al.,* 2007) Furthermore, they are used in pharmaceutical industry, medical diagnosis, detergent industry, and in the dehairing process for leather production (Ito *et al.,* 1998; Gessesse *et al.,* 2003; Genckal and Tari, 2006; Saeki *et al.,* 2007).

1.8.2. Alkaline Amylases

Horikoshi, (1971) was the first to show the production of extracellular alkaline amylases in an alkaliphilic *Bacillus* species, strain No A-40-2 (ATCC 21592). This enzyme has optimum activity at pH 10 –10.5 and shows 50% of the activity remaining at pH 9 and 11.5. This enzyme hydrolyses about 70 % w/w of starch in the manner of ά-amylase (Horikoshi, 1971; Horikoshi, 2008). Recently, a Ca^{2+}- free amylase (AmyK38) requiring Na^{+} ions for its activity was discovered from an alkaliphilic *Bacillus* sp. KSM-K38 that is highly resistant to chelating reagents and chemical oxidants. A haloalkaliphilic archaeon, *Natronococcus* sp. strain Ah-36, produced an extracellular amylase. The amylase exhibited maximal activity at pH 8.7 and 55°C in the presence 2.5 M NaCl. Moreover, the gene has been cloned and expressed in another halophilic archaeon *Haloferax volcanii.* However, no industrial application has been found to date (Horikoshi, 2008).

1.8.3. Alkaline Pullulanases

Extracellular alkaline pullulanase of *Bacillus* sp. 202-1 has an optimum pH at 8.5 – 9 and furthermore, is stable for 24 hours at pH 6.5 –11.0 and 4°C. On the other hand this enzyme is most active at 55°C, and is stable at up to 50°C for 15 min in the absence of substrate. A new type of alkaline pullulanase, having an optimum pH of around 10 – 10.5 was isolated from the alkaliphilic *Bacillus* sp. KSM-1876 during the course of screening for alkaline cellulases as detergent additives. The alkaline pullulanase is a good candidate to use as an additive to dish-washing detergents, but this application has not yet been developed (Horikoshi, 2008).

1.8.4. Other Enzymes

Several other enzymes from alkaliphilic or alkalitolerant organisms have been studied but have not yet found commercial applications. One of them is an alkaline ß-1, 3-glucanase, which is active at pH values higher than 8. Many extracellular alkaline lipases have also been isolated from a variety of different alkaliphiles. These enzymes may be used as additives to detergents in the near future (Horikoshi, 2008).

1.9. Molecular Biology Techniques

The investigations of prokaryotic biodiversity has been hindered for long time because of the difficulty of assessing the true microbial diversity by microscopic and cultural methods. It has been reported that less than 0.1% of the total microbial population of a natural habitat can be successfully isolated in pure culture. This is due to the bacteria being highly selective with their growth requirements. Hence, a variety of media must to be employed to obtain diverse microbial populations (Hill *et al.,* 2000).

Therefore, every process which avoids the need to cultivate microorganisms before examining their characteristics and diversity, will have the potential to become a powerful tool in microbial ecology (Marilley *et al.,* 1998). Compared to traditional visual and microscopic diagnostic methods, molecular methods have a higher specificity and sensitivity and therefore may be useful to find infections pre-symptoms and identify the target diseases without time-consuming isolation and culturing of pathogens (Martin *et al.* 2000).

Characterization and identification of an organism by molecular techniques requires only a gene sequence and not a functioning cell. Commonly 16S rRNA genes or gene fragments are used that can be selectively amplified by PCR from total genomic DNA obtained directly from environmental samples without the need to cultivate microorganisms. Figure1.6 shows the sequential steps required to characterize an environmental sample by comparative rRNA analysis (Amann *et al.,* 1995).

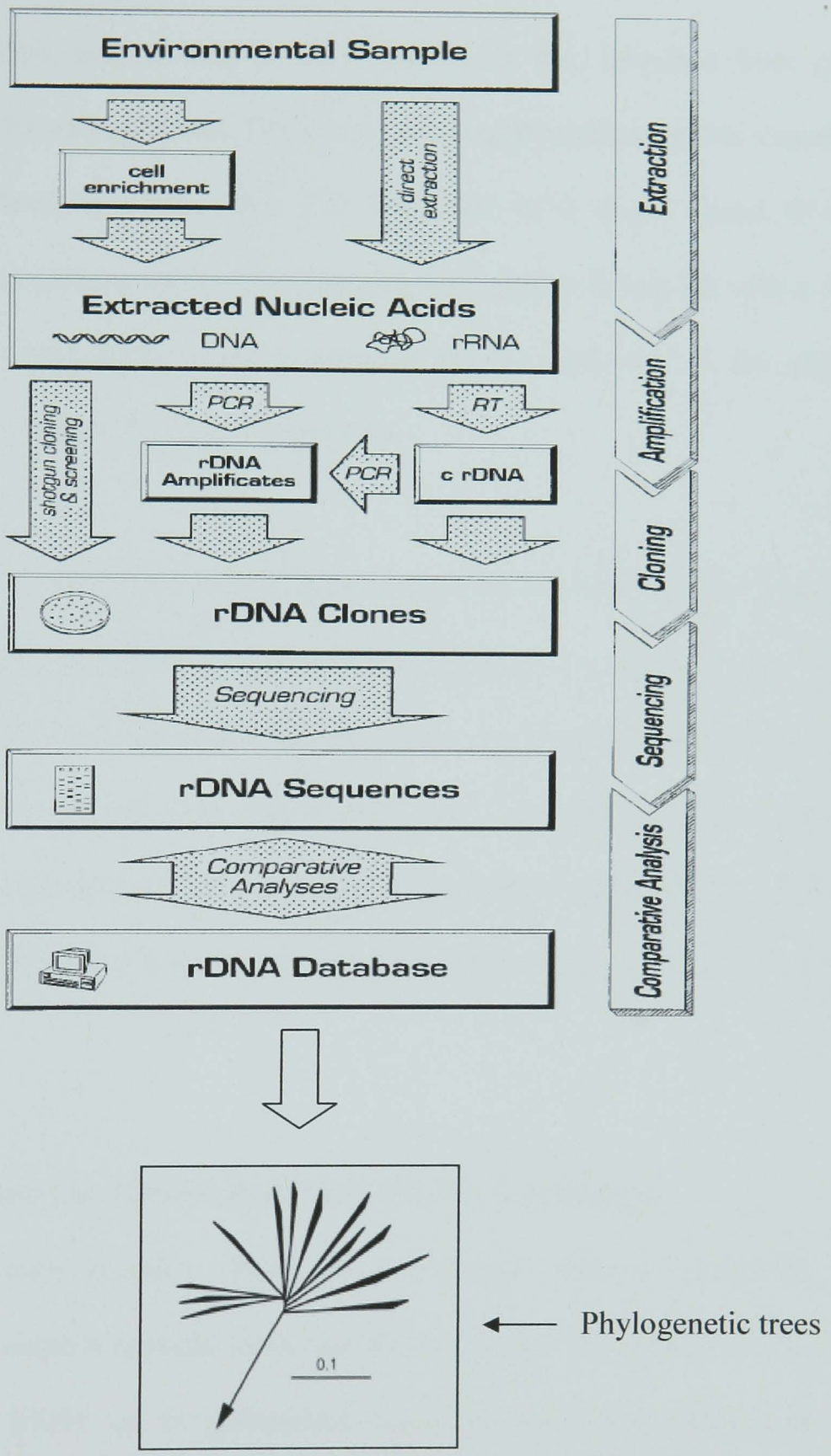


Figure1.6: Flow chart showing the different possibilities to characterize an environmental sample by comparative rRNA sequence analysis. Modified from, Amann *et al.*, (1995).

1.9.1. Deoxyribonucleic Acid (DNA)

Deoxyribonucleic acid (DNA) is the molecule of life, inherited from generation to generation in living organisms. DNA is a very long threadlike double stranded molecule made from deoxyribonucleotides. The backbone of a single strand DNA molecule consists of deoxyribose molecules (nitrogen base molecule bonded with a ribose sugar) linked by phosphodiester bridges between the 3′- hydroxyl of the sugar with 5′- hydroxyl of the adjacent sugar (Figure 1.7).

The building blocks of the DNA chemical structure are four nitrogen bases i.e. guanine (G), adenine (A), thymine (T) and cytosine(C). The two strands of the double helix of the DNA run in opposite directions. However, the sugar-phosphate backbone of each strand forms the outside of the double helix, while the nitrogen bases are the inner part. Furthermore, each pair of nitrogen bases is connected together by hydrogen bounds i.e. the thymine is always linked with adenine, whereas guanine is always linked with cytosine.

1.9.2. Polymerase Chain Reaction (PCR) Technique

Polymerase chain reaction (PCR) is a powerful tool in molecular biology to exponentially amplify specific DNA sequences (Lexa *et al.,* 2001; Fenollar *et al.,* 2006; Yeung *et al.,* 2009), i.e. by polymerase chain reaction, large amounts of target DNA sequences can be amplified from tiny amounts of DNA (Figure 1.8) (Vierstraete, 1999).

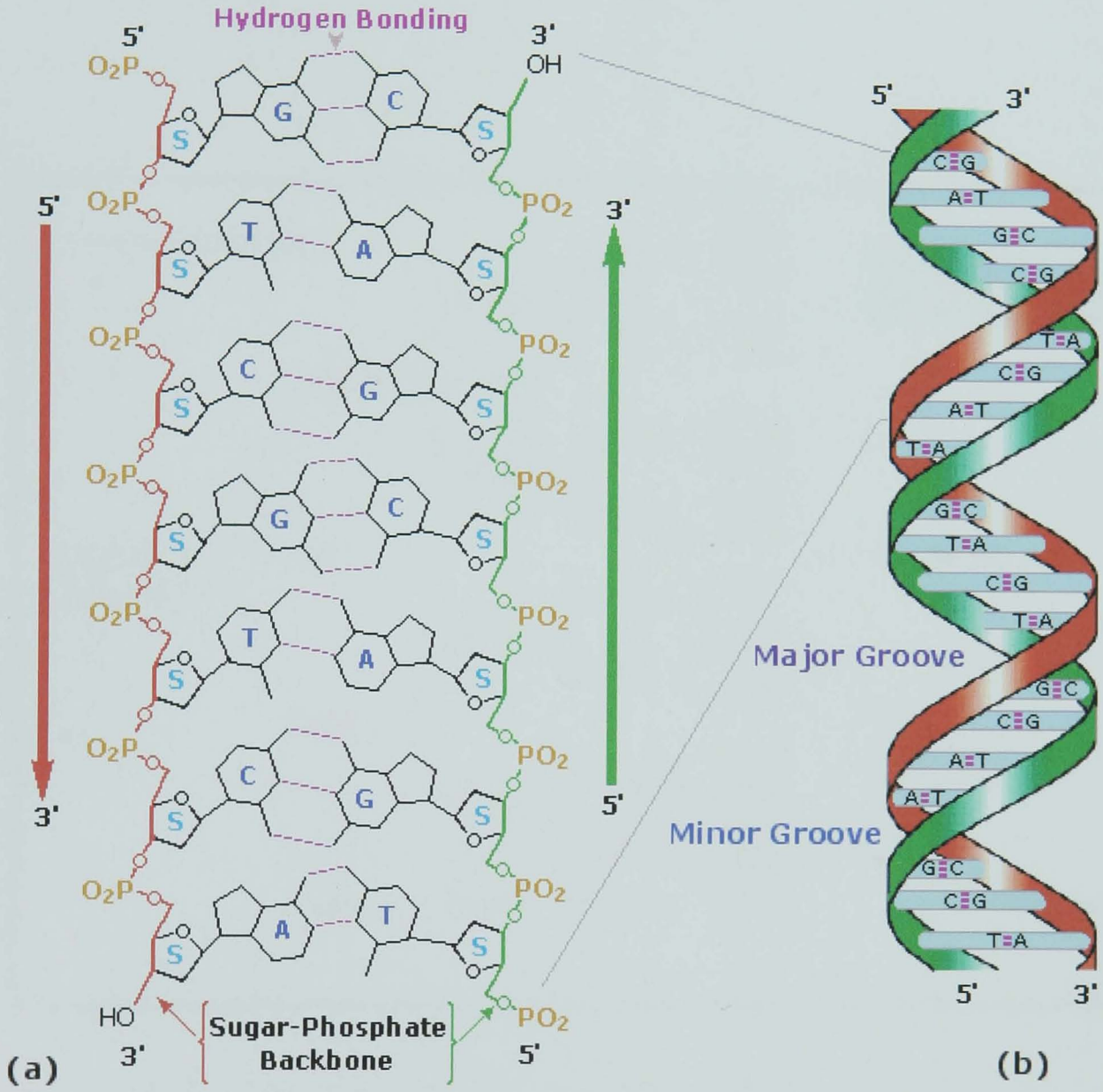


Figure 1.7: Structure of double stranded DNA illustrating the sugar-phosphate backbone of each strand is on the outside of the double helix, but the nitrogen bases are forming the inside part. Each pair of nitrogen bases are held together by hydrogen bounds. The base pairs links, as adenine always paired with thymine and cytosine with guanine in double stranded DNA molecule. From www.cem.msu.edu/~reusch/VirtualText/nucacids.htm - 59k

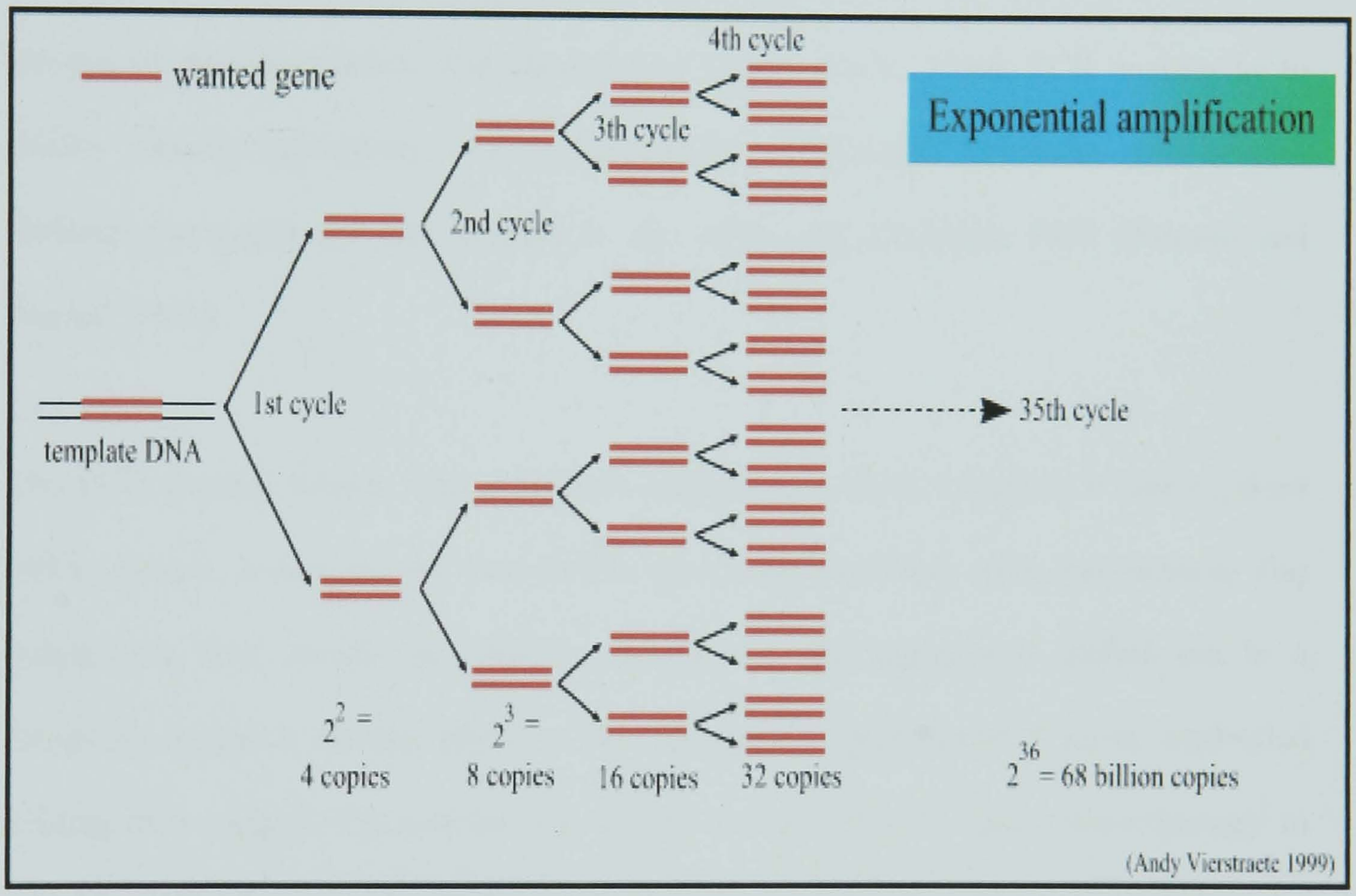


Figure1.8: Scheme of exponential amplification of DNA copies of the target region by polymerase chain reaction. Taken from, Vierstraete (1999). http://allserv.rug.ac.be/~avierstr/principles/pcr.html.

As described in Saiki *et al.,* (1985), Kary Mullis was the first to invent and introduce a polymerase chain reaction in 1983. In comparison to traditional diagnosis methods, PCR has a number of advantages: first, PCR is a molecular biological method for amplifying DNA without using a living organism i.e. organisms do not need to be cultured prior to their detection in this case. Second, this technique is a highly sensitive and specific methodology for detection of nucleic acids and a useful tool for quantification of the amount of specific nucleic acid present in a target sample. Third, PCR enables us to swiftly identify pathogens without knowledge of complex taxonomy and several different pathogens can be detected in one tube using multiplex PCR (Henson and French, 1993).

The PCR process begins with a mixture containing a DNA template, a pair of short DNA primers, a pool of the four dNTPs, and a heat-resistant DNA polymerase, *Taq* polymerase (*Taq* stands for *Thermus aquaticus*). The reaction is carried out in a computer-regulated heating block, a thermal cycler, which permits rapid, controlled heating and cooling. The primers are chosen so that they are base-complementary to opposite ends ($\grave{3}$ end and $\grave{5}$ end) of either strand of a short stretch of DNA containing the gene region of interest. The reaction is first heated to 94°C to denature the double-stranded DNA into two single strands. This is followed by a lower temperature step (45-65°C), at which temperature the primers anneal to their respective complementary DNA sequence in the template. The reaction is finally heated to 72°C, at which temperature the *Taq* polymerase replicates the primed DNA template (extension) using the dNTPs (*Taq* enzyme exhibits relative stability at DNA-melting temperatures, which eliminates the need for enzyme replenishment after each cycle of synthesis i.e., reduces PCR costs

and allows automated thermal cycling). At the end of one cycle, the region between the two primers has been copied once, producing two copies of original gene region (Figure 1.8). Normally PCR consists of twenty to thirty cycles, each cycle includes the three essential steps: melting of target DNA (denaturation), annealing of two oligonucleotide primers to the denatured DNA strands, and primer extension by a thermostable DNA polymerase (Henson and French, 1993). The full details of the PCR method are as follows:

1. The double-stranded DNA has to be heated to 94 – 96°C in order to separate the strands. This step is called melting; it breaks apart the hydrogen bonds that connect the two DNA strands. Prior to the first cycle, the DNA is often melted for an extended time to ensure that both the template DNA and the primers have completely separated and it is now single-strand only, this cycle requires 1 to 2 minutes.

2. After separating the DNA strands, the temperature is lowered so the primers can attach themselves to the single DNA strands. This step is called annealing. The temperature of this stage depends on the primers and is usually 5°C below their melting temperature and within the range of 45 – 60°C. A wrong temperature during the annealing step can lead to primers not binding to the template DNA at all, or binding at random. The time required for this step is between 1 to 2 minutes.

3. The DNA-Polymerase has to fill in the missing strands. It starts at the annealed primer and works its way along the DNA strands. This step is called elongation. The elongation temperature depends on the DNA polymerase. The time for this step depends both on the DNA polymerase itself and on the length of the DNA fragment to be amplified.

However, the success of PCR analysis is highly dependent on the selection of appropriate primers (Lexa *et al.,* 2001). A useful primer must have various specific properties; it must be specific to the region that needs to be replicated, it must join with enough energy to support the experimental conditions and it must not allow the formation of structures that might weaken the reaction. Several cycles of amplification are performed in the same vial so that enough copies of the desired sequence are reproduced for analysis (Figure 1.9).

1.9.3. 16S ribosomal RNA Gene (16S rRNA gene)

Since the 1980s, 16S rRNA gene sequencing has been used to detect possible phylogenetic relationships between different bacteria to produce a molecular based classification of bacteria from various sources, such as environmental or clinical specimens (Cai *et al.,* 2003; Mignard and Flandrois, 2006 ; Clarridge, 2004).

Carl Woese during the 1970's and 1980s developed the idea that molecular sequences of highly conserved molecules could be used to detect possible phylogenetic relationships between bacterial groups (Pace, 1997). Olsen *et al.,* (1986) reported that three types of rRNA molecules are found in the microbial ribosomes including, 5S rRNA, 16S rRNA (~1600 nucleotides) and 23S rRNA (3000 ~ nucleotides). The first attempts to use rRNA to characterize microbes were carried out by extracting the 5S rRNA molecules. However, the information content in the approximately 120-nucleotide long molecule is relatively small and therefore it was abandoned in favour of the 1,500 nucleotide long 16S rRNA gene.

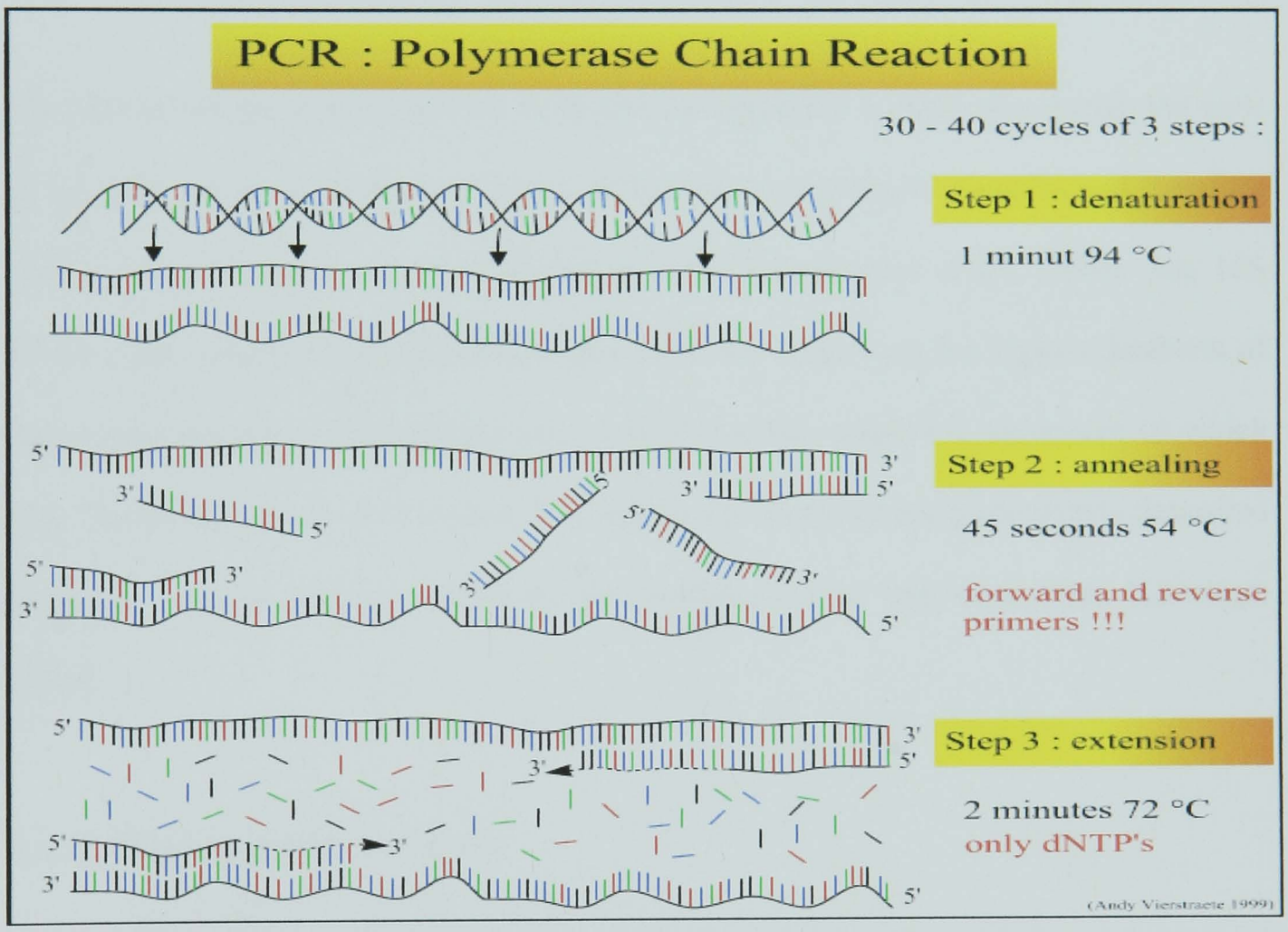


Figure 1.9: Schematic diagram illustrating the three processing steps of PCR (Vierstraele, 1999), ugent.be/~avierstr/principles/pcr

16S rRNA gene sequence is composed of both variable and conserved regions which often contain a high degree of species specificity. The gene is large enough, with statistically relevant sequence information (Figure1.10). 16S rRNA gene is also suitable for phylogenetic studies of distantly related organisms and for the analysis of phylogenetic relationship between phyla and families (Amann *et al.,* 1995).

The 16S rRNA gene is suitable for PCR and consequently is easily sequenced, the ends of the gene are highly conserved across all bacterial and archaeal domains and therefore almost the entire gene can be amplified by PCR (Giovanonni *et al.,* 1990). The 16S rRNA gene sequence has been determined for many strains and the biggest databank of nucleotide sequences (Gen Bank) has up to 20 million deposited sequences of which over 90,000 are of 16S rRNA gene. This means that there are many previously deposited sequences against which to compare the sequence of an unknown strain (Clarridge, 2004).

1.9.4. Phylogenetic Analyses

Construction of phylogenetic trees has become a very useful tool for gathering all information regarding the evolutionary relationship between the newly obtained sequences and analysis of the historical evolutionary relationships between different groups of organisms (Olsen, 1987; Cantarel *et al.,* 2006). Phylogenetic trees play a fundamental role in allowing us to observe how closely or distantly a particular DNA sequence relates to other sequences, and allows the characterisation of organisms on the basis of sequence similarity (Bull and Wichman, 2001).

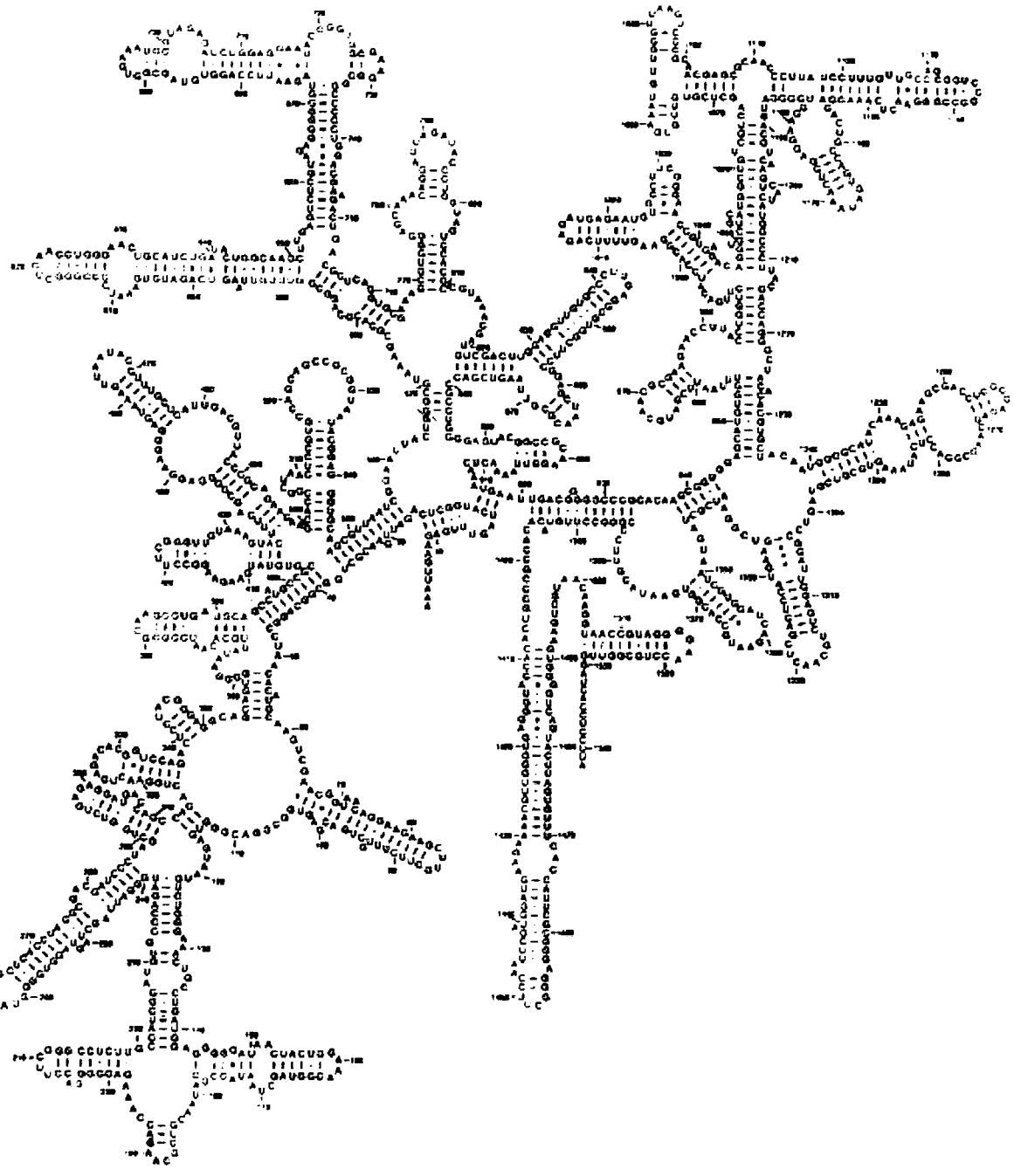


Figure 1.10: Secondary structure of 16S rRNA gene sequencing (used with the permission of Applied Biosystems, Forest City, CA). Taken from, Patel (2001).

Thus phylogenetic analyses are based on the comparison of the ribosomal sequences with previously identified ones, that are already obtainable in large databases accessible worldwide (Maidak *et al.*, 1999).

1.10. Aims of Project

Extremophilic microorganisms are now known to be widespread and are often found in environments that are not extreme (Grant *et al*, 1990; Horikoshi, 1999 and 2008). In an effort to isolate unusual alkaliphilic bacteria (i.e. bacteria with optimum growth at pH 9 or above), liquid and sediment samples were taken from two rivers in the Derbyshire Peak District. These rivers are not extreme environments and our aim is to isolate an extremophile from a not extreme habitat. Enrichment cultures of river water were set up at pH 10 to select for alkaliphilic bacteria.

The bacterial strains isolated in pH 10 medium were further characterised to categorise them as alkaliphilic or alkalitolerant (Chapter 3). Two strains were identified using 16S rDNA sequencing (Chapter 4) and the bioenergetics of growth of the two species was examined in Chapter 5. In the final results chapter, the role of Na^+ and K^+ in supporting growth at pH 10 was examined and a range of enzyme activities were measured to better understand the physiology of alkaliphilic bacteria.

Materials and Methods

Chapter Two

2.1. Sample Collection

A set of ten samples were collected from two unpolluted rivers in the Derbyshire Peak District about 20 miles south west of Sheffield. Samples 1 to 5 were collected on the 1/1/06 from the edge of the River Bradford between the villages of Youlgreave and Alport, and samples 6 to 10 were collected on 2/1/06 from the edge of the River Lathkill between the village of Over Haddon and the Conksbury Bridge. As much sediment as possible was collected along with the river water into autoclaved Bijou bottles. On returning to the laboratory, the pH of each sample was measured using a Mettler Toledo MP225 pH meter.

Each sample was given a designated name starting with my initials (i.e. MA) and either B for Bradford River or K for Lathkill River e.g. MAB1, MAB4, MAK7 and MAK10 and then was stored in the fridge at 4°C until required.

2.2. Isolation and Selection of Alkaliphiles

Isolation of alkaliphilic microorganisms was carried out using Horikoshi medium (liquid and plates) which contained the ingredients shown in Table 2.1 in section 2.4.1 with pH values 7 – 10 (Horikoshi, 1999). Initial isolation of alkaliphilic microorganisms was carried out as follows:

Each river sample was thoroughly mixed and then 5 ml of water and sediment was added into 10 x 250 ml conical flasks plugged with cotton wool containing 50 ml of pH 9 Horikoshi medium. All flasks were then placed on a rotary shaker at 250 rpm in the 25°C room and left to incubate overnight. The river samples were again thoroughly

mixed and then 0.5 ml from each was aseptically spread on Horikoshi medium pH 9 plates in duplicate which had been dried in the oven at 50°C for 20 minutes. One set of plates was incubated at 25°C and the other set at 30°C. 1 ml from each liquid culture which grew at pH 9 and 25°C was inoculated into separate 250 ml conical flasks plugged with cotton wool containing 50 ml of pH 10 Horikoshi medium and then incubated at 25°C on a rotary shaker at 250 rpm overnight.

Each flask of pH 10 Horikoshi medium from the overnight cultures at 25°C that showed growth was streaked out on the pH 10 Horikoshi medium plates in duplicate, and then returned (both flasks and plates) to incubate at 25°C. Routine sub-culture and streak plating techniques as necessary were repeated several more times at high pH values until single colonies were obtained which showed good growth at pH 10.

2.3. *Bacillus* Isolate

During the work a contaminant appeared in cultures of MAK7 in pH 10 Horikoshi medium and was subsequently purified as described in section 2.5, and then was identified as an alkaliphilic strain of the genus *Bacillus*.

2.4. Media Used and Growth Conditions of Alkaliphiles

All media were prepared using distilled water and unless stated otherwise sterility was achieved by autoclaving for 20 min at 121°C (15 lbs in^{-1}).

2.4.1. Horikoshi Medium: This medium was prepared using a slightly modified method as described by Horikoshi, (1999) in Table 2.1 below:

Ingredient	g litre^{-1}
Yeast extract	5
Tryptone	5
Glucose	10
KH_2PO_4	1
$Mg_2SO_4.7H_2O$	0.2
Na_2CO_3	10

Table 2.1: *Composition of normal Horikoshi medium for alkaliphilic microorganisms*

To produce 1 litre of the Horikoshi medium at different pH values, all the ingredients shown in Table 2.1, with the exception of glucose, $KH_2\ PO_4$ and Na_2CO_3, were added and dissolved in 700 ml of distilled water in a 1 litre Duran. Then 50 ml of 1 M solution either HEPES buffer (for pH 7 and 8.5) or CAPS buffer (for pH 10) was added and gently mixed to give 750 ml of main solution. Two separate solutions were prepared by adding 10 g of Glucose and 1 g of KH_2PO_4 to 100 and 50 ml of distilled water respectively. A separate solution was prepared by adding 10 g of sodium carbonate to 80 ml of distilled water, the pH was adjusted as necessary using either 1 M H_2SO_4 and or 1 M NaOH and the volume was made up to 100 ml with distilled water. All four solutions were separately autoclaved, this is necessary to avoid the precipitation and caramelisation (brown colour) of the media at high pH. Once cooled the four solutions were combined aseptically and gently inverted to mix and give 1 litre of Horikoshi

liquid medium. The pH value of liquid media was checked after autoclaving and shown not to alter by more than 0.05 pH units.

The same procedure mentioned above was employed when Horikoshi solid medium was required, but in this case 20 g of bacteriological agar no.1 was added to the main solution before making it up to 750 ml with distilled water. After autoclaving all solutions were allowed to cool in a water bath to approximately 50°C before the addition three solutions to the main medium to provide 1 litre of Horikoshi solid medium. Then plates were poured and allowed to solidify at room temperature and then stored in the fridge until required.

2.4.2. M 9 minimal Medium

Ingredient	Weight / distilled water
M 9 minimal salts (Sigma M-6030)	10 g in 980 ml of distilled water
Glucose	9 ml of (15 g in 45 ml)
NH_4Cl	9 ml of (5 g in 45 ml)
$MgSO_47H_2O$	1 ml of 1 M $MgSO47H_2O$
$CaCl_2$	1 ml of 100 mM $CaCl_2$

***Table 2.2**: Composition of M 9 Minimal medium*

M9 minimal medium was prepared by dissolving 10 g of M9 minimal salts in 900 ml distilled water, then the pH was adjusted to10 using either 1 M H_2SO_4 and or 1 M NaOH and the volume was made up to 980 ml with distilled water and autoclaved. Other ingredients were also separately dissolved in distilled water as shown in Table 2.2 and

then autoclaved. After autoclaving, all solutions were allowed to cool to approximately 50°C, combined aseptically and gently inverted to mix and give 1 litre of basal M 9 minimal medium. The pH value of liquid media was checked after autoclaving and shown not alter by more than 0.05 pH units.

2.4.3. Luria-Bertani (LB) Medium:

Luria-Bertani (LB) medium consisted of 5 g yeast extract, 10 g tryptone, 10 g NaCl in 1000 ml of distilled water to produce liquid medium. For solid medium 15 g of bacteriological agar no.1 was added per litre. To produce a selective medium, filter sterilised ampicillin was also added to Luria-Bertani (LB) medium after autoclaving, either at 100 $\mu g\ ml^{-1}$ in solid medium or at 50 $\mu g\ ml^{-1}$ in liquid medium.

2.5. Purity of the Strains of Alkaliphiles

The purity of strains was monitored by streaking a loopful of the culture on agar plates of pH 10 Horikoshi medium. After incubation the plates were first examined for colony morphology and then single colonies were picked off and observed as a wet mount using a phase contrast microscope.

2.6. Maintenance of the Alkaliphilic Strains

All isolated strains were maintained by adding 2 ml of overnight culture (active inoculum) to 2 ml of sterile glycerol (50% v/v) and kept in a freezer at – 20°C. When required, the frozen cells were thawed at room temperature and inoculated into 250 ml conical flasks plugged with cotton wool containing 50 ml of Horikoshi medium of the

same pH, and incubated overnight at 25°C on a rotary shaker at 250 rpm. In addition, each strain of alkaliphilic bacteria was maintained by streaking on three pH 10 Horikoshi medium plates and then incubated for 24 – 48 h at 25°C once every two months. When pure colonies had developed on the plates a piece of parafilm was stretched around each plate's edge and then one set of plates was stored at room temperature and the other set in the fridge until required. Sub-culturing of the strains was also performed routinely once every two weeks to minimize contamination over time by adding 1 ml of overnight culture into 250 ml conical flasks containing 50 ml of Horikoshi medium of the same pH and incubated at 25°C on a rotary shaker at 250 rpm and then kept at room temperature until required.

2.7. Identification of Alkaliphiles

2.7.1. Cell Morphology

Gram stain was conducted on the overnight cultures and checked under the light microscope (Kirkpatrick *et al.*, 1993). A droplet of water was placed onto a slide and an inoculating loop was used to transfer some cells into the water droplet. The slide was allowed to dry and then was heat-fixed by passing through a Bunsen flame. The cells were then washed in an excess of crystal violet solution and then treated with iodine solution as a mordant for approximately one minute. Ethanol was then added to decolourise the stain (for about 30 seconds). The cells were then counterstained with safranin solution for 1 minute and the excess stain was removed. The slides were examined under a light microscope at 1000 times magnification (oil immersion). Gram positive cells appear purple, whereas Gram negative cells will be pink.

2.7.2. Motility of Bacteria

The motility of the bacteria was examined by using a hanging drop preparation and then viewing the slide under the light microscope (Allen and Baumann 1971).

2.8. Effect of Medium pH on Growth of Bacteria and Growth Curve Determination

The effect of pH on the growth of bacteria was determined using overnight cultures in Horikoshi medium. 1 ml of active inoculum from each strain was inoculated into Horikoshi medium pH 7, pH 8.5 and pH 10 and the optical density (OD) at 600 nm was measured using the Unicam Helisα spectrophotometer against distilled water blank in 1 ml plastic cuvettes. To produce a growth curve, 4x 250 ml conical flasks containing 50 ml of each pH value Horikoshi medium were each inoculated with 1 ml of the same pH adapted cells from an overnight culture. The OD_{600} was measured against a water blank immediately after inoculation then every two hours over an incubation period at 25°C on a rotary shaker at 250 rpm. The growth curves were plotted against time of incubation.

2.9. Growth Rate and Growth Yield of Alkaliphilic *Bacillus* sp. and MAK7 in Comparison with *Bacillus subtilis*

To produce a growth rate, 1 ml of cells from an overnight culture grown in Horikoshi medium of the appropriate pH was inoculated into 250 ml conical flasks containing 50 ml Horikoshi medium pH 7 for *Bacillus subtilis* and pH 10 for both *Bacillus* sp. and MAK7. The optical density (OD) at 600 nm was measured using the Unicam Helisα spectrophotometer against distilled water blank immediately after inoculation and then

every hour thereafter. *Bacillus* sp. and MAK7 were inoculated at 25°C and *Bacillus subtilis* was inoculated at 37°C, in all cases on a rotary shaker at 250 rpm. The growth rate was plotted against time of incubation.

To determine growth yield (bacterial dry weight), duplicate 5 ml cell samples from overnight cultures of each strain grown in Horikoshi medium were transferred into 15 ml Falcon tubes and then harvested by centrifugation in a bench top centrifuge at 3000 g for 10 minutes. The supernatant was discarded immediately and then each pellet was resuspended in 5 ml of 0.15 M NaCl and centrifuged at 3000 *g* for 10 minutes and then the supernatant was discarded. The pellets were again resuspended in 5 ml of 0.015 M NaCl and centrifuged at 3000 *g* for 10 minutes. The supernatant was poured off immediately and then each pellet was resuspended in 2 ml of distilled water and then transferred to pre-weighed aluminium caps. The caps were placed in a glass Petri dish lid, covered with a glass beaker and then dried to constant weight at 105°C overnight. The caps were allowed to cool to room temperature in a desiccator in order to minimise water absorption from the atmosphere, before being reweighed on an analytical balance that was sensitive to 0.01 g. The bacterial dry weight was calculated by subtracting the cap weight after drying from the original weight to give g dry weight per 5 ml of cells and then divided by 5 to get g ml^{-1} dry weight.

2.10. Measurement of Respiration Rate of Bacterial Cells (Oxygen Uptake)

Respiration rate was measured using a modified Clarke oxygen electrode (Hansatech Scientific Instruments, Kings Lynn, UK) with a Servoscribe 1S potentiometric chart recorder as described by Delieu and Walker (1972). Schematic drawings of the oxygen electrode unit are shown in Figures 2.1 and 2.2. The electrode consists of platinum wire sealed in plastic as the cathode and an anode of circular silver wire bathed in a saturated potassium chloride (KCl) solution which forms a bridge between the electrodes. The electrodes were separated from the reaction mixture (chamber) by an oxygen- permeable Teflon membrane. The reaction mixture in the Perspex container was stirred constantly with a small magnetic stirring rod.

When a voltage was applied across the two electrodes using the polarising meter the platinum electrode became negative with respect to the reference electrode and the oxygen in the solution is thought to undergo electrolytic reduction at the cathode.

$$4H^+ + O_2 + 4e^- \longleftrightarrow 2H_2O$$

The flow of current in the circuit when the polarising volts were set between 0.5 and 0.8 V varied in liner relationship to the partial pressure of oxygen in solution. The current flowing was measured by connecting the electrode to a sensitive potentiometric chart recorder. The reaction chamber of oxygen electrode was maintained at a constant temperature similar to the culture temperature (25°C), by circulating water from a temperature controlled water bath (Figures 2.1 and 2.2).

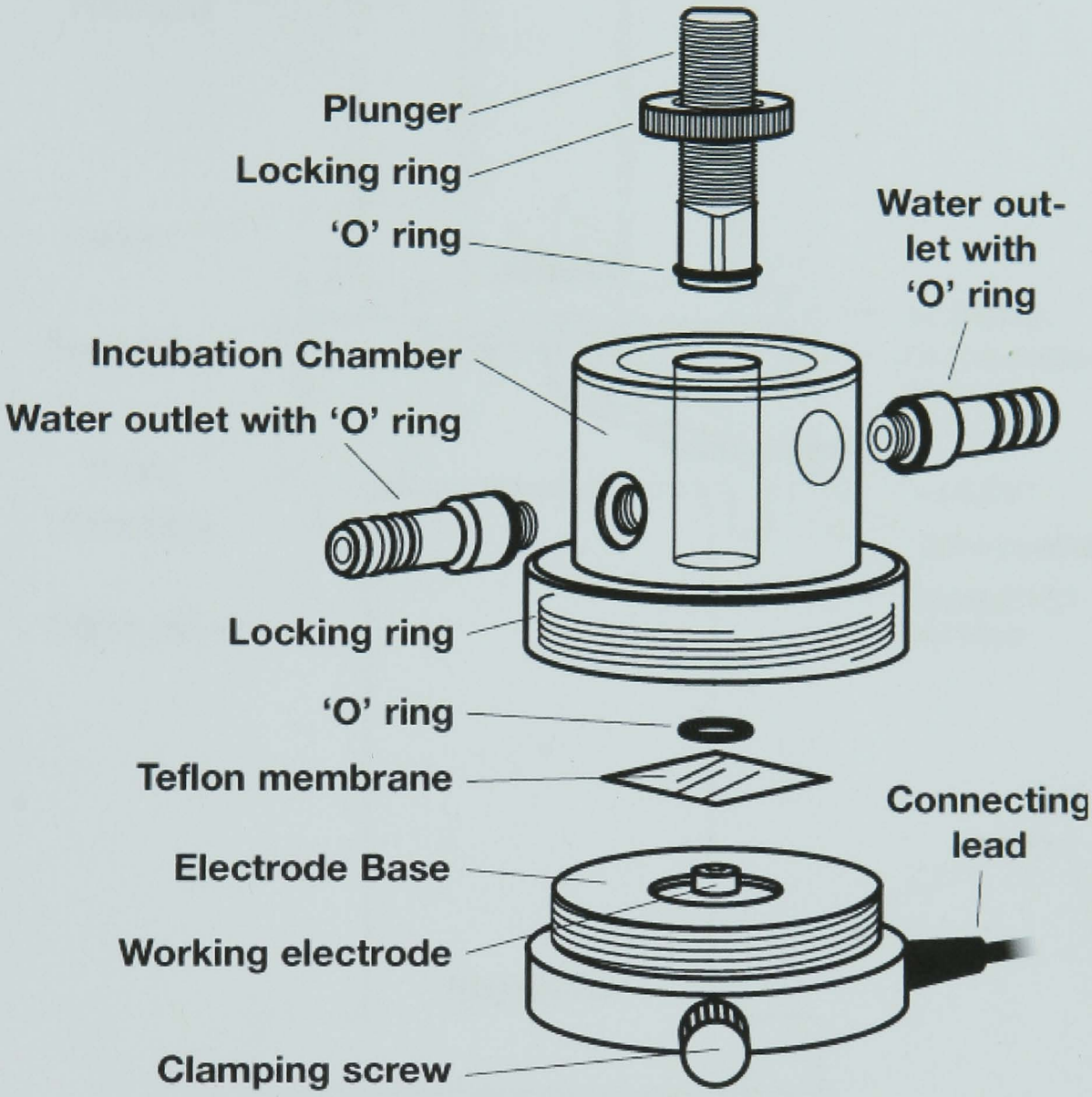


Figure 2.1: Schematic diagram illustrating the key components of an oxygen electrode. Figure from http:// www.rankbrothers.co.uk/prod 1exp.htm.

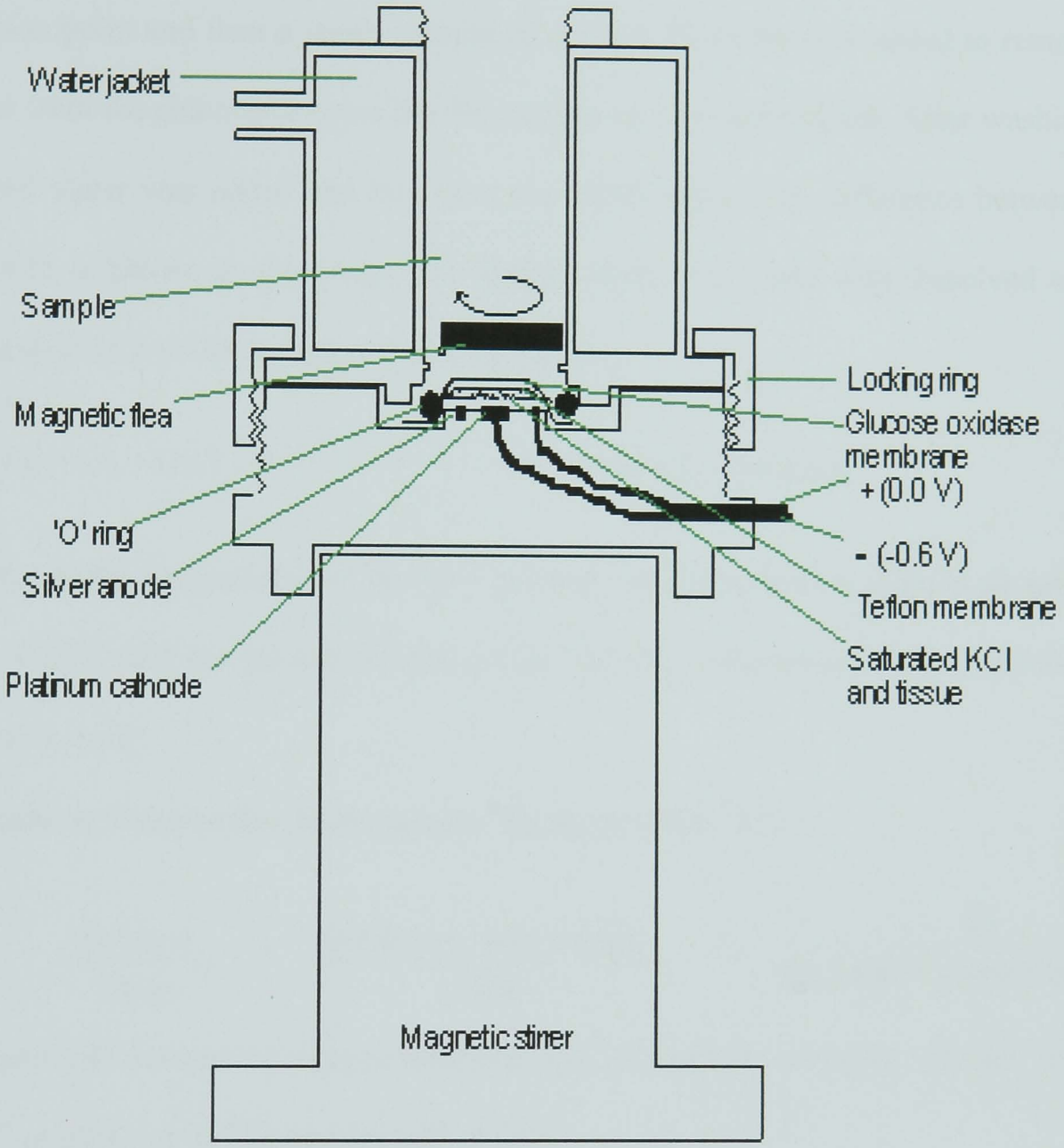


Figure 2.2: A diagram of the oxygen electrode showing more details of the electrode components. Only a Teflon membrane was used in the current work and the tissue was cigarette paper.

Figure from http:// www.lsbu.ac.uk/biology/enzyme/oxelectrode.html

The calibration of the oxygen electrode was achieved by placing 2 ml of distilled water in the reaction chamber for 15 minutes and setting the chart recorder to 100% oxygen saturation point and then a small amount of sodium dithionite was added to remove all oxygen from the chamber to give the 0% oxygen concentration point. After washing, air saturated water was added and this gave the 100% value. The difference between 0% and 100% is known as the range. The sodium dithionite reacts with dissolved oxygen and removes it from the solution as shown below:

$$Na_2S_2O_2 + O_2 + H_2O \longleftrightarrow NaHSO_4 + NaHSO_3$$

To calculate the respiration rate we need to know the concentration of O_2 in air saturated water at 25°C and the protein content of the bacterial suspension. Then the following equation is used:

O_2 uptake or Respiration Rate (µmoles O_2 mg protein^{-1} h^{-1}) =

$$\frac{\text{standard}}{\text{range}} \times \frac{\text{Number of units change}}{\text{Time}} \times \frac{60}{\text{mg protein present in sample}}$$

- **Standard:** Amount of oxygen solubility in 2 ml medium (sample) = 0.660 µmoles ml^{-1} at 30°C or 0.722 µmoles ml^{-1} at 25°C
- **Range:** Units taken from calibration (0 – 100%)
- **Number of units:** Number of units are read directly from the chart recorder, it is normal to draw best fit straight line over 5 min
- **Time:** The length of time in minutes for which the sample was measured
- **60:** This converts the time from minutes to hours
- **Protein present in sample (mg):** this relates to amount of protein in a sample of 2 ml of cells from Bradford assay (see section 2.11).

To prepare cells for measurements in the oxygen electrode, the OD_{600} of all cultures was measured and 20 ml of cells from each sample (grown overnight at pH 7, pH 8.5, and pH 10 in Horikoshi medium) were transferred to a 50 ml Falcon tube and harvested by centrifugation at 3000 *g* for 10 minutes. Each bacterial pellet was then resuspended in 10 ml of fresh Horikoshi medium of the same pH. The cells were also normally concentrated twofold and the protein content was determined (section 2.11). The viability of the cultures grown overnight was measured by adding 0.5 ml of concentrated cells to 1.5 ml of fresh medium of the same pH in the chamber of oxygen electrode (keeping the overall volume at 2 ml to allow standardisation of the results) and the plunger placed on top and was left for 5 minutes without illumination to induce oxygen uptake. The plunger was removed and the sample unloaded using a Pasteur pipette. Distilled water was used to clean both the chamber and the plunger. When the effect of increasing external salinity on the respiration rate was studied the same procedure was employed except that the washing step was carried out with sodium free pH 10 Horikoshi medium. Then the cells were resuspended in different concentrations of sodium chloride for the measurements in the oxygen electrode.

2.11. Determination of Protein Content

2.11.1. Determination of Standard Curve

A standard curve was produced using Bovine serum albumin (BSA) in the range of 0 - 100 µg by dissolving 250 mg of BSA in 50 ml of distilled water. The final concentration of protein in the stock solution was 5 mg ml^{-1} (5 µg μl^{-1}). The stock

solution was used to make a range of protein concentrations by a series of dilutions as shown in Table 2.3.

Tube number	Volume of BSA Stock solution (µl)	Volume of distilled water (µl)	Total volume in each tube (µl)	Amount of Protein (µg)
1	0	100	100	0 (Blank)
2	2.5	97.5	100	12.5
3	5	95	100	25
4	7.5	92.5	100	37.5
5	10	90	100	50
6	15	85	100	75
7	20	80	100	100

***Table 2.3**: Components in test tubes which were needed to make a standard protein curve*

Three replicates were made for each test tube above. To each test tube, 3 ml of Bradford Reagent (Sigma) was added then thoroughly mixed and left on the bench for 5 min. Optical density was measured for each sample at 595 nm using a 3 ml glass cuvette. The blank was used to zero the Unicam Helisα spectrophotometer. The protein standard curve (Appendix A) was plotted from which protein concentration for each sample can be determined. Standard error for each sample were calculated and shown on the curve.

2.11.2. Determination of Sample Protein

Soluble protein in bacterial cells was measured using the method described by Bradford (1976). Bovine serum albumin (BSA) was used as the standard as described in section 2.11.1.

Samples were prepared for protein determination as follows:

- 0.1 ml from the same concentrated cells which were prepared for respiration rate measurements (see section 2.10) was placed into a clean test tube and then 0.9 ml of 1M NaOH was added. The sample was then thoroughly mixed and heated at 90°C for 10 minutes.
- The test tube was cooled on ice for 5 minutes and dried outside and then centrifuged for 10 minutes in bench centrifuge at full speed (3000 *g*).
- 0.3 ml of the resulting supernatant was added to 3 ml of Bradford's reagent in a fresh test tube and the contents were thoroughly mixed. After 5 minutes incubation at room temperature the optical density was measured in the Unicam Helisα spectrophotometer against 0.1 ml water plus 3 ml of Bradford's reagent (blank) at 595 nm.

The protein content of the samples was determined by reading (μg protein) from the standard curve (Appendix A), divided by 0.3 to get μg protein ml^{-1} and then multiplied by 10 to take into account the dilution by NaOH.

2.12. Effect of Sodium Concentration on the Growth of Cells

To prepare sodium free Horikoshi medium, the pH of medium was adjusted to pH 10 as necessary using either 1 M HCl or 1 M KOH instead of NaOH and 200 mM potassium carbonate buffer was used instead of sodium carbonate with different concentrations of sodium chloride (0 - 1000 mM). All other Horikoshi medium ingredients were as shown in Table 2.1.

An overnight culture (50 ml) grown in Horikoshi medium to mid-exponential phase at pH 10 was transferred into a 50 ml Falcon tube and then harvested by centrifugation in a bench top centrifuge (3000 *g*) for 15 minutes. The pellet was resuspended (washed) three times in 50 ml of sodium free Horikoshi medium of the same pH and then 1 ml of cells was added into a number of 250 ml conical flasks containing 50 ml of slightly modified version of normal Horikoshi medium (Na^+- free medium) with different concentrations of sodium chloride (0 - 1000 mM). The optical density (OD) at 600 nm was measured in the Unicam Helis α spectrophotometer against distilled water blank, immediately after inoculation, and then all flasks were incubated for 24, 48 and 72 h at 25°C on a rotary shaker at 250 rpm, the OD at 600 nm was measured daily and used as growth parameter.

2.13. Effect of Potassium Concentration on the Growth of Cells

The same procedure described in section 2.12 was employed, but in this case potassium chloride was added to final concentration of 0, 0.05, 0.1, 0.5, 1, 5 and 10 mM in pH 10 Horikoshi medium. Sodium carbonate buffer was used at 200 mM and NaH_2PO_4 instead of KH_2PO_4. A number of conical flasks were incubated for 24, 48 and 72 h at 25°C on a rotary shaker at 250 rpm, the OD at 600 nm was measured daily and used as growth parameter.

2.14. Effect of Sodium Concentration on the Respiration Rate of Cells

The effect of increasing external salinity in the media on the oxygen consumption of both obligate alkaliphilic bacteria (MAK7 and *Bacillus*) was determined using a slightly

modified version of normal Horikoshi medium and a modified Clarke type oxygen electrode as described in section 2.10. To prepare Na^+- free medium, the pH of medium was adjusted to pH 10 as necessary using either 1 M HCl or 1 M KOH instead of NaOH and 200 mM potassium carbonate buffer was used instead of sodium carbonate with different concentrations of sodium chloride (0, 25, 50, 100, 200 and 400 mM).

To prepare cells for measurements in the oxygen electrode, 40 ml of overnight culture grown at pH 10 in normal Horikoshi medium to mid-exponential phase were transferred into a 50 ml Falcon tube and harvested by centrifugation in a bench top centrifuge at 3000 *g* for 15 minutes and the pellet was resuspended (washed) three times in 20 ml of sodium free Horikoshi medium (pH 10). The respiration rate of the cells was measured in the oxygen electrode by adding 0.1 ml of concentrated cells to 1.9 ml of fresh sodium free Horikoshi medium with different NaCl concentrations and 1.9 ml of normal Horikoshi medium was also used as control (keeping the overall volume at 2 ml to allow standardisation of the results). A Bradford, assay (see section 2.11) was performed on the concentrated cell suspensions to determine the protein concentration for use in calculating the respiration rate of the cells.

2.15. Effect of Potassium Concentration on the Respiration Rate of Cells

The same procedure described in section 2.14 was employed, but in this case potassium chloride was added to final concentration of 0, 25, 50, 100, 200 and 400 mM in pH 10

Horikoshi medium. Sodium carbonate buffer was used at 200 mM and NaH_2PO_4 was used instead of KH_2PO_4.

2.16. Effect of Different Carbon Sources on the Growth of Bacteria

Carbon source utilization was examined using either modified Horikoshi medium (without yeast extract and tryptone) or M9 minimal medium as the basal medium at pH 10. In the first set of experiments five carbohydrates (glucose, sucrose, fructose, mannitol, and glycerol) and sodium acetate were tested by preparing sterile solutions and aseptically adding them separately to numerous of 250 ml conical flasks containing 50 ml of the basal medium as sole carbon sources at a final concentration of 1% (w/v). In the second set of experiments six amino acids (glutamine, lysine, aspartate, methionine, proline and alanine) were aseptically added as carbon sources at a final concentration of 1% (w/v). The flasks were inoculated with 1 ml from each strain and then incubated in a 25°C constant temperature room with orbital shaker at 250 rpm overnight. The growth of three strains of MAB4, MAK7 and MAK10 was measured using the Unicam Helisα spectrophotometer at a wavelength of 600 nm in 1 ml plastic cuvettes against a water blank.

2.17. Silicone Oil Technique

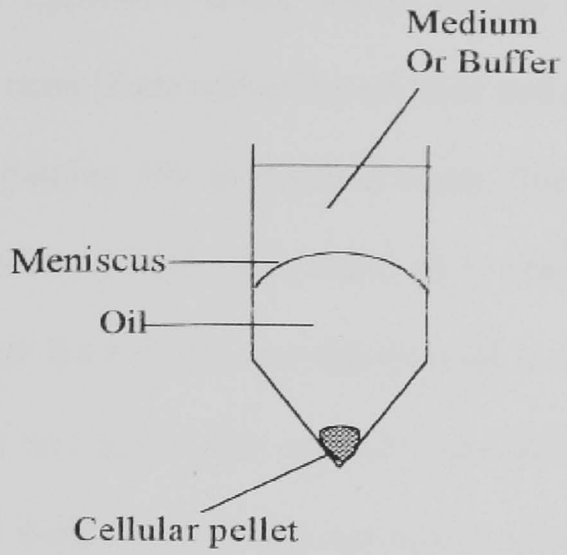
Centrifugation through silicone oil was used to completely separate cells from medium (Gimmler and Schirling, 1978). A range of oils with different densities were produced by mixing individual silicone oils with different densities (Dow Corning 200/1 grade oil was mixed with Dow Corning 550 grade oil). Cell suspensions of the density to be used

in experiments (with no isotopes added) were used to find the oil which is dense enough to restrict mixing with the medium, but which allows the cells to pass through the oil during centrifugation and produce a pellet (Figure 2.3).

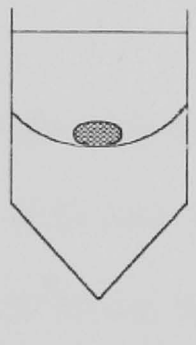
2.17.1. Determination of Intracellular Volume (ICV) of Bacteria

Estimation of cell volume was carried out using the silicone oil technique based on the method described by Rottenberg, (1979).

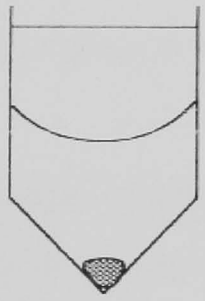
Two 1 ml samples of concentrated cells (20 ml of cells from an overnight culture grown in Horikoshi medium at different pH values were transferred to a 50 ml Falcon tube and harvested by centrifugation at 3000 *g* for 10 minutes and then the bacterial pellet was resuspended in 8 ml of fresh Horikoshi medium of the same pH) were placed in 1.5 ml Eppendorf tubes. 10 µl of $^{3}H_2O$ (1850 kBq ml^{-1}) were added to one sample of concentrated cells to give 18.5 kBq ml^{-1} and 8 µl of ^{14}C-dextran (MW = 70000, 1850 kBq ml^{-1}) were added to second sample of concentrated cells to give 14.8 kBq ml^{-1}. Both samples were vortexed thoroughly. After 5 minutes incubation at room temperature the 1.5 ml Eppendorf tubes were vortexed again, triplicate samples (300 µl) were taken from each 1.5 ml Eppendorf tube and layered onto 300 µl of the silicone oil mixture (right density) in fresh 1.5 ml Eppendorf tubes. The samples were then centrifuged for 1 minute at 13000 rpm (11340 *g*) in a bench top microcentrifuge (MSE, Micro-Centaur). From the aqueous supernatant fraction, triplicate samples (50 µl) were taken and placed into separate scintillation vials containing 5 ml of FluoranSafe scintillation fluid (VWR) and labelled appropriately.



1. Oil is not dense enough



2. Oil is too dense



3. Oil is in the right density

Figure 2.3: Silicone oil density selection for intracellular volume, membrane potential and internal pH determinations. After centrifugation a pellet was formed and then medium remained on top of silicone oil of the correct density (3), silicone oil of the incorrect density (1 and 2).

The tips of the 1.5 ml Eppendorf tubes, which contain the pellet of bacteria, were carefully cut off using a razor blade within the oil layer and placed cut end down into 1.5 ml Eppendorf tubes containing 300 µl distilled water. They were then centrifuged for approximately 15 seconds at 13000 rpm (11340 *g*) to remove the pellet from the tips which was then discarded. Each pellet was resuspended in the water and then the whole sample was pipetted out and placed into separate scintillation vials containing 5 ml of FluoranSafe scintillation fluid (labelled appropriately) and then dispersed using a vortex agitator. All the vials were placed in racks and counted for 5 minutes per vial in a Beckman LS 1801 Liquid Scintillation Counter.

The $^{3}H_2O$ was evenly distributed throughout the pellet, whereas the ^{14}C- dextran was only found in the spaces between the cells and the pellet due to its high molecular weight (Figure 2.4). The pellet volume (PV) and the extracellular volume (ECV) were calculated from the ratio of $^{3}H_2O$ and ^{14}C-dextran in the pellet and supernatant fractions respectively using the following equations given in Hard and Gilmour, (1996).

$$\text{Pellet volume (PV) } (\mu l) = \frac{^{3}H_2O \text{ dpm in pellet}}{^{3}H_2O \text{ dpm in supernatant} \times 6} \times 300$$

$$\text{Extracellular volume (ECV) } (\mu l) = \frac{^{14}C - \text{dextran dpm in pellet}}{^{14}C - \text{dextran dpmin supernatant} \times 6} \times 300$$

The intracellular volume (ICV) was calculated by subtracting the ECV from the total pellet volume (PV) (Rottenberg, 1979).

* dpm = Disintegrations per minute

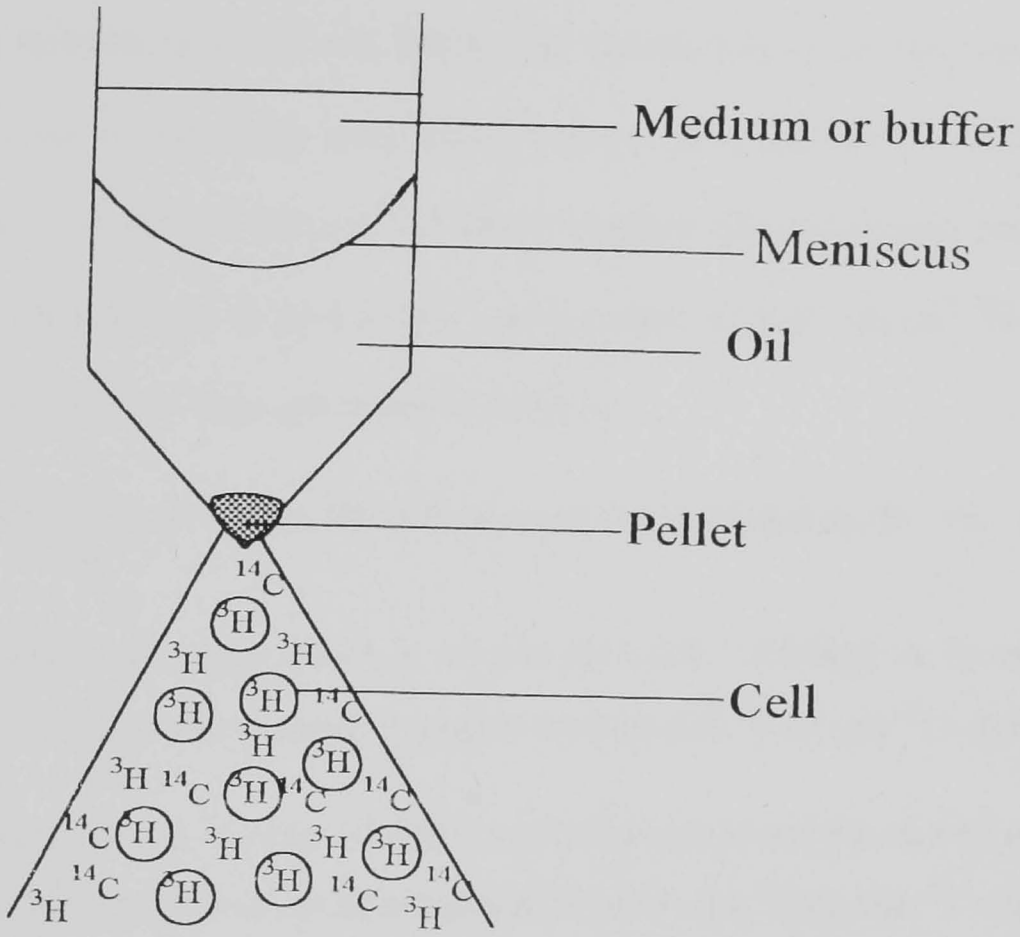


Figure 2.4: Intracellular volume (ICV) determination using $^{3}H_2O$ and ^{14}C- dextran. The $^{3}H_2O$ distributes throughout the pellet and ^{14}C- dextran is only found in the spaces between cells in the pellet.

2.17.2. The Measurement of Membrane Potential ($\Delta\Psi$) in Bacterial Cells

Estimation of membrane potential was carried out using the silicone oil technique as described by Rottenberg, (1979 and 1989). The experimental procedure was similar to the method used to determine intracellular volume (ICV) (section 2.17.1), with the exception that 5 µl of 9250 kBq ml^{-1} ^3H-TPP^+ (tetraphenylphosphonium) were added to 1 ml of concentrated cells to give a final concentration of 18.4 kBq ml^{-1} ^3H-TPP^+. The membrane potential was then calculated as follows:

1) Dpm ^3H-TPP^+ in 1 µl = Dpm ^3H-TPP^+ of supernatant divided by 50 $\Rightarrow$ **A** (a_o)

2) ^3H-TPP^+ within the pellet which is outside the cells = multiply **A** by extracellular volume in µl (calculated from parallel samples treated with ^3H_2O and ^{14}C- dextran) $\Rightarrow$ **B**

3) Dpm μl^{-1} cell volume = Dpm ^3H-TPP^+ in pellet minus **B** and divided by intracellular volume in µl (calculated from parallel samples treated with ^3H_2O and ^{14}C- dextran) $\Rightarrow$ **C** (a_i)

4) Ratio of $\frac{C}{A}$ = concentration of ^3H-TPP^+ inside the cells (a_i) / concentration of ^3H-TPP^+ outside cells (a_o)

Using the Nernst equation $\longrightarrow$ $\Delta\Psi\ (mV) = -\frac{RT}{ZF}\ \ln\frac{ai}{ao}$

Where:-

R = 8.3143 Joules mol^{-1} K^{-1} **T** = 303 K (25°C)

F = 96.487 Joules ml^{-1} mV^{-1} **Z** = 1 (charge on ionic species)

At 25°C and converting from ln to $\log_{10}$ (x 2.303) $\longrightarrow$ $\Delta\Psi\,(mV) = -58.8 \times \log\frac{ai}{ao}$

2.17.3. Determination of Internal pH (pH_i) of Bacterial Cells

The principle of measurement of internal pH in small bacterial cells is based on the ability of weak acids or bases to penetrate the cell membrane of microorganisms (Waddell and Butler, 1959; Rottenberg, 1979 and 1989; Kashket, 1985). In order to obtain a measurable accumulation, a weak acid (^{14}C- benzoic acid) was used when the internal pH was higher than the external pH (external pH is lower than pH 7). On the other hand a weak base (^{14}C-methylamine) was used when the internal pH was lower than the external pH (external pH is higher than pH 7).

The silicone oil method was used in an identical manner to that used for intracellular volume (ICV) calculation (section 2.17.1) and membrane potential determination (section 2.17.2), except that 5 µl of ^{14}C-methylamine were added to 1 ml of cell suspension (sample) to give a final concentration of 18.5 kBq ml^{-1}. The calculations were exactly the same as those used in section 2.17.2 to calculate the ratio a_i / a_o. To determine the internal pH from the a_i / a_o ratio calculated from ^{14}C-methylamine, two equations can be used.

If the pK of the probe is more than 1.5 units above the external pH then:

$$\Delta \text{ pH} = -\log \left(\frac{a_i}{a_o} \right)$$

If the pK of the probe is less than 1.5 units above the external pH then:

$$\text{pHi} = -\log \left[\frac{a_i}{a_o} \left(10^{-pk} + 10^{-pHo} \right) - 10^{-pk} \right]$$

pK of methylamine = 10.6

When a weak acid such as benzoic acid was used, there are also two equation involved in the calculations.

If the pK of the weak acid is more than 1.5 units below the external pH then the following equation is utilized.

$$\Delta \text{ pH} = \log \left(\frac{a_i}{a_o} \right)$$

If the pK of the weak acid is less than 1.5 units below the external pH this alternative equation is used.

$$\text{pHi} = \log \left[\frac{a_i}{a_o} \left(10^{\text{pk}} + 10^{\text{pHo}} \right) - 10^{\text{pk}} \right]$$

pK of benzoic acid = 4.2

The equation used to calculate the proton motive force (PMF) is:-

Proton motive force (ΔP) = $\Delta \Psi$ - (2.3 RT/F) Δ pH

See section 2.17.2 for definitions of R, T and F

2.17.4. Measurement of Isotope Uptake over Time

In order to determine uptake of each isotope by MAK7 and *Bacillus sp.* a time course experiment was carried out. Essentially the silicone oil method (section 2.17) was carried out using only one isotope. An appropriate volume of isotope was added to five 1

ml samples of cells and the silicone oil procedure carried out at 1, 5, 10, 15 and 30 minute intervals. Thirty scintillation vials containing 5 ml of FluoranSafe scintillation fluid and samples were labelled appropriately and then dispersed using a vortex agitator. All the vials were placed in racks and counted for 5 minutes per vial in a Beckman LS 1801 Liquid Scintillation Counter. The average disintegration per minute (dpm) for the pellet and supernatant triplicates per time point were calculated and plotted against time.

2.18. Determination of Intracellular Enzyme Activities of Bacteria

2.18.1. Preparation of Cell Free Extract

50 ml culture of bacteria grown in normal Horikoshi medium at pH 7, 8.5 and 10 were transferred into 50 ml Falcon tubes and harvested by centrifugation at full speed (3000 *g*) in a bench top centrifuge for 15 minutes. The bacterial pellet was resuspended in 4 ml of fresh growth medium of the same pH. 4 x 1ml aliquots of this 4 ml resuspension were placed into Eppendorf tubes and then were placed immediately on ice until required. Bacterial cells were disrupted using an ultrasonic disintegrator (MSE Soniprep 150). The sonication vessel was surrounded by an ice-water mixture and the cells disrupted by sonication for 2 x 20 seconds with 10 seconds breaks between each 20 seconds of sonication to allow cooling. The probe (1 cm diameter) was used at a power setting of 7 microns and then cells debris were removed by centrifugation at 13000 *g* for 60 seconds at 4°C. The supernatant fluid obtained was referred to as the crude cell free extract (CFE). It was kept on ice until used for measurements. A Bradford assay was performed, as described in section 2.11, on CFE to determine the protein concentration for use in calculating the specific activity of the enzymes.

2.18.2. General Assay Conditions

Continuous assay of enzyme activity were carried out using a Unicam Helisα spectrophotometer, the temperature of the cuvette was maintained at room temperature (20 – 25°C). Both glass and Quartz 4 ml cuvettes with 1 cm light path were used in all cases. For all assays the reaction rate was initially linear and proportional to the amount of extract present. Any activity measured before addition of the substrate was subtracted from the reaction rate.

2.18.3. Determination of Malate Dehydrogenase Enzyme Activity (EC 1.1.1.37)

Malate dehydrogenase is an enzyme found in the TCA cycle (citric acid cycle) and it catalyses the following reaction:

$$\text{L-Malate} + NAD^+ \longleftrightarrow \text{Oxaloacetate} + NADH + H^+$$

The assay was based on the method described by Reeves *et al.*, (1971).

The assay mixture contained:

2.0 ml	Defined growth medium (50 mM Tris- HCl buffer pH 7, 8.5 and 10)
0.05 ml	1.5 mM NADH
0.2 ml	7.5 mM Oxaloacetate pH 7.5
100 µl	Cell-free extract

Distilled water to a final volume of 3.0 ml.

All reagents, with the exception of oxaloacetate, were added to the 3 ml cuvette and then background rate of reaction measured at 340 nm for 1 minute. The reaction was started by addition of oxaloacetate to the same 3 ml cuvette and represents the decrease in absorbance at 340 nm min^{-1}. Enzyme activity is expressed as µmoles NADH oxidised min^{-1}. mg $protein^{-1}$. The extinction coefficient of NADH at 340 nm is 6.22 x 10^3 litre $mole^{-1}$ cm^{-1}.

2.18.4. Determination of Fumarase Enzyme Activity (EC 4.2.1.2)

Fumarate + H_2O ⟷ Malate

The method of Hill and Bradshaw, (1969) was used to detect the formation of fumarate from malate.

The reaction mixture contained:

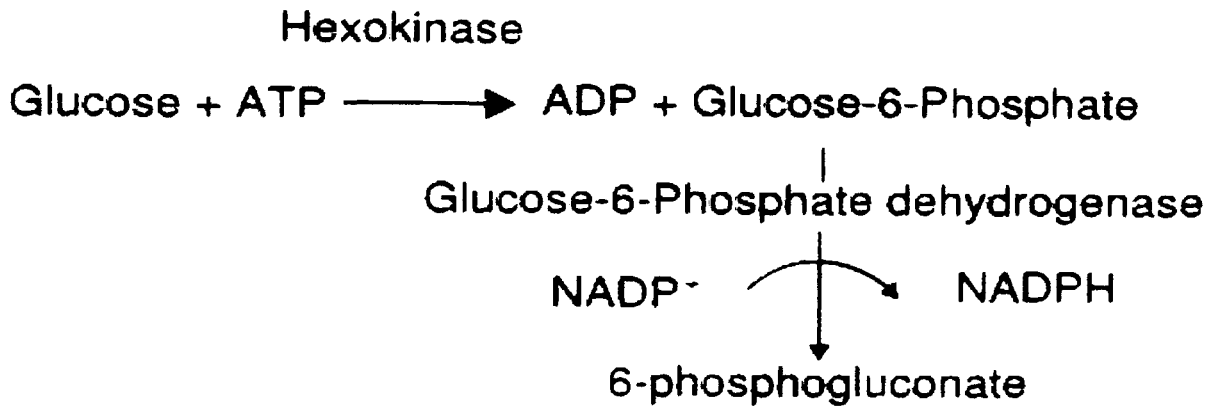
1.5 ml	Defined growth medium (50 mM Tris- HCl buffer pH 7, 8.5, 9, 9.5 and 10)
0.1 ml	1 M L – malate (sodium salt)
0.025 ml	Cell- free extract

Distilled water to a final volume of 3.0 ml.

The reaction was started by addition of 0.1 ml L – malate and the increase in absorbance at 240 nm (with deuterium lamp and quartz cuvette) was followed against water blank. Enzyme activity is expressed as µmole fumarate produced min^{-1}. mg $protein^{-1}$. The extinction coefficient of fumarate at 240 nm is 2.44 L μmol^{-1} min^{-1}.

2.18.5. Determination of Hexokinase Enzyme Activity (EC 2.7.1.2)

Hexokinase catalyses the phosphorylation of glucose to glucose - 6- phosphate using the gamma phosphate of ATP as shown below. This phosphorylation provides the activation energy required for glycolysis.



The volume of reagents used for the hexokinase assay are below listed

Volume	Reagent
300 µl	1M KCl
300 µl	1 M D (+) glucose
150 µl	150 mM $MgSO_4$
1.5 ml	200 mM Tris-HCl (adjust to pH7.5 with 1M HCl)
90 µl	50 mM PEP
150 µl	100 mM ATP
100 µl	15 mM NADH
5 µl	2 mg ml^{-1} pyruvate kinase
5 µl	5 mg ml^{-1} L-lactic dehydrogenase
100 µl	Cell free extract
200 µl	Water (adjusted to 3 ml)

This assay was carried out according to the malate dehydrogenase assay (section 2.18.3). All the reagents were added excluding the glucose and ATP. This was run in the Unicam

Helisα spectrophotometer for 2 mins to monitor any background NADH oxidation. ATP was then added to the reaction mixture and run for 3 mins to observe background ATPase activity. Finally glucose was added to the sample and the decrease in absorbance at 340 nm was read against a water blank for a further 3 minutes.

2.18.6. Effect of Sodium Concentration on Malate Dehydrogenase Enzyme Activity

The effect of sodium concentration on malate dehydrogenase enzyme activity in both alkaliphilic strains was determined using sodium free Horikoshi medium and a slightly modified method as described by Reeves *et al.,* (1971), see section 2.18.3.

To prepare cells for measuring the effect of sodium concentration on malate dehydrogenase enzyme activity, 50 ml of cells from an overnight culture grown in normal pH 10 Horikoshi medium to middle exponential phase were transferred into a 50 ml Falcon tube and then harvested by centrifugation in a bench top centrifuge at 3000 *g* for 15 minutes. The bacterial pellet was resuspended (washed) three times in 50 ml of fresh sodium free Horikoshi medium of the same pH (pH 10) and then centrifuged at 3000 *g* for 15 minutes and the bacterial pellet was resuspended in 4 ml of sodium free Horikoshi medium pH 10. Four x 1ml aliquots of this 4 ml resuspension were then sonicated as described in section 2.18.1 to produce a crude cell free extract (CFE). A Bradford assay was performed as described in section 2.11 on the CFE to determine the protein concentration for use in calculating the malate dehydrogenase enzyme specific activity.

The same procedure described in section 2.18.3 to determine malate dehydrogenase enzyme activity was employed, but in this case 2 ml of sodium chloride was used to give final concentrations of 0, 50, 100, 200, 400, 800 and 1000 mM with pH 10 instead of the Defined growth medium.

2.19. Sensitivity to Antibiotics

Estimation of the sensitivity of both strains (MAK7 and *Bacillus*) to antibiotics was carried out using standard disk method. Four antimicrobial susceptibility test disks were obtained from Oxoid: Streptomycin 25 µg, Tetracycline 50 µg, Neomycin 30 µg and Penicillin G 10 units. 0.2 ml of the fresh culture was spread on pH 10 normal Horikoshi medium plates using a sterile glass spreader and then left for 10 minutes to dry. The four antimicrobial disks were put on the bacterial film (one in each quarter of the plate). Three plates were used to perform the test and a plate without any antibiotics was used as a control. The plates were incubated (uninverted) at 25°C overnight. Inhibition zones were measured by ruler in mm and then the mean and standard error of the inhibition zone for each antibiotic was calculated.

2.20. Anaerobic Growth

Anaerobic growth of bacteria was carried out using fresh overnight cultures in normal Horikoshi medium pH 10. Triplicate test tubes completely filled by normal Horikoshi medium pH 10 were inoculated with 1 ml of active inoculum from each strain and then placed in an anaerobic jar and incubated overnight at 25°C. The optical density (OD) at 600 nm was measured using the Unicam Helisα spectrophotometer against distilled water blank to check if anaerobic growth was possible.

2. 21. Molecular Identification Techniques

2.21.1. Genomic DNA Extraction

2.21.1.1. Environmental sample (MAK7 strain)

Extraction of genomic DNA from MAK7 was carried out using the commercially available PowerSoil DNA kit (MoBio Laboratories Inc, California) following the manufacturers guidelines (Appendix B) with the following exception. 50 ml of bacterial cells from an overnight culture at pH 10 in Horikoshi medium were transferred into a 50 ml Falcon tube and then harvested by centrifugation in a bench top centrifuge at 3000 *g* for 10 minutes and then 0.25 g of bacterial pellet was added to the 2 ml PowerBead tubes provided instead of 0.25 g soil sample.

Successful extraction of genomic DNA was verified by resolving 2 µl of genomic DNA with 5 µl of Orange G loading dye by gel electrophoresis (section 2.21.2) against 1 µl of GeneRuler 1 kb ladder (Fermentas International Inc, Canada). 1.5 ml Eppendorf tubes containing genomic DNA were labelled and stored at – 20°C until required

2.21.1.2. *Bacillus* strain

Genomic DNA of *Bacillus* strains was extracted for 16S rDNA analysis using the CTAB method as described by Chen *et al.*, (2001) as follows:-

Four 0.5 ml samples of bacterial cells from an overnight culture at pH 10 in Horikoshi medium were harvested by centrifugation at 11340 *g* for 10 minutes. The supernatant was poured off immediately and then each pellet was resuspended in 250 µl of CTAB buffer [2% (w/v) cetyltrimethylammonium bromide (CTAB), 2% (v/v) *ß*-

mercaptoethanol, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 10 mM EDTA (ethylene diamine tetraacetic acid)].

The samples were then combined into two 1.5 ml Eppendorf tubes, each containing 500 µl, and incubated in a bench hot block at 65°C for 1 hour. The genomic DNA was extracted using an equal volume (500 µl) of phenol-chloroform-isoamylalcohol (25:24:1, v/v) and then centrifuged at 11340 *g* for 5 minutes at the room temperature. The aqueous layer (top layer) was carefully transferred into a fresh 1.5 ml Eppendorf tube and 500 µl of chloroform was added into the same tube and centrifuged at 11340 *g* for 5 minutes at room temperature. The top layer was transferred into a fresh Eppendorf tube and then 1/10 total volume of 3 M sodium acetate (pH 5.2) was added into the same tube. DNA was precipitated with 2.5 volumes of 100% (v/v) cold ethanol and incubated at – 20°C for 15 minutes and then pelleted by centrifugation at 11340 *g* for 15 minutes at 4°C, and the supernatant was discarded immediately. The pellet was gently washed with 1 ml of 70% cold ethanol and precipitated by centrifugation at 11340 *g* for 15 minutes at 4°C and the supernatant was carefully poured off immediately. The genomic DNA pellet was air dried for 5 - 10 minutes and then was dissolved (resuspended) in 50 µl of MilliQ water (Ultrapure water) and incubated in a hot block at 55°C for 1-2 hours. Successful extraction of genomic DNA was verified by resolving 2 µl of genomic DNA with 5 µl of Orange G loading dye by gel electrophoresis (section 2.21.2) against 1 µl of GeneRuler 1 kb ladder (Fermentas International Inc, Canada). 1.5 ml Eppendorf tubes containing genomic DNA were labelled and stored at –20°C until required.

2.21.2. Agarose Gel electrophoresis

All DNA samples were analysed (separated) by gel electrophoresis using 0.8 – 1.3% gels. To prepare gels, 0.8 - 1.3 g low melting point agarose (Roche) were added to 250 ml flask containing 2 ml of 50 X TAE (Appendix C) and 98 ml distilled water. This mixture was heated in the microwave until the agarose had melted. The solution was allowed to cool before 5 µl ethidium bromide (BioRad # 161-0.433) was added and then the gel poured to set in the BioRad Subcell GT electrophoretic tank with 14, 20 or 30 well comb. Once the gel had set, it was covered with 1 X TAE buffer (20 ml of 50 X TAE made up to 1000 ml distilled water) and run at 80 V for 60 minutes using the BioRad Power Pack 300. Visualisation of gel was performed in the Uvitec "Uvidoc" mounted camera system to ensure the genomic DNA was extracted successfully and that RNA was absent. If the gel shows smearing this indicates the presence of RNA, so it would be removed by adding 1 µl of RNAse to all of the genomic DNA preparations with incubation at 4°C overnight.

2.21.3 Polymerase Chain Reaction (PCR) Amplification of 16S rDNA

Following extraction of total genomic DNA from a particular microorganism, 16S rRNA gene was amplified from the total DNA extracted as described by Chen *et al*, (2001) using polymerase chain reaction (PCR) with two universal bacterial primers: Forward primer (f D1: 5'CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCA G 3') and Reverse primer (r D1: 5' CCC GGG ATC CAA GCT TAC GGC TAC CTT GTT ACG ACT T 3') designed to target the conserved regions of the 16S rRNA gene (Weisburg *et al.*, 1991). The reaction mixture contained the following reagents in a 0.2

ml thin walled PCR tube: 39 µl of distilled water, 5 µl of 10 X Buffer, 2.5 µl of 50 mM $MgCl_2$, 0.5 µl of Forward primer, 0.5 µl of Reverse primer, 1 µl of 25 mM dNTPs, 1 µl of genomic DNA and 0.5 µl of Taq DNA polymerase (Bioline).

Amplification were carried out in a MyCycler thermal cycler (BioRad Laboratories, inc., USA) and began with an initial denaturation step for 3 minutes at 94°C followed by 30 cycles of 1 minute denaturation at 94°C, primer annealing 1 minute at 50°C and extension at 72°C for 2 minutes followed by a final extension at 72°C for 5 minutes.
2 µl of PCR reaction was added to 5 µl of Orange G loading dye and run on a 1% agarose gel against 1 µl of 1 kb GeneRuler ladder (Fermentas) to confirm the correct sized product had been amplified.

2.21.4. Purification of PCR Products

The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Ltd., UK) by following the kit protocol, which was designed to purify single or double stranded DNA fragments from PCR and other enzymatic reactions. Five volumes of Buffer (PBI) were added to 1 volume of PCR sample in sterile 1.5 ml tube (250 µl of PBI buffer were pipetted into 50 µl of the DNA). Then it was pipetted into a QIAquick spin column and placed in a 2 ml collection tube which was then centrifuged at 11340 *g* for 60 seconds and the flow through was discarded and the QIAquick spin column was placed again in the same tube. The PCR product was washed with 0.75 ml of buffer PE in the QIAquick spin column and centrifuged at 11340 *g* for 60 seconds and then the flow through was discarded. The QIAquick spin column was moved to a fresh 1.5 ml Eppendorf tube and then the DNA was precipitated by adding 50 µl of buffer EB (10

mM Tris-Cl, pH 8.5) to the centre of the QIAquick membrane and centrifuged at 11340 *g* for 60 seconds. The product was run on a 1% agarose gel against 1 µl of 1 kb GeneRuler as a marker ladder to ensure that the correct sized product had been purified.

2.21.5 Cloning of PCR Products and Transformation of *E.coli*

The purified PCR products were ligated into the cloning vector pCR2.1 (Invitrogen) using the following reaction: 4.5 µl insert, 1 µl pCR2.1 vector, 1 µl T4 DNA ligase, 1 µl ligase buffer and 2.5 µl MilliQ water. The ligation mixture was incubated at 4°C overnight and used to transform competent *E. coli* DH5α cells. 100 µl aliquots of competent *E. coli* DH5α cells were defrosted on ice for 10 minutes before all 10 µl of the ligation mixture was carefully pipetted on top of the cells and very gently mixed. The mixture was incubated on ice for 30 minutes and then heat shocked at 42°C for 90 seconds followed by a 5 minute incubation period on ice. 100 µl SOC medium (Appendix C) was added and the mixture was incubated at 37°C for 60 minutes. 80 µl of 100 mM X-gal (5 -bromo - 4- chloro -3-indolyl - ß-D-galactopyranoside), 40 µl of 100 mM IPTG (Isopropyl ß-D-1-thiogalactopyranoside) and 10 µl of 100 mg ml^{-1} ampicillin were added to the mixture before plating onto LB medium plates and incubating at 37°C overnight.

Individual white colonies were picked from the overnight selective plate with sterile pipette tips and used to inoculate 4 ml of LB ampicillin selective medium (50 µg ml^{-1}) and then incubated with shaking at 250 rpm at 37°C overnight. Plasmid DNA was extracted using the Qiagen Mini-Prep kit as per the manufacturer's protocol. Restriction

digests were carried out for 2 hours at 37°C to test whether the insert had ligated into the vector, using: 2 µl of purified plasmid DNA, 1 µl of *Eco*R1 enzyme, 1 µl of 10X reaction buffer and 6 µl of MilliQ water in PCR tube. All 10 µl of the mixture was analysed by gel electrophoresis against 1 µl of 1 kb DNA ladder (Fermentas). Only digests giving two products (at 1.5 kb and 3.9 kb) were deemed as successful. The plasmid preparations containing the correct sized insert (vectors containing the PCR inserts) were sent to Sheffield University Medical School for DNA sequencing.

2.21.6. Phylogenetic (DNA Sequence) Analysis

For the phylogenetic placement, 16S rRNA gene sequences were processed by The National Collection of Industrial, Marine and Food Bacteria (NCIMB) using the MicroSeq database and the EMBL public database.

2.22. Statistics

Most of the experiments in this study were carried out in triplicate and error bars represent standard errors of the means. If no error bars are shown, they were smaller than symbol used to represent the mean. For experiments carried out in duplicate, both values plus the average are shown.

Isolation and Initial Characterisation of Alkaliphilic Strains from the River Bradford and the River Lathkill

Chapter Three

3.1. Introduction

Hydrogen ion concentration is probably one of the most fundamental factors affecting the growth and reproduction of microorganisms (Kroll, 1990; Padan *et al.,* 2001). pH influences the ionic state of many metabolites and it also affects the availability of inorganic ions such as Fe^{2+}, Ca^{2+} and Mg^{2+}, which may become insoluble and precipitate particularly at alkaline pH (Kroll, 1990). Furthermore, hydrogen ion concentration greatly affects the stability of macromolecules and it affects how they function in biological processes and motility (Kroll, 1990; Hicks and Krulwich, 1995). Nevertheless, the pH range at which organisms are known to grow is from less than pH 1 to approximately pH 12.5 (Kroll, 1990).

Most microorganisms (non-extremophilic) grow within the range of external pH values from 5.5 – 9, but their optimal growth normally lies within the narrow range of pH 7.4 – 7.8 (Padan *et al.,* 2005). Microorganisms capable of growth outside these limits, but which show optimal growth within this range, are classified as acid- or alkali-tolerant organisms (Kroll, 1990). As noted in Chapter 1, bacteria that live at extreme pH values (alkaliphilic or acidophilic) must maintain a cytoplasmic pH that is compatible with optimal functional and structural integrity of the cytoplasmic proteins that support growth (Padan *et al.,* 2005). Therefore, it is very likely that the internal pH of such acidophiles and alkaliphiles is much closer to pH 7 than the prevailing external pH.

Alkaliphilic bacteria are widely distributed and are usually isolated from alkaline environments, although there have been reports on isolation of alkaliphilic bacteria from

environments which may not be alkaline, for example, pH neutral environments such as garden soil and faeces and even acidic soils (Horikoshi, 1996 and 1999; Grant *et al*, 1990). These observations show a strong correlation between temporary increases in alkalinity and biological activity of alkaliphiles such as ammonification, sulphate reduction, or photosynthesis (Grant *et al*, 1990). Life of alkaliphilic microorganisms in extreme environments has been studied intensively focusing attention on the diversity of organisms and regulatory mechanisms involved (Yumoto *et al*., 2000).

In this chapter, the isolation of bacteria capable of growth at pH 10 was demonstrated using water samples from the River Bradford and the River Lathkill. Selected strains of the isolated bacteria were further studied based on their ability to grow well at pH 10.

3.2. Results and Discussion

3.2.1. Isolation and Selection of Microorganisms

In order to isolate and grow native alkaliphilic bacteria, an alkaline (high pH) medium must be used. Therefore, Horikoshi medium, (see Table 2.1), was selected for the initial isolation of strains (Horikoshi, 1999). Ten environmental samples of water and sediment with a variety of original pH values (Table 3.1) were collected from different places in both the Bradford and Lathkill rivers in the Derbyshire Peak District (see section 2.1).

Sample source	Bradford River					Lathkill River				
Sample No.	1	2	3	4	5	6	7	8	9	10
Original pH	7.8	8.3	8.3	8.2	8.3	8.0	8.2	7.5	7.9	8.1

Table 3.1: Original pH measurements of samples 1 to 5, the samples were collected from the edge of the River Bradford between the villages of Youlgreave and Alport. Samples 6 to 10 were collected from the edge of the River Lathkill between the village of Over Haddon and the Conksbury Bridge. The pH of the samples was measured using a Mettler Toledo MP225 pH meter.

To isolate alkaliphilic bacteria (i.e. bacteria with optimum growth at pH 9 or above), from the river samples, repeated applications of the serial sub-culture and streak plating techniques were used, with alkaline Horikoshi medium (liquid and plates), until single colonies were obtained which showed best growth at pH 10. Additionally, both morphology and motility of cells were examined microscopically using Gram stain reaction and hanging drop preparation respectively to find out more information about the bacterial isolates (see section 2.7).

Using these techniques, three strains of bacteria were isolated and designated as MAB4, MAK7 and MAK10. The cells of these strains showed most resistance to alkaline pH and subsequently were successfully grown at pH 10 in Horikoshi medium at an early stage in the adaptation process.

3.2.2. Basic Characteristics of Strains MAB4, MAK7 and MAK10

Table 3.2 presents the basic characteristics of MAB4, MAK7 and MAK10 strains grown in pH 10 liquid Horikoshi medium at 25°C overnight. It was found that all three strains were non-motile and consisted of single cells. The cell shape of MAB4 and MAK7 strains was oval, whereas cells of MAK10 strain were short rods. The cell wall of MAB4 and MAK10 isolates had a Gram-negative structure, whereas the cell wall of MAK7 strain had a Gram-positive structure.

On solid Horikoshi medium the colony morphology of all three strains was observed to be smooth, shiny, semi-translucent, circular, regular, entire, and low convex. Furthermore, MAB4 strain had orange colonies, whereas both MAK7 and MAK10 strains had white colonies on plates.

Name of Bacterium	Gram stain reaction	Motile	Cell- shape	Colony colour
MAB4	Negative	No	oval-shaped	orange
MAK7	Positive	No	oval-shaped	white
MAK10	Negative	No	rod-shaped	white

Table 3.2: The basic characteristics of MAB4, MAK7 and MAK10 strains. Cells were grown in pH 10 liquid Horikoshi medium and were shaken at 250 rpm in a 25°C constant temperature room overnight.

Examination of growth temperature range showed that for all three strains, no growth was observed at 37°C, and the growth at 25°C was better than at 30°C.

3.2.3. Growth Characteristics of Strains MAB4, MAK7 and MAK10 at Different pH Values

Growth curves were produced for MAB4, MAK7 and MAK10 to observe the effect of external pH on specific growth rates at pH 7, pH 8.5 and pH 10 in Horikoshi medium. Bacterial growth was quantified by direct optical density (OD) at 600 nm measured with Unicam Helisα spectrophotometer.

3.2.3.1. Pre-adapted period

Soon after isolation at early stage in the adaptation process, growth curves were produced for all three strains at pH 7, pH 8.5 and pH 10 (Figures 3.1, 3.2 and 3.3). It was found that MAK10 strain grew well at all pH values tested. The same observation was reported for alkaliphilic *Bacillus firmus* OF4 which grew at least as well at pH 10.5 as at pH 7.5 in both batch and continuous culture on malate containing medium (Sturr, *et al.*, 1994). This association was also found for a facultative alkaliphilic strain that was isolated from the alkaline washwaters of edible olives. This bacterium grows from pH 7.0 to 10.5, with optimal growth from pH 8 to 9 (Ntougias and Russell, 2000). Similar results were found when a *Bacillus* isolate was examined under buffered conditions over the pH range from pH 7 to pH 10 (Garnova, *et al.*, 2004; Olivera *et al.*, 2005). Therefore, MAK10 strain was classified as a facultative alkaliphile.

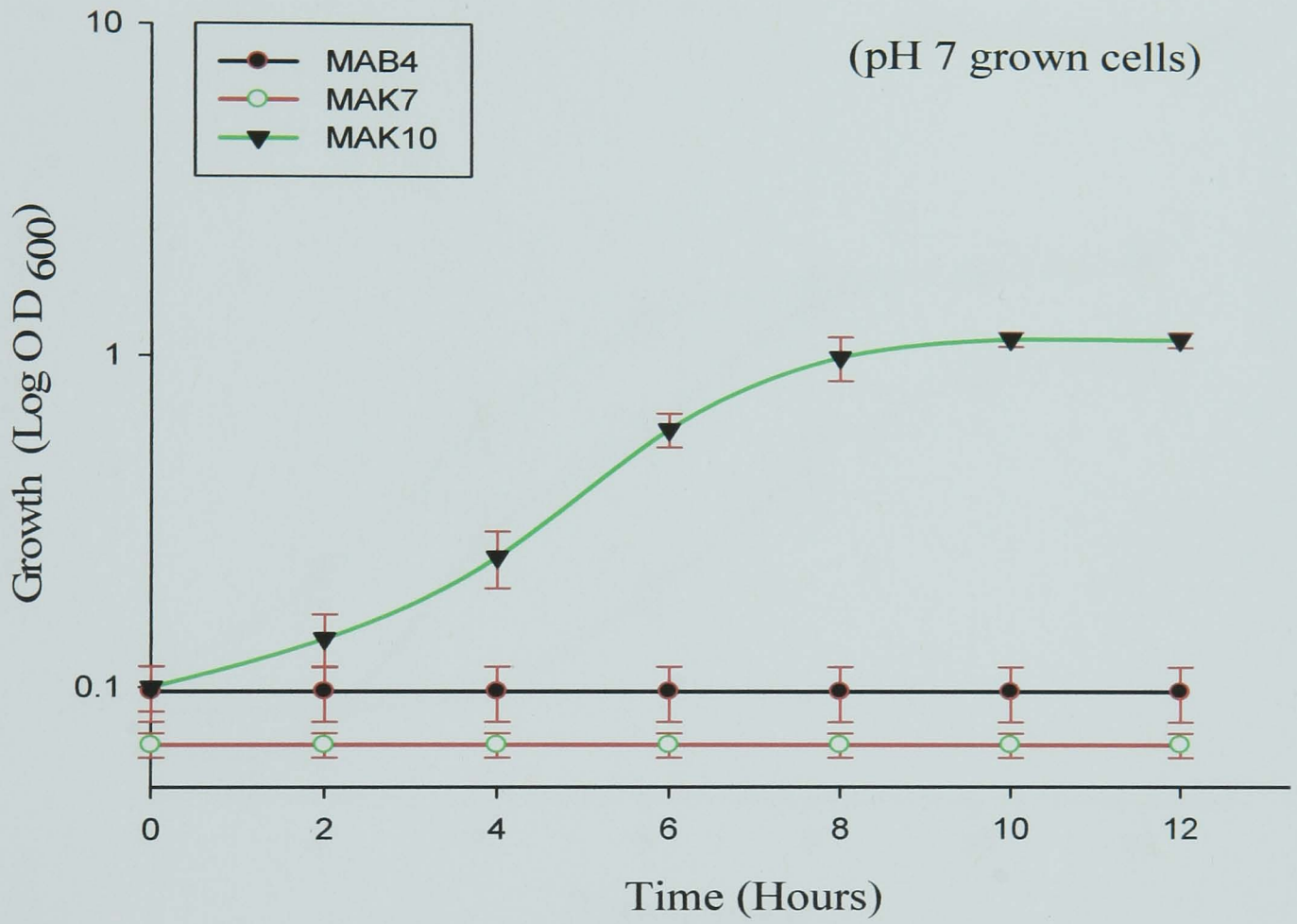
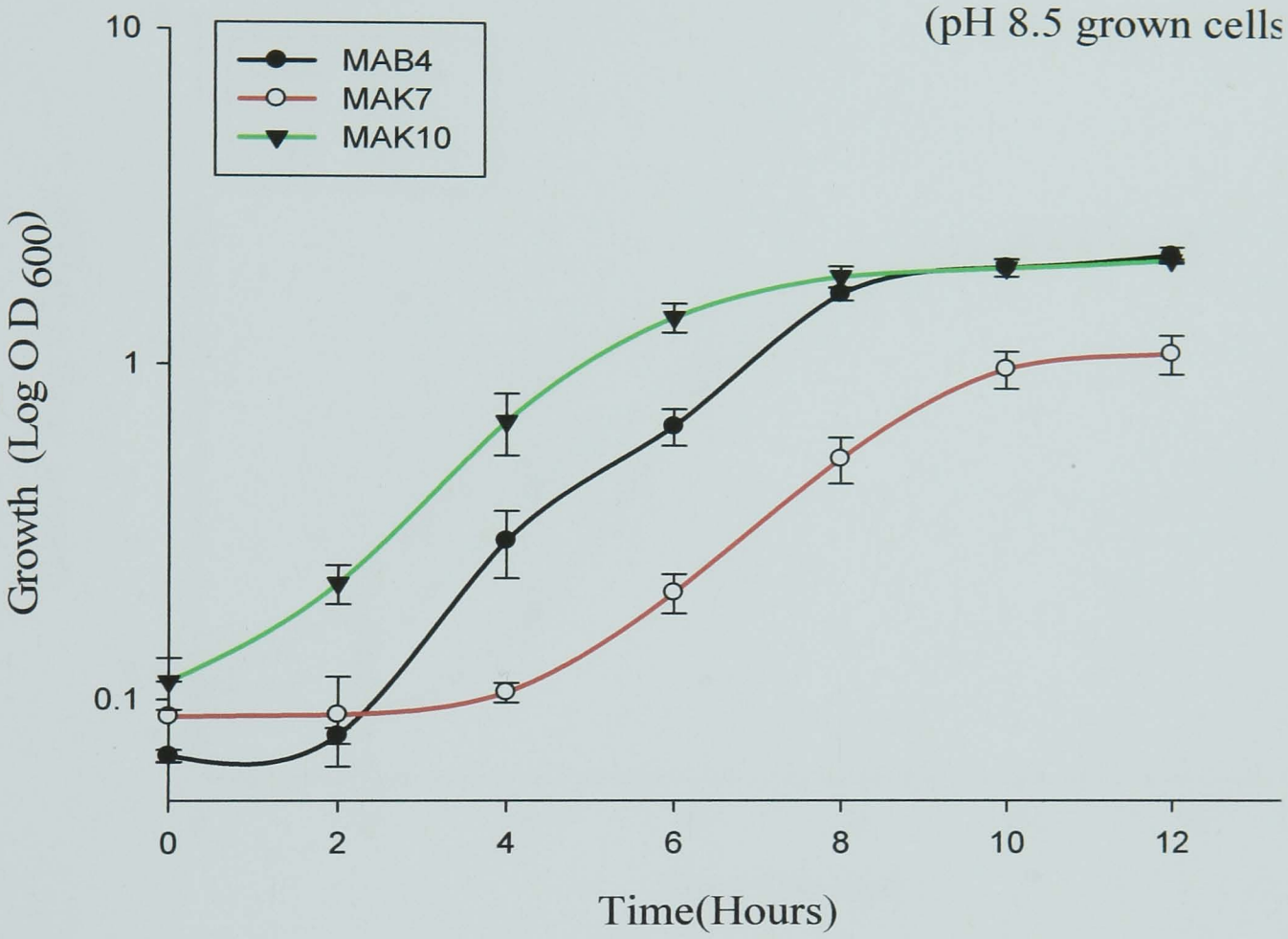
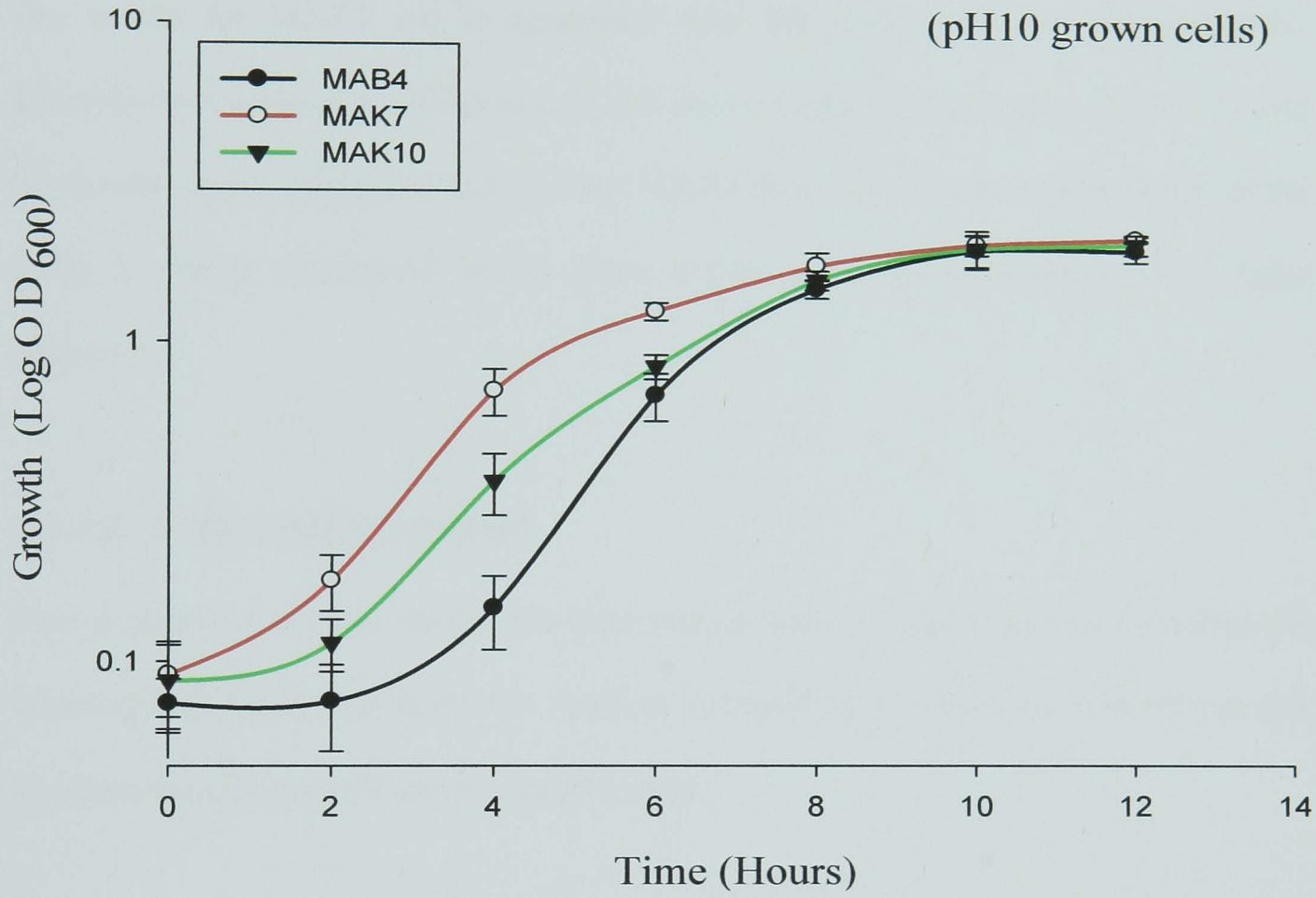


Figure 3.1: Growth curves for MAB4, MAK7 and MAK10 strains before the fully adapted period. Cells were grown in Horikoshi medium at pH 7, incubated in a 25°C constant temperature room on an orbital shaker at 250 rpm overnight. The OD for each strain was measured at 600 nm. Data points are the mean of three replicates plus or minus standard error.



Figures 3.2: Growth curves for MAB4, MAK7 and MAK10 strains before the fully adapted period. Cells were grown in Horikoshi medium at pH 8.5, incubated in a 25°C constant temperature room on an orbital shaker at 250 rpm overnight. The OD for each strain was measured at 600 nm. Data points are the mean of three replicates plus or minus standard error.



Figures 3.3: Growth curves for MAB4, MAK7 and MAK10 strains before fully adapted period. Cells were grown in Horikoshi medium at pH 10, incubated in a 25°C constant temperature room on an orbital shaker at 250 rpm overnight. The OD for each strain was measured at 600 nm. Data points are the mean of three replicates plus or minus standard error.

However, the other two strains (MAB4 and MAK7) although growing well at pH 10, did not grow at pH 7. Furthermore, MAK7 strain showed the poorest growth of the three strains at pH 8.5 and highest growth at pH 10 (Figures 3.1, 3.2 and 3.3).

The results for MAK7 are in agreement with the results reported for alkaliphilic actinomycetes isolated from desert soil, that showed optimum growth at pH 9.5 –10 and no growth at pH 7 (Hozzein, *et al.,* 2004). MAK7 strain appears from these initial results to be the most alkaliphilic of the three strains and was classified as an obligate alkaliphile.

3.2.3.2. Fully adapted period

Over a period of several weeks, the three strains were adapted to grow at external pH values of 7, 8.5 and 10 in Horikoshi medium by repeated sub-culturing. After this period of adaptation, the growth curves were repeated.

It was found that MAB4, MAK7 and MAK10 now grew well at all pH values tested (pH 7, pH 8.5 and pH 10; Figures 3.4, 3.5 and 3.6). It is clear from these findings that repeated sub-culturing in the laboratory has allowed both MAB4 and MAK7 strains to adapt to grow at external pH 7 although both MAB4 and MAK7 were isolated from samples with an original pH of 8.2 (see Table 3.1). This illustrates the ability of microorganisms to adapt to grow and survive at a wide range of environmental pH (see sections 1.6 and 1.7).

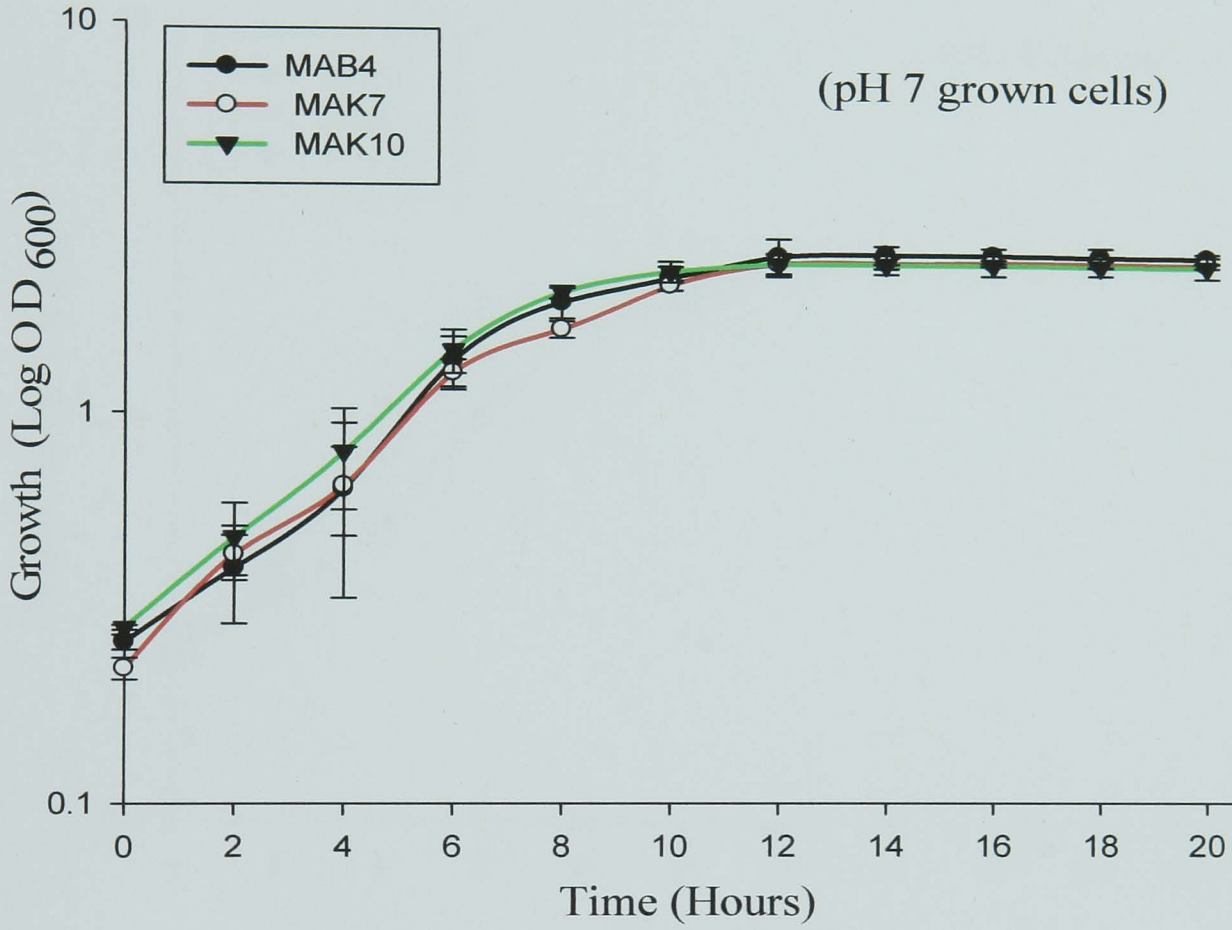


Figure 3.4: Growth curves for MAB4, MAK7 and MAK10 strains after repeated sub-culture period (fully adapted period). Cells were grown in Horikoshi medium at pH 7, incubated in a 25°C constant temperature room on an orbital shaker at 250 rpm overnight. The OD for each strain was measured at 600 nm. Data points are the mean of three replicates plus or minus standard error.

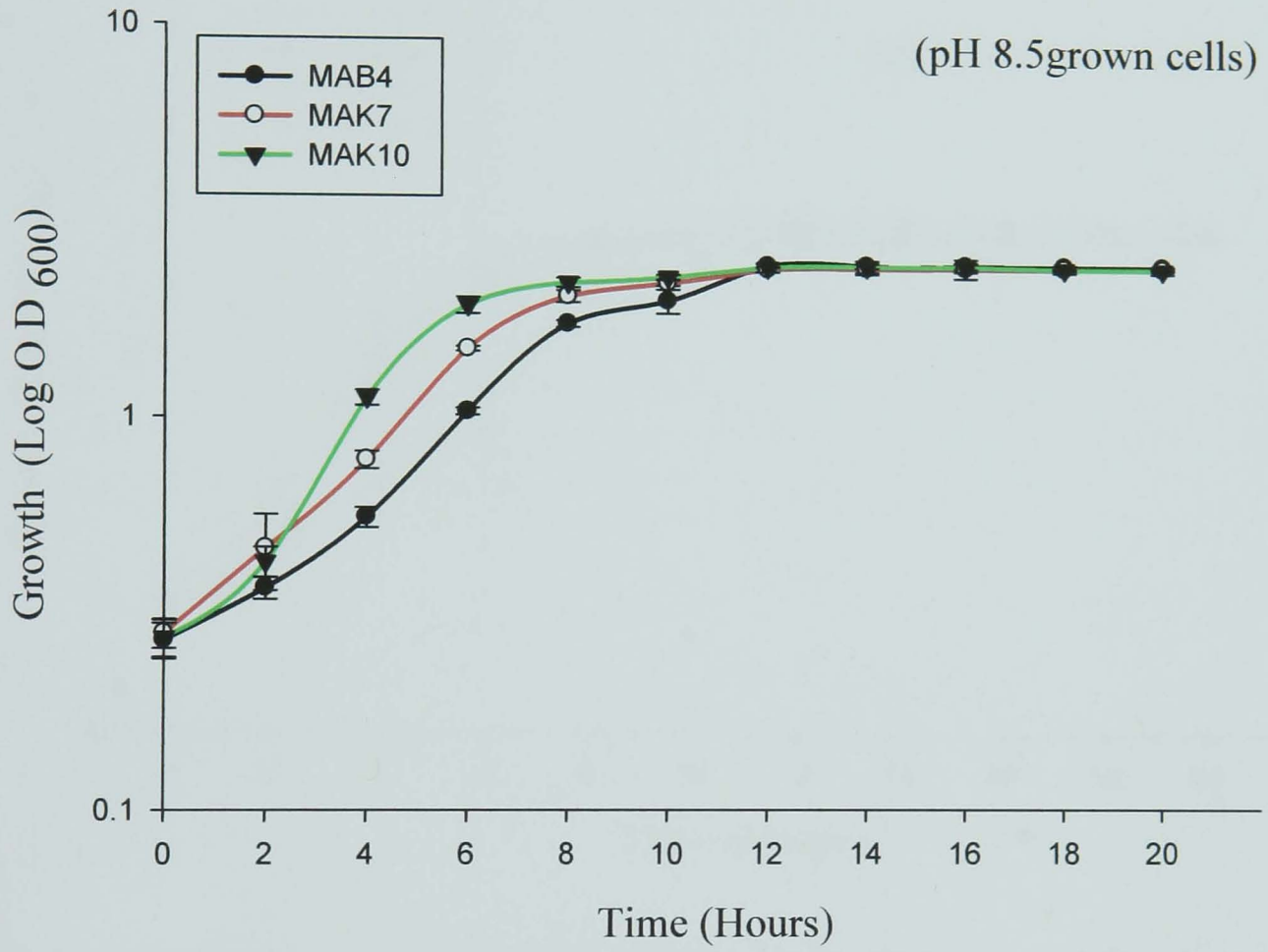


Figure 3.5: Growth curves for MAB4, MAK7 and MAK10 strains after repeated sub-culture period (fully adapted period). Cells were grown in Horikoshi medium at pH 8.5, incubated in a 25°C constant temperature room on an orbital shaker at 250 rpm overnight. The OD for each strain was measured at 600 nm. Data points are the mean of three replicates plus or minus standard error.

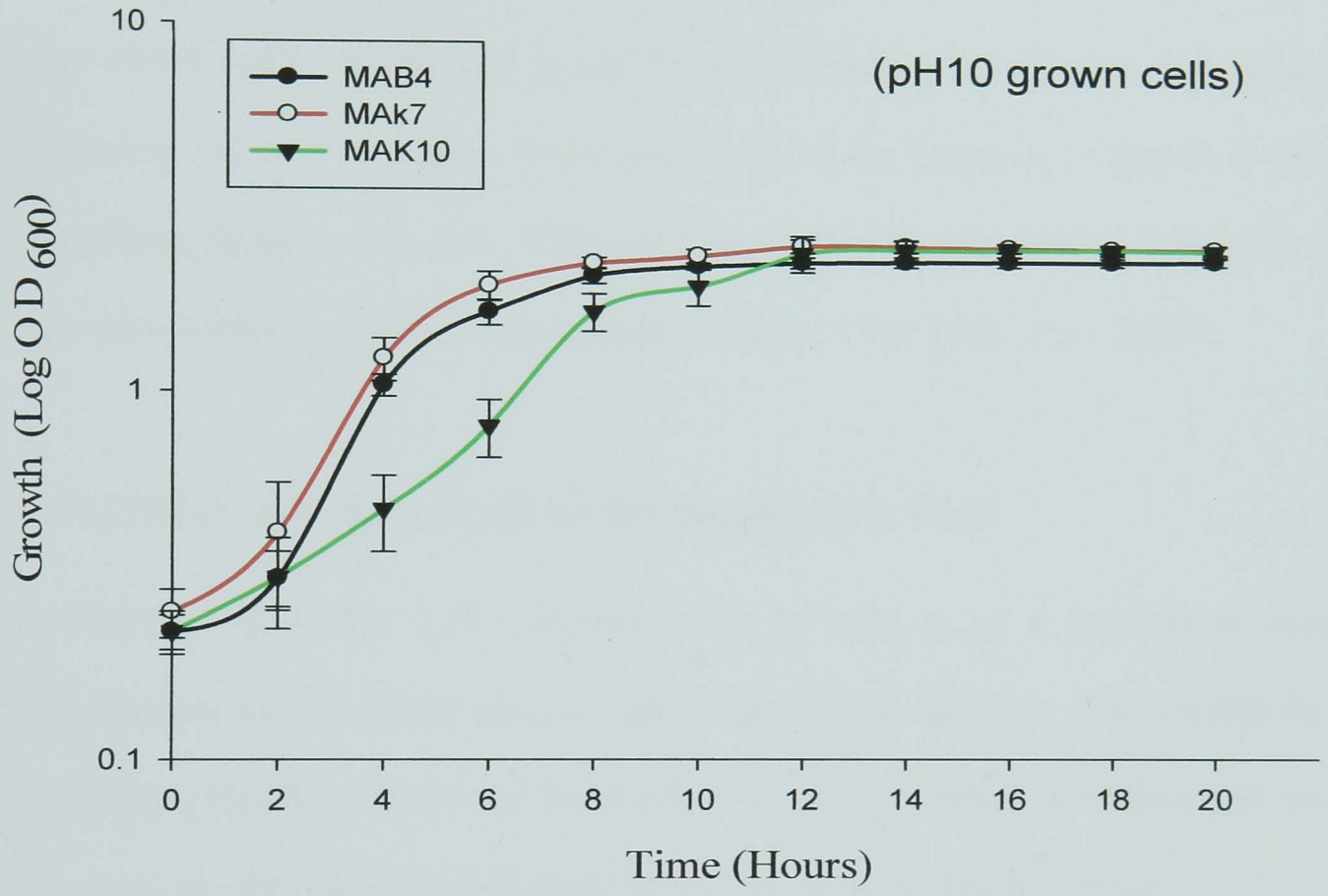


Figure 3.6: Growth curves for MAB4, MAK7 and MAK10 strains after repeated sub-culture period (fully adapted period). Cells were grown in Horikoshi medium at pH 10, incubated in a 25°C constant temperature room on an orbital shaker at 250 rpm overnight. The OD for each strain was measured at 600 nm. Data points are the mean of three replicates plus or minus standard error.

The ability of MAB4, MAK7 and MAK10 to grow and survive at external pH values below 7 and above 10 in Horikoshi medium was examined before adaptation and after the adaptation period. However, none of the strains showed significant growth outside the pH 7 – 10 range. These findings are similar to those of Zhilina *et al.*, (2005) who reported that an obligate extremely alkaliphilic organism was isolated with a pH growth range of 8.0 –10.2, an optimum at pH 9.0 and no growth or cellulose decomposition occurred at pH 10.5 or higher. There are reports in the literature of growth at pH 11 (Fe-reducing bacteria; Ye *et al.*, 2004) and up to pH 12.5 (alkaliphiles isolated from a mine water containment dam below ground in Soth Africa; Takai *et al.*, 2001).

3.2.4. Effect of External pH on the Respiration Rate

As described in section 2.10, respiration rate of bacteria, as a function of oxygen concentration, was measured using a Clarke-type oxygen electrode. It was tested for all three strains (MAB4, MAK7 and MAK10) to observe the effect of external pH on the rate of uptake of oxygen over pH range of pH 7 to 10 in Horikoshi medium.

3.2.4.1. Pre-adapted period

Soon after isolation, cells of MAB4, MAK7 and MAK10 were grown overnight at 25°C in Horikoshi medium at different pH values and then the oxygen consumption was determined (Table 3.3). It can be seen that at this early stage in the adaptation process, pH 7 is optimum pH for respiration of both MAB4 and MAK10 strains; in both cases the rate of O_2 uptake was decreased significantly when the external pH was increased to pH 8.5 and 10.

Respiration Rate (Mean ± S.D) (μmoles mg protein^{-1} h^{-1})			
Growth pH / Strains	pH 7	pH 8.5	pH10
MAB4	5.16 ± 1.32	0.55 ± 0.13	0.17 ± 0.06
MAK7	1.46 ± 0.28	1.60 ± 0.41	5.42 ± 0.98
MAK10	6.98 ± 1.59	0.08 ± 0.03	0.10 ± 0.03

Table 3.3: Respiration rate for MAB4, MAK7 and MAK10 at pH 7, 8.5 and 10, before fully adapted period. 20 ml of cells from each strain grown at pH 7, pH 8.5, and pH 10 in Horikoshi medium overnight at 25°C on an orbital shaker at 250 rpm were harvested by centrifugation and resuspended in 10 ml of fresh Horikoshi medium of the same pH. The O_2 uptake was measured as described in section 2.10. Data points are the mean of three replicates plus or minus standard error.

For MAB4 strain the decrease was from 5.16 μmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 7 to 0.17 μ moles O_2 taken up mg^{-1} cell protein h^{-1} at pH 10. For MAK10 strain the decrease was from 6.98 μmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 7 to 0.10 μmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 10 (Table 3.3).

However, this association was not found with MAK7, Table 3.3 shows that the rate of oxygen uptake increased with increasing external pH. As a consequence MAK7 showed the highest respiration rate of the three stains at pH 10 (5.42 μmoles mg $protein^{-1}$ h^{-1}) and showed the lowest rate of uptake of oxygen with pH 7 (1.46 μmoles mg^{-1} protein h^{-1}). From these data, MAK 7 again looks to be the most alkaliphilic strain. These results are in agreement with those of Ohta *et al.,* (1975), who observed that oxygen consumption was highest at pH 10 for alkaliphilic *Bacillus* sp., isolated from an indigo ball, which also had pH 10 as the optimum pH for growth. A similar result was observed with strains of ammonia-oxidizing bacteria grown at pH 10, but the pH profile of the respiratory activity was broader, with limits at 6.5 – 7.0 and 11.0 with an optimum at 9.5 –10 (Sorokin, *et al,* 2001b). Overall, it is clear that there were significant differences between the three isolates in the response of their respiration rate to external pH stress.

3.2.4.2. Fully adapted period

Once all three strains (MAB4, MAK7 and MAK10) were fully adapted to grow at different external pH values of pH 7, pH 8.5 and pH 10 in Horikoshi medium over a period of several weeks, respiration rates were measured again using the same procedure as described in section 3.2.4.1.

Table 3.4 shows that the respiration rate of MAB4 and MAK10 was highest at neutral pH and that the respiration rate decreased with increasing external pH for these two strains. There was a significant fall in the rate of O_2 uptake from 30.45 μmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 7 to 5.244 μmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH10 for MAB4 strain. There was also a dramatic drop in the respiration rate of MAK10 strain from 22.89 μmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 7 to 4.69 μmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 10 (Table 3.4). In contrast, the respiration rate of MAK7 strain was not significantly changed with increasing external pH. It was highest at pH 10 (16.27 μmoles mg $protein^{-1}$ h^{-1}) and it slightly decreased to 14.71 μmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 7.

In general, the respiration rates of all three strains at all pH values tested (pH 7, pH 8.5 and pH 10) during the fully adapted period were much higher than the respiration rates measured during the pre-adapted period (compare Tables 3.3 and 3.4). This may be due to an ability of the cells to adapt more fully to different external pH stress by repeated sub-culturing over several weeks. Nevertheless, the highest respiration rate at pH 10 is shown by the MAK7 strain and MAK 7 again looks to be the most alkaliphilic bacterium of the three strains.

Respiration Rate (Mean ± S.D) (μmoles mg protein^{-1} h^{-1})			
Growth pH / Strains	pH 7	pH 8.5	pH10
MAB4	30.45 ± 1.08	12.64 ± 1.30	05.24 ± 1.25
MAK7	14.71 ± 1.20	14.17 ± 1.15	16.27 ± 0.67
MAK10	22.89 ± 0.89	05.98 ± 0.65	04.69 ± 1.21

Table 3.4: Respiration rate for MAB4, MAK7 and MAK10 at pH 7, 8.5 and 10, after fully adapted period. 20 ml of cells from each strain grown at pH 7, pH 8.5, and pH 10 in Horikoshi medium overnight at 25°C on an orbital shaker at 250 rpm were harvested by centrifugation and resuspended in 10 ml of fresh Horikoshi medium of the same pH. The O_2 uptake was measured as described in section 2.10. Data points are the mean of three replicates plus or minus standard error.

3.2.5. Effect of Different Carbon Sources on Growth of Cells

When the sole carbon and energy source in the medium is changed the redox potential within the cell can change and this results in changes in the carbon-flux within the cell (Snoep, *et al.,* 1992). It is obvious bacteria play important roles in the cycling of energy and materials within food chains and therefore, they have been received a great deal of attention to identify the substrates available for bacterial growth and in making accurate measurements of key factors such as the cellular carbon and nitrogen contents and also the gross growth efficiency (Goldman and Dennett, 2000). These observations were extended by El- Banna, (2006), who found that antimicrobial substances produced by some bacterial species were influenced by a range of carbon sources. For example, galactose and glucose strongly enhanced the antimicrobial activity of *Corynebacterium kutscheri* and *C. xerosis* respectively, whereas both ribose and lactose repressed their activity.

To investigate the most suitable carbon source (s) for the growth of alkaliphilic bacteria, pH 10 adapted cells of the three strains (MAB4, MAK7 and MAK10) were grown with a range of carbohydrates, sodium acetate and a variety of amino acids at a final concentration of 1% (w/v). Each compound was provided as the sole carbon source as described in section 2.16, and a full list of compounds used is given in Table 3.5.

The idea was to identify a defined medium (all components known) to replace the rich Horikoshi medium which contains, in addition to the basic mineral salts, 5 g l^{-1} yeast extract and 5 g l^{-1} tryptone as described in section 2.4.1. M9 minimal medium (see section 2.4.2) was chosen as the basal medium.

Category of Carbon Source	Carbon Source Name
Simple sugar	glucose, fructose and sucrose
Hexahydric alcohol	mannitol
Commonly used carbon source	sodium acetate (trihydrate form)
Three-carbon alcohol	glycerol
Small charged amino acid	aspartate
Neutral, hydrophilic amino acid	glutamine
hydrophobic amino acid	alanine
Non-polar amino acid	methionine
Positively charged amino acid	lysine
Conformationally important amino acid	proline

Table 3.5: A summary of the type of carbon sources tested as sole sources of carbon for growth of MAB4, MAK7 and MAK10. All carbon sources were made up in basal medium at pH 10 to a final concentration of 1% (w/v).

However, it was found that both Horikoshi medium with the omission of both yeast extract and tryptone and M 9 minimal medium did not support the growth of the strains, which indicated that MAB4, MAK7 and MAK10 require some carbohydrates and amino acids or even vitamins that are present in yeast extract or tryptone or both for their growth

The next step was to identify whether the yeast extract or tryptone or both of them was essential for growth, therefore, one of the two components (yeast extract or tryptone) was omitted from the Horikoshi medium. It was found that Horikoshi medium failed to support the growth of all three strains when the yeast extract was removed from Horikoshi medium even though tryptone was still present. On the other hand, Horikoshi medium containing yeast extract without tryptone allowed normal growth of MAB4, MAK7 and MAK10 strains. Ntougias and Russell, (2000) also reported that yeast extract could support the growth of facultative alkaliphilic bacteria, and in particular Garnova *et al.*, (2004) observed that yeast extract was required for growth of obligately anaerobic alkaliphilic bacteria at pH 9.0.

From these results, it was concluded that the compounds required as carbon sources could be one or more carbohydrates or amino acids present in yeast extract more so than tryptone. Tables 3.6 and 3.7 show typical chemical analysis of yeast extract and tryptone respectively.

In order to identify whether the growth factors were amino acids or carbohydrates or sodium acetate, these chemicals were added to both Horikoshi medium without yeast extract or tryptone and M9 minimal medium as the basal medium at pH 10 as described in section 2.16.

YEAST EXTRACT*

Cat. 1702

Peptones

Description

Yeast Extract is a water soluble extract of selected autolyzed yeast cells. It is rich in vitamins, especially B-complexes, amino acids and other growth factors. It is used in many microbiological culture media formulations as an excellent growth source.

CHEMICAL CHARACTERISTICS

	SPECIFICATIONS	TYPICAL ANALYSIS
Amino Nitrogen (AN)	Minimum 4.5%	5.40%
Total Nitrogen (TN)	Minimum 10%	10.70%
AN/TN Ratio	N/A	50.46%
Loss on drying	Maximum 5%	3.30%
Ash	Maximum 15%	9.50%
pH (2% solution)	6.0 - 7.2	6.8

ELEMENTAL PROFILE

	TYPICAL ANALYSIS
Calcium	0.10%
Magnesium	0.10%
Potassium	5.70%
Sodium	0.30%

AMINO ACIDS

	Total (g/100g)		Total (g/100g)		Total (g/100g)
Alanine	8.70	Histidine	2.0	Proline	4.0
Arginine	5.00	Isoleucine	5.60	Serine	4.70
Aspartic acid	9.70	Leucine	7.60	Threonine	4.40
Cystine	0.80	Lysine	8.0	Tryptophan	1.20
Glutamic acid	16.10	Methionine	1.30	Tyrosine	2.30
Glycine	4.90	Phenylalanine	3.80	Valine	5.80

GROWTH SUPPORTING PROPERTIES

Yeast Extract Agar	Satisfactory

MICROBIOLOGICAL TEST

Standard plate count	Less than 5000 col/g
Yeasts and molds	Less than 100 col/g
Coliforms	Negative
Salmonella	Negative

* See Comparative Table of all Peptones on pg 33

Table 3.6: Typical analysis of Yeast Extract, Token from: www.condalab.com/pdf/1702.p

TRYPTONE*

Cat. 1612

Peptones

Description

Tryptone is a pancreatic digest of casein containing all amino acids found in casein as well as larger peptide fractions. It is an excellent nutrient for use in culture media for producing antibiotics, toxins, enzymes and other biological products. This product is widely used in the pharmaceutical and veterinary industries and the diagnostic culture media industry.

CHEMICAL CHARACTERISTICS

	SPECIFICATIONS	TYPICAL ANALYSIS
Amino Nitrogen (AN)	Minimum 3.9%	4.20%
Total Nitrogen (TN)	Minimum 10%	13.31%
AN/TN Ratio	N/A	31.70%
Loss on drying	Maximum 6%	3.30%
Ash	Maximum 15%	6.00%
pH (2% solution)	6.5 - 7.5	6.8

ELEMENTAL PROFILE

	TYPICAL ANALYSIS
Calcium	0.019%
Magnesium	0.0065%
Potassium	0.95%
Sodium	2.10%

AMINO ACIDS

	Total (g/100g)		Total (g/100g)		Total (g/100g)
Alanine	2.87	Histidine	2.29	Proline	8.65
Arginine	3.31	Isoleucine	4.48	Serine	5.08
Aspartic acid	6.52	Leucine	7.63	Threonine	3.91
Cystine	0.40	Lysine	6.51	Tryptophan	1.05
Glutamic acid	18.70	Methionine	2.35	Tyrosine	1.86
Glycine	1.79	Phenylalanine	4.09	Valine	5.51

GROWTH SUPPORTING PROPERTIES

Peptone Agar	Satisfactory

MICROBIOLOGICAL TEST

Standard plate count	Less than 5000 col/g
Yeasts and molds	Less than 100 col/g
Coliforms	Negative
Salmonella	Negative

* See Comparative Table of all Peptones on pg 33

Table 3.7: Typical analysis of Tryptone, Token from: www.condalab.com/pdf/1612.pdf

However, poor growth was obtained on all of the listed carbohydrates, amino acids and sodium acetate when they were added separately as sole carbon sources (Table 3.5). In contrast, good growth was observed when amino acids, carbohydrates and sodium acetate were added together to the basal medium. It appears that a mixture of carbon sources (such as found in yeast extract and tryptone) is required for good growth of all three strains at pH 10. It is likely that other components of the yeast extract or tryptone (e.g. amino acids or vitamins) are also required for optimum growth at pH 10.

3.2.6. Further Characterisation of Strain MAK7

Based on the results described in this chapter, MAK7 strain was selected as the most alkaliphilic bacterium isolated from the river samples. Therefore, MAK7 was used for further study in this work to find out more about the mechanisms employed to allow such good growth at pH 10. The ability of pH 10 Horikoshi medium grown MAK7 cells to grow under anaerobic conditions was also examined and the bacterium was found to be strictly aerobic and did not display anaerobic growth. The cell morphology of MAK7 grown at 25°C overnight in Horikoshi medium at pH 10 was examined using a phase contrast light microscope (Olympus Bx61 Upright) (Figure 3.7).

3.2.7 Isolation of Laboratory Strain of *Bacillus*

During the work in the laboratory, a contaminant appeared in cultures of MAK7 strain at pH 10 in Horikoshi medium and was subsequently purified by repeated application of the serial sub-culture and streak plating techniques using alkaline Horikoshi medium at high pH values until single colonies were obtained. Both morphology and motility of the

contaminant were examined microscopically using Gram stain reaction and hanging drop preparation. The cells of the contaminant were Gram-positive, nonmotile, single and rod-shaped (Figure 3.8). The colonies of the contaminant strain were also examined on solid Horikoshi medium and were found to be smooth, semi-translucent, circular, regular, entire, and low convex.

The contaminant strain was unable to grow anaerobically on Horikoshi medium at pH 10, it was found to be strictly aerobic. It seemed clear that the contaminant was a *Bacillus* sp. and this was subsequently confirmed by 16S rDNA sequencing (see Chapter 4).

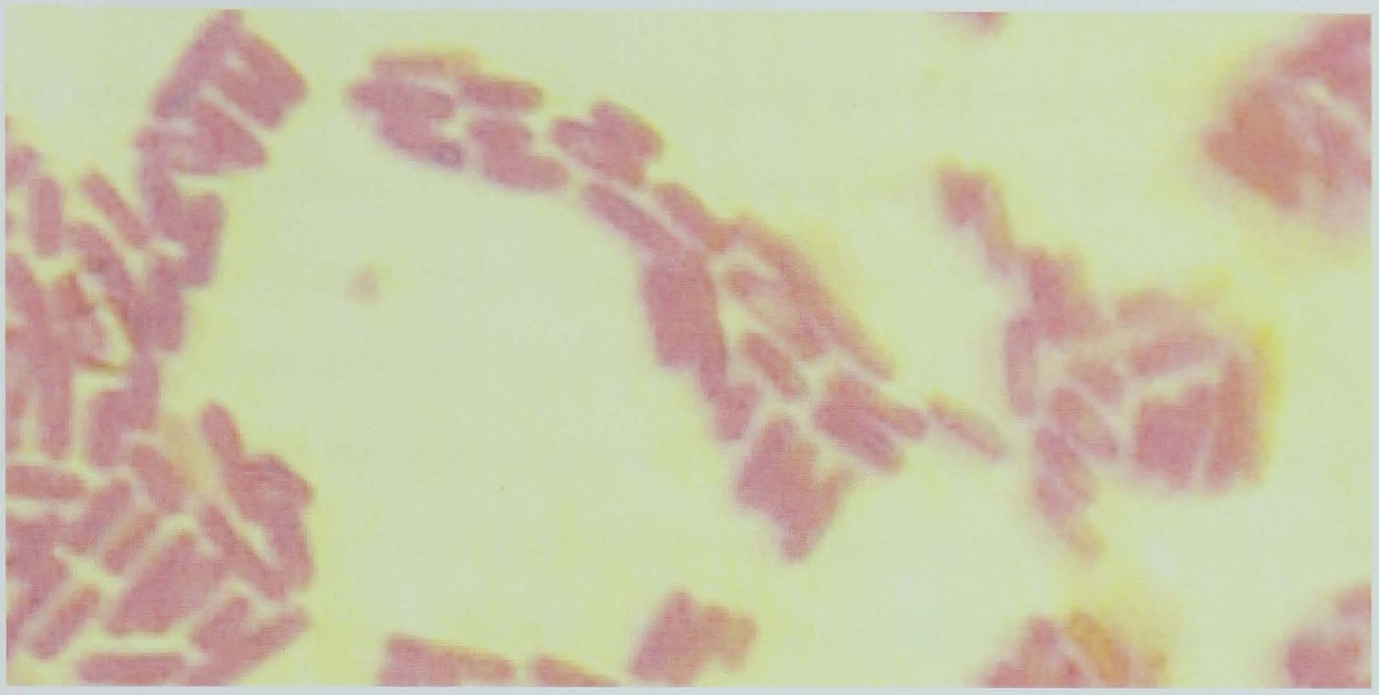


Figure 3.7: Phase contrast photomicrograph of MAK7 strain. Cells were grown at Horikoshi medium pH 10 overnight at 25°C and micrograph was taken using an Olympus Bx61 Upright microscope. Magnification was x 1000.

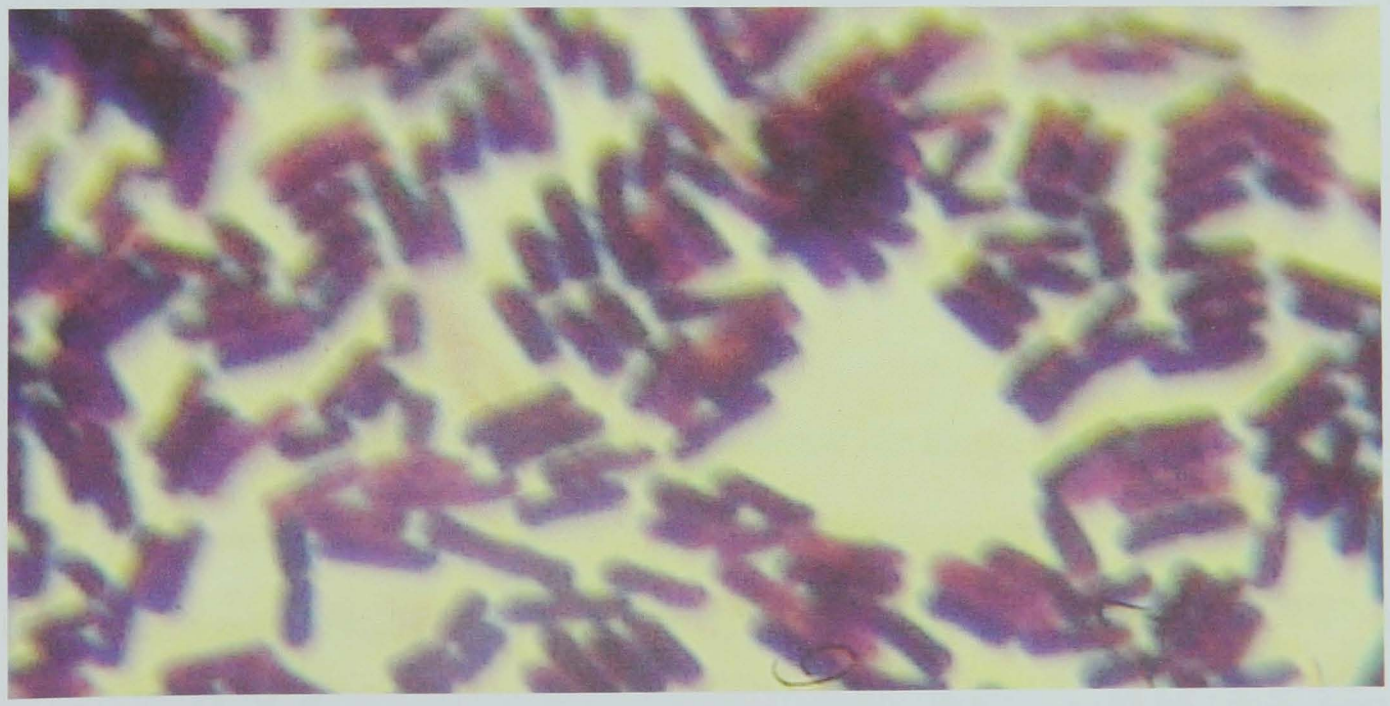


Figure 3.8: Phase contrast photomicrograph of the contaminant strain, subsequently identified as *Bacillus cereus* (see Chapter 4). The cells were grown in Horikoshi medium at pH 10 overnight at 25°C and the micrograph was taken using the Olympus Bx61 Upright microscope. Magnification was x 1000.

3.3. Conclusions

Ten samples of liquid and sediment were collected from two rivers in the Peak District and enrichment cultures were set up under alkaline conditions at pH 10 in Horikoshi medium at 25°C to grow and isolate alkaliphilic bacteria. Three strains (MAB4, MAK7 and MAK10) were chosen for further studies due to their good growth at pH 10. MAB4 and MAK10 strains were Gram-negative, whereas MAK7 strain was Gram-positive. All three strains were non-motile and consisted of single cells. MAB4 and MAK7 strains were oval-shaped, whereas MAK10 was rod-shaped. The MAK10 strain was able to cope with changes in external pH and grew well at all pH values tested (pH 7, 8.5 and 10) and was classified as a facultative alkaliphile.

At an early stage in the adaptation process, the MAB4 and MAK7 strains did not grow at pH 7 and moreover, MAK7 showed the highest growth at pH 10 and the poorest growth of the three strains at pH 8.5. However, repeated sub-culturing in the laboratory for several weeks allowed both MAB4 and MAK7 strains to adapt to grow at pH 7.

In both adaptation periods, the influence of external pH on the respiration rates of the three strains was also examined. The highest and lowest respiration rates of the three stains were found with pH 10 and pH 7 grown cells of MAK7 strain respectively. While MAB4 and MAK10 showed very low respiration rates at pH 8.5 and pH 10. On the basis of these characteristics, the MAK7 strain was classified as an aerobic obligately alkaliphilic bacterium and was selected for further study.

Carbon source utilization was examined for all three strains (MAB4, MAK7 and MAK10) using either Horikoshi medium (minus yeast extract and tryptone) or M9 minimal medium as the basal medium. The following conclusions were made from these experiments, all which were conducted at pH 10:

1. The best growth was found in Horikoshi medium containing both yeast extract and tryptone. If only one is added, better growth is found with yeast extract than with tryptone.

2. Poor growth was obtained on all of the carbohydrates, and amino acids and sodium acetate when they were added separately as sole carbon sources.

3. It appears that a mixture of carbon sources (found in yeast extract and tryptone) is required for good growth of all three strains at pH 10. It is likely that other components of the yeast extract/tryptone (e.g. amino acids or vitamins) are also required for optimum growth at pH 10. The requirement for a mixture of carbon sources may be related the nutrient (oligotrophic) conditions in their river habitat or it may be a particular requirement for growth at pH 10.

In conclusion, it was decided to carry out further work using the MAK7 strain, which showed the best growth at pH 10. It was further decided that the bioenergetic work on determination of membrane potential, internal pH and some physiological studies would be carried out on both MAK7 and the *Bacillus* sp. contaminant to allow a comparison between MAK7 as natural environmental river sample and the *Bacillus* strain. In the next chapter, both MAK7 and the contaminant strain were identified using 16S rDNA sequencing.

Identification of Environmental Strain MAK7 and Laboratory Isolate *Bacillus* sp. Using 16S rDNA Sequencing

Chapter Four

4.1. Introduction

As previously mentioned, the study of microbial biodiversity has been severely limited and delayed for many years by relying on microorganisms that can be cultured in the laboratory, which represent only a tiny fraction of the microbial biodiversity in the environment. It has been estimated that less than 0.1% of the soil microbial community can be successfully isolated in pure culture (Hill *et al.,* 2000). This situation has arisen because the identification of microorganisms in most microbiology laboratories has usually been achieved by traditional identification techniques including, Gram staining, morphology, culture requirements and biochemical reactions (Woo *et al.*, 2003). However, conventional identification techniques have two main drawbacks. First, these methods can not be used to identify non-cultivable or even slow growing bacteria, which are characteristics of many anaerobic bacteria. Second, bacteria which exhibit unusual biochemical characteristics also tend to be difficult to identify (Lau *et al.*, 2002). Over the last decade, however, recombinant DNA and molecular phylogenetic identification techniques have provided methods for analysis of natural microbial diversity without the need to cultivate bacteria. Molecular identification methods overcome the problems that may arise from the isolation and culturing of bacteria such as time consumption and uncertainty (Dojka *et al.,* 2000; Spratt, 2004; Janda and Abbott, 2007; Simmon *et al.,* 2008).

Since the discovery of polymerase chain reaction (PCR), DNA sequencing has been used as an important tool for phylogenetic characterisation and classification of bacteria. It has been shown that the 16S ribosomal RNA (16S rRNA) gene is highly conserved

within a species and among species of the same genus (Woo *et al.*, 2001; Cai *et al.*, 2003; Mignard and Flandrois, 2006). The 16S rRNA gene sequences have been selected and used to study bacterial phylogeny and taxonomy because this gene is present in all bacteria and is thus a universal target for bacterial identification. 16S rRNA gene is also chosen because it contains regions of conserved, variable and hypervarible sequence. Finally, 16S rRNA a 1500 bp sized gene is also relatively easily sequenced but large enough to contain sufficient information for identification and phylogenetic analyses of organisms (Patel, 2001; Drancourt *et al.*, 2000). Furthermore, there is no evidence of gene transfer of rRNA genes between different species and consequently this gene provides correct information regarding evolutionary relationships (Pace, 1997).

During the investigations of alkaliphilic bacteria isolated from river samples (see Chapter 3), a laboratory contaminant was also isolated due to its good growth at pH 10. This contaminant showed the characteristics of bacterial species belonging to the genus *Bacillus*. The genus *Bacillus* has been well characterised and a number of *Bacillus* species have had their 16S rRNA gene sequenced and also DNA:DNA hybridisation experiments have been carried out to examine the relatedness of a number of *Bacillus* species (Janda and Abbott, 2007). On the basis of phenotypic characteristics, DNA-DNA relatedness and phylogenetic evidence based on the 16*S* rRNA gene sequences, more than ten new alkaliphilic *Bacillus* species were proposed by Yumoto *et al.*, (1998) and Yumoto *et al.*, (2000).

Due to this wealth of information about the *Bacillus* genus, it was decided to identify the laboratory contaminant alongside one of the strains isolated from river samples

described in Chapter 3. The strain MAK7 was chosen for identification because it was the most obligate extremely alkaliphilic bacterium of the three strains examined. It showed the best growth at pH 10, poorest growth at pH 8.5 and initially no growth at pH 7. Furthermore, MAK7 exhibited highest respiration rate at pH 10 and poorest respiration rate at pH 7.

In this chapter, MAK7 and the laboratory strain of *Bacillus* were identified to the genus level by 16S rRNA gene sequencing. The 16S rRNA gene was amplified by the polymerase chain reaction (PCR), then ligated into the cloning vector pCR 2.1 and sequenced. The sequence of the PCR product (~ 1.5 kb) was compared with known 16S rRNA gene sequences in the MicroSeq database and the EMBL public database by multiple sequence alignment as described in section 2.21.6. Phylogenetic trees were also constructed for both MAK7 and the laboratory strain of *Bacillus* to predict their genetic relatedness.

4.2. Results and Discussion

4.2.1. Extraction of Genomic DNA

A DNA extraction protocol was established in order to obtain high molecular weight genomic DNA from cells grown overnight at pH 10, the DNA extraction process generally involves cell breakage by digesting cell walls, centrifugation to remove the cell fragments and debris and then nucleic acid precipitation and purification. Cell breakage is normally achieved using detergents such as sodium dodecylsulphate (SDS) or cetylmethylammonium bromide (CTAB) (Puchooa, 2004). After a number of experiments, total genomic DNA was successfully extracted from environmental strain MAK7 and laboratory strain of *Bacillus* using the PowerSoil DNA kit (MoBio Laboratories Inc, California) (see section 2.21.1.1) and CTAB method (see section 2.21.1.2) respectively.

Figure 4.1 shows that genomic DNA was successfully extracted from environmental strain MAK7 (lane 2) and laboratory strain of *Bacillus* (lane 3). Moreover, the purity of the DNA is evidence that both the CTAB protocol and PowerSoil DNA kit effectively removed proteins, polysaccharides and other contaminating molecules. This result also showed the absence of RNA and presence of high molecular weight DNA in large enough amounts for conventional PCR.

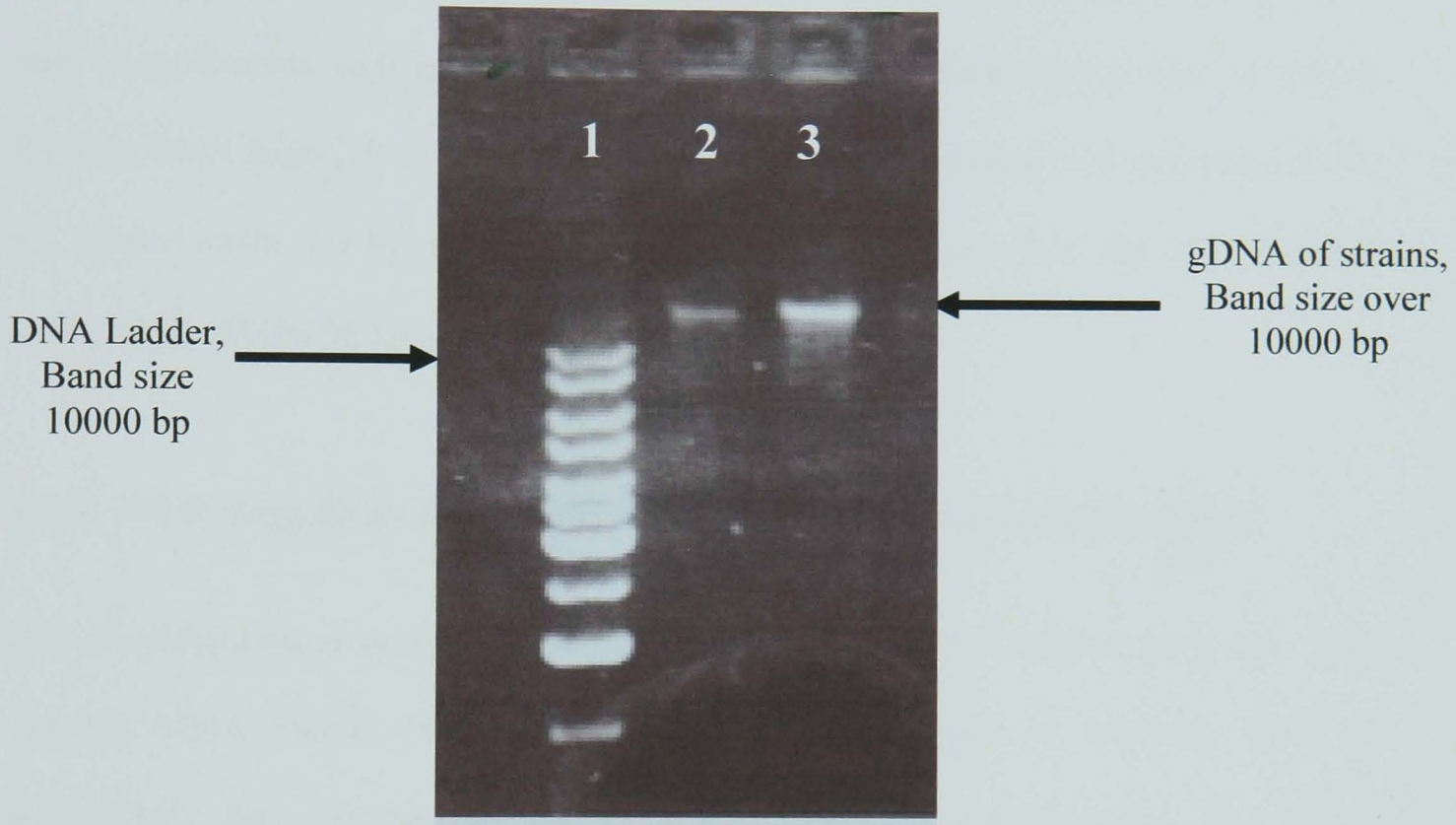


Figure 4.1: Agarose gel (1%) electrophoresis with ethidium bromide showing 10 regularly spaced bands of 1 kb DNA ladder (lane 1) and total genomic DNA extraction with a size over 10000 base pairs from both environmental strain MAK7 (lane 2) and laboratory strain of *Bacillus* (lane 3). Total genomic DNA was extracted from environmental strain MAK7 and laboratory strain of *Bacillus* using PowerSoil DNA kit and CTAB method respectively.

Figure 4.2 represents the standard HyperLadder I that was used in this study as molecular weight marker. HyperLadder I, produces a pattern of 14 regularly spaced bands, ranging from 200 to 10,000 bp. The 1,000 and 10,000 bp bands have the highest intensity to allow easy identification. HyperLadder I has been specially designed for easy quantification and as a convenient marker for determining the size of double stranded DNA from 200 to 10,000 bp. The size of each band is an exact multiple of 100 bp. HyperLadder I was used in all further experiments to allow the size of DNA molecules to be determined.

4.2.2. PCR Amplification of 16S rRNA Gene of Bacterial Isolates

PCR amplification protocol was established to amplify the 16S rRNA gene from total genomic DNA extract (see section 2.21.3), that was used as template DNA in polymerase chain reaction (PCR) assay using the universal bacterial 16S rDNA primers specific for the domain Bacteria (Chen *et al.*, 2001). Universal primers: forward primer 5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT-3' designed to target the conserved regions of the 16S rRNA gene, were utilized (Weisburg *et al.*, 1991). Therefore, these primers should allow the amplification of the 16S rRNA gene from both the environmental strain MAK7 and laboratory strain of *Bacillus*.

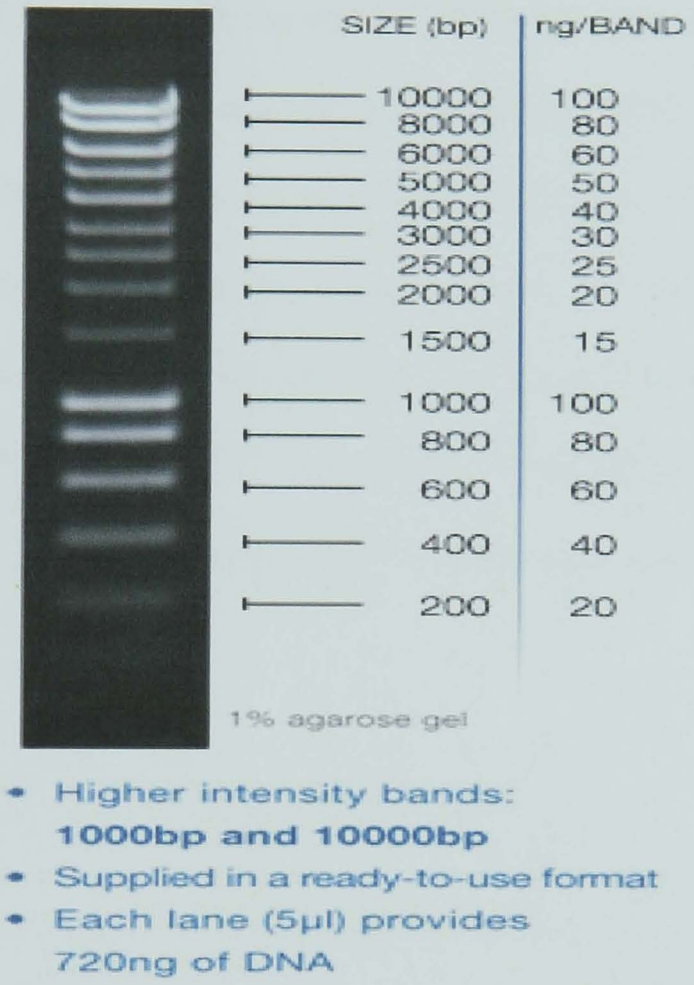


Figure 4.2: Standard HyperLadder I produces a pattern of 14 regularly spaced bands (10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 800, 600, 400 and 200 bp).

Figure 4.3 shows the result of 16S rDNA PCR amplification experiment with both MAK7 and *Bacillus* strains (lanes 2 and 3 respectively) compared with standard 1 kb DNA ladder (lane1). It is clear from these findings that the 16S rRNA genes were successfully amplified from the genome of both environmental strain MAK7 and laboratory strain of *Bacillus*, with the size of 16S rRNA gene as expected around 1.5 kb. Furthermore, a purification step was also performed on PCR products (16S rRNA gene) using QIAquick PCR purification kit protocol (see section 2.21.4). The PCR purification process removes compounds that may be present in the PCR products such as primers and dNTPs that may inhibit subsequent cloning and sequencing reactions. The result of this experiment indicated that PCR clean up was successfully completed and inhibitory compounds present in the PCR products were completely removed (Figure 4.4).

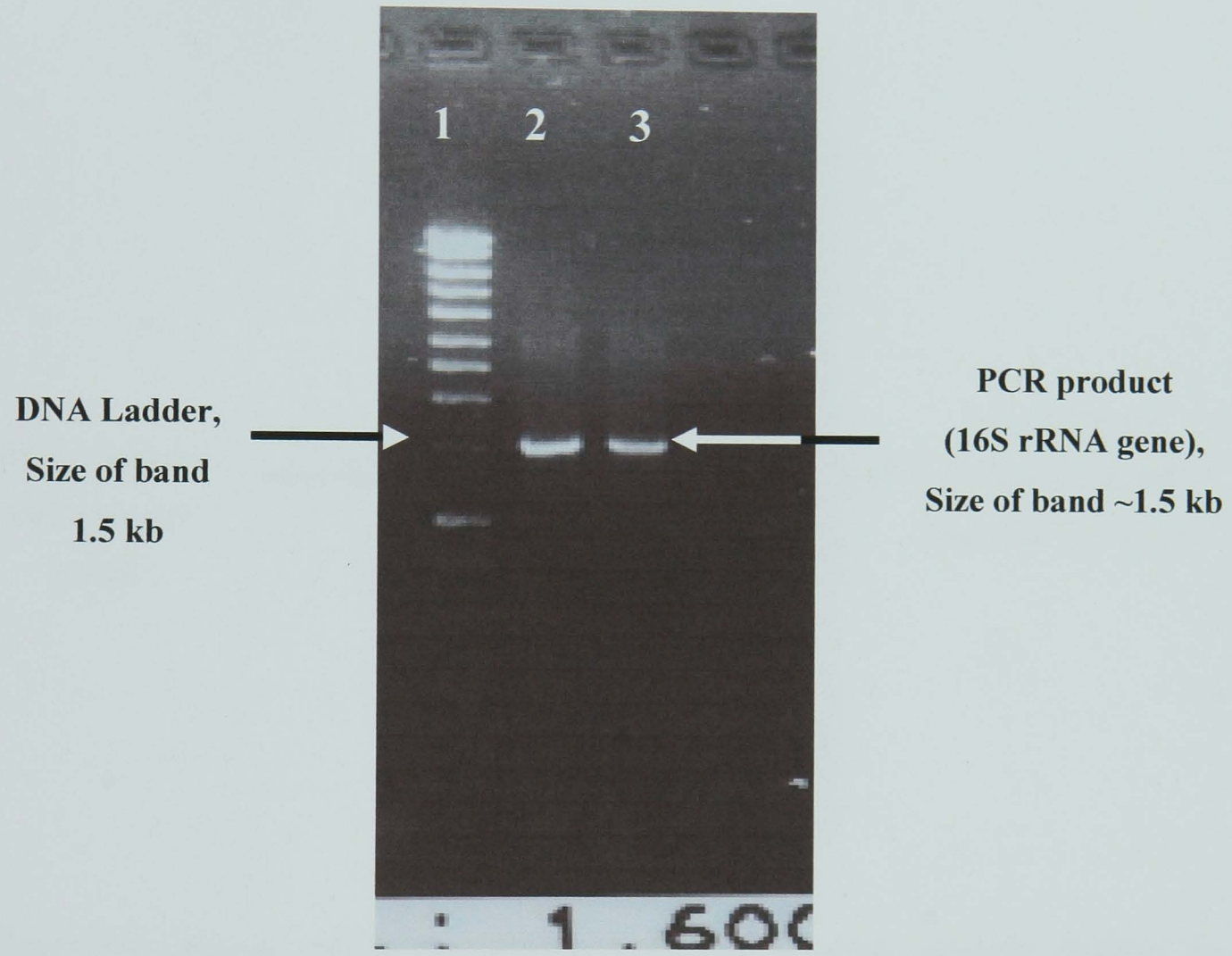


Figure 4.3: Agarose gel (1%) electrophoresis with ethidium bromide showing the resolution of an approximate 1500 base pair product (16S rRNA gene) from the PCR involving the universal bacterial primers using 1 µl of total genomic DNA from environmental strain MAK7 (lane 2) and laboratory strain of *Bacillus* (lane 3). Lane 1 shows the 1 kb DNA ladder.

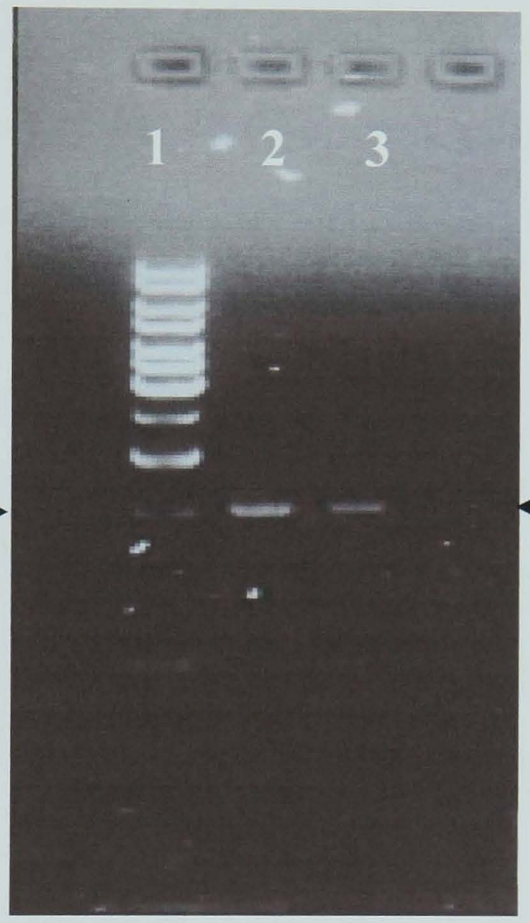
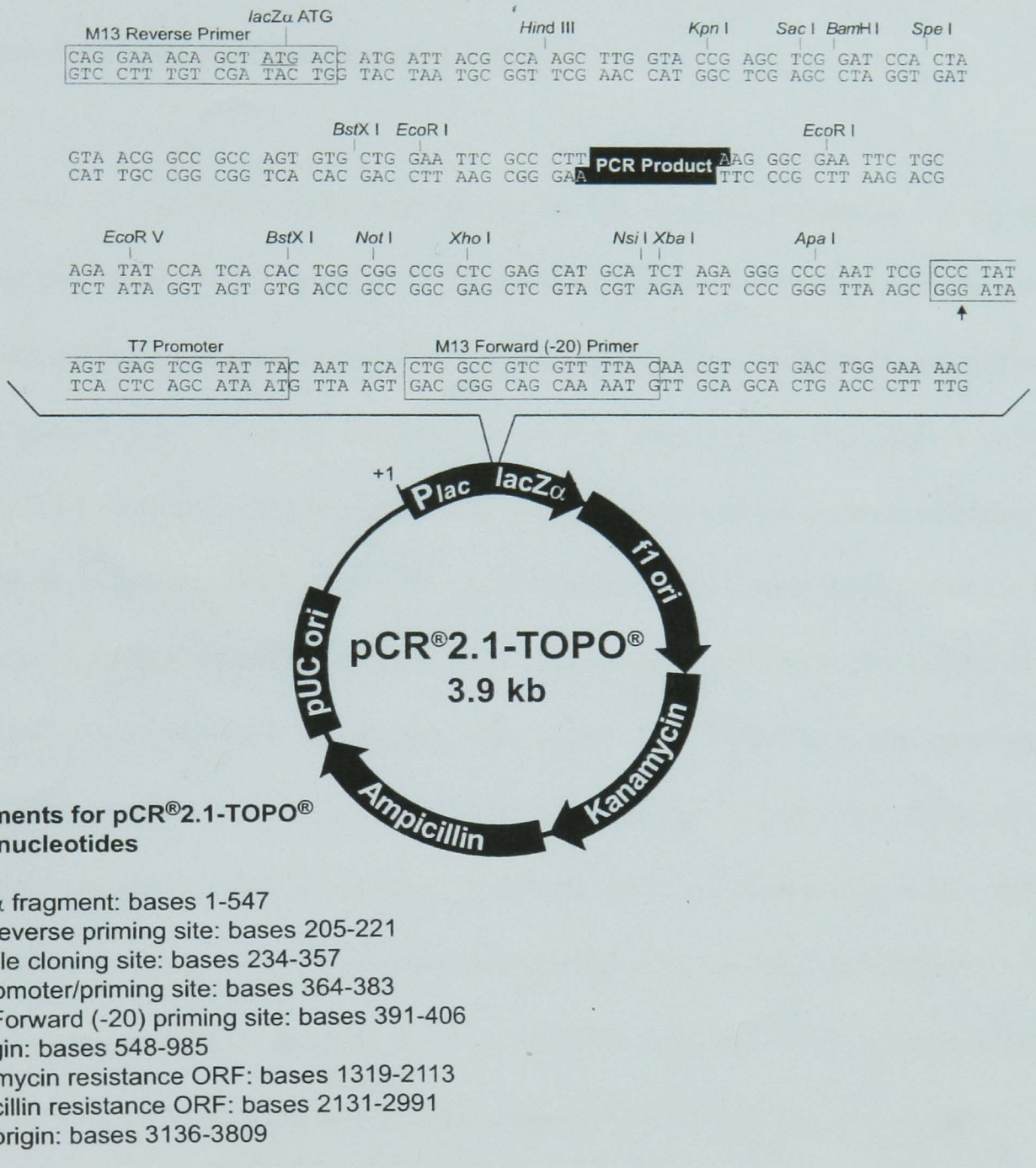


Figure 4.4: Agarose gel (1%) electrophoresis with ethidium bromide showing the resolution of 1.5 kb purified product of 16S rRNA gene from the polymerase chain reaction (PCR) involving the universal bacterial primers using 1 µl of total genomic DNA from environmental strain MAK7 (lane 2) and laboratory strain of *Bacillus* (lane 3) and Lane 1 shows the 1 kb ladder.

4.2.3. Cloning of PCR Products and Transformation of *E. coli*

16S rRNA gene sequences can be obtained directly from PCR products, however, the sequence returned from direct sequencing of PCR products is normally only a maximum of 800-1000 bp, significantly shorter than the full 1500 bp. Therefore, for almost all analyses of microbial ecosystems the amplified 16S rRNA gene has to be inserted into a plasmid (vector) to obtain a longer sequence for better comparison with other organisms in the databases. However, one of the most important steps directly affecting the results of molecular phylogenetic techniques is the ligation step that involves the linking together of the genomic DNA fragment generated by PCR reactions and the specific plasmid with antibiotic resistance that have been cut open at specific locations using restriction enzymes (endonucleases) (Figure 4.5). DNA ligase is then used to insert the PCR product into the vector using the sticky ends of the vector DNA.

In this study the plasmid vector pCR2.1 was used to perform the ligation step of 16S rRNA gene of both environmental strain MAK7 and laboratory strain of *Bacillus*. This is because the vector pCR2.1 is a small (3.9 kb) double-stranded plasmid and contains the essential information to support its autonomous replication in host cells. Also, this vector carries a selectable marker (ampicillin-resistance) to allow easy identification and recovery of host cells harbouring the plasmid. Finally, the vector pCR2.1 has a small region, called the *multiple cloning site* (the polylinker region), that contains unique cutting sites for a range of restriction enzymes. These unique sites occur only once in the vector and provide useful positions to insert DNA fragments from the source organism (Forbes *et al.*, 2007).



Comments for pCR®2.1-TOPO®
3931 nucleotides

LacZα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Figure 4.5: The vector data for pCR2.1 indicating the ligation site for the TA cloning system and the available restriction sites. Information extracted from the Invitrogen TA cloning kit manual.

Cloning in *Escherichia coli* DH5α cells is the most widely used method to amplify the PCR product within the pCR2.1 vector. Therefore, the ligation mix (the vector containing the 16S rRNA insert) was used to transform competent *E. coli* DH5α cells via heat shock method as described in section 2.21.5.

Transformed *E. coli* DH5α cells were plated on LB medium containing 50 μg ml^{-1} ampicillin and incubated overnight at 37 °C and subsequently only bacteria which have a copy of the plasmid can survive on LB ampicillin media. The colonies were not allowed to grow greater than 2 mm in diameter to prevent smearing. In this project colony density of 150 - 200 colonies per plate was proven to be optimal for accurate selection of clones. Both X-gal and IPTG, that was added to the medium, were used to screen for colonies containing a recombinant plasmid as the cloning site in the vector is located in the middle of the plasmid's lacZά gene (see Figure 4.5). If the host cell receives a recombinant plasmid containing a 16S rRNA insert in the lacZά gene, then the resulting transformant colony is white. In contrast, if the plasmid does not contain a 16S rRNA insert then the lacZά gene will be transcribed (giving a functional ß-galactosidase) and the X-gal substrate will be used by the *E. coli* DH5α cells and these colonies will be blue. White single colonies are selected and each white colony added to a small volume of liquid growth media containing ampicillin that will allow only the transformed cells to grow. This allows the generation of a large number of plasmid copies and then the plasmid was isolated as described in section 2.21.5.

At this point it is necessary to carry out a restriction digest in order to confirm that the plasmid does have the correct insert, restriction enzymes (endonucleases) were used to

achieve this step. Restriction enzymes are found naturally in bacteria and mass produced by industry in purified forms for use in research and are named for the cellular strain from which they are isolated. In effect, each restriction enzyme recognizes and cuts double stranded DNA at a specific sequence. For example *Eco*R1 is a restriction enzyme (from *E. coli*) cuts the sequence 5'...GAATTC...3' between G and A, and it cuts the sequence 3...CTTTAAG...5' between A and G. Given the fact that we know the sequence of the plasmid and where the 16S rRNA gene target should have inserted we can choose the correct restriction enzyme to allow us to see if 16S rRNA gene target is inserted properly.

However, as already mentioned, the vector pCR2.1 (3.9 kb in length) was used and it has *Eco*R1 sites either side of the region where the 16S rRNA gene should have inserted. Therefore, an *Eco*R1 digest of pCR2.1 with 16S rRNA gene inserted should produce 3.9 kb and 1.5 kb bands when run on an agarose gel. There may also be a 5.4 kb band, which would be undigested vector which contained the correct sized insert. Figure 4.6 shows the results of this experiment confirming the successful insertion of the 16S rRNA gene into the vector pCR 2.1 in both environmental strain MAK7 (lane 2) and laboratory strain of *Bacillus* (lane 3). Each digest produced two products (vector pCR 2.1 and 16S rRNA gene) at 3.9 kb and 1.5 kb band respectively (Figure 4.6).

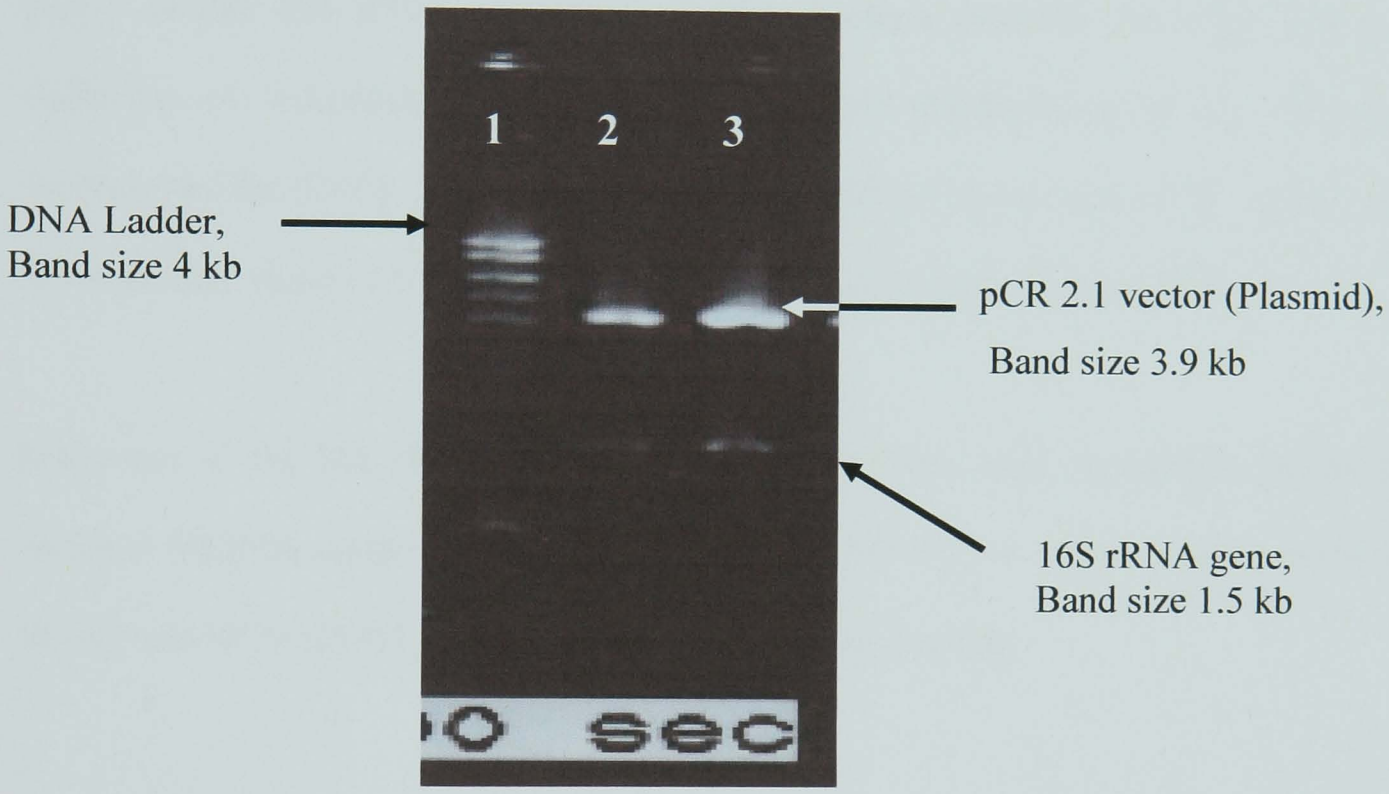


Figure 4.6: Agarose gel (1%) electrophoresis with ethidium bromide showing the restriction endonuclease analysis of vector pCR2.1 digested with *Eco*R1 for environmental strain MAK7 (lane 2) and laboratory strain of *Bacillus* (lane 3). Lane 1 shows the 1-kb DNA ladder. The upper band indicates the pCR 2.1 vector (Plasmid), with size 3.9 kb and the lower band indicates the 16S rRNA gene with size 1.5 kb.

4.2.4. Sequencing of 16S rDNA Gene of Bacterial Isolates

16S rDNA gene sequencing is a powerful tool that has been used to identify phylogenetic relationships between bacteria. The 16S rRNA gene sequences of both environmental strain MAK7 and laboratory strain of *Bacillus* were aligned with the highly similar 16S rDNA sequences of other bacteria available from the National Collection of Industrial, Marine and Food Bacteria (NCIMB) using the MicroSeq database and the EMBL public database. The 16S rRNA gene sequences for MAK7 and the laboratory strain of *Bacillus* are shown in Figures 4.7 and 4.8 respectively.

Sequences of the 16S rRNA genes obtained in this work were deposited with the in National NCIMB under entry name NCSQ25435_MAK7 for environmental strain of MAK7 and NCSQ25435_BCA for laboratory strain of *Bacillus.*

ACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATCTGAGGGAGCTTGCTC
CCAAAGATTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGA
TAACTTCGGGAAACCGGAGCTAATACCGGATAATCCCTTTCCTCACATGAGGAAAGGCT
GAAAGACGGTTTCGGCTGTCACTTACAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGA
GGTAACGGCTCACCAAGGCCACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATG
GACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACT
CTGTTGTTAGGGAAGAACAAGTATCGGAGTAACTGCCGGTACCTTGACGGTACCTAACC
AG

Figures 4.7: 16S rRNA gene sequence of aerobic environmental strain of MAK7 (entry name NCSQ25435_MAK7). Number of base pairs = 474

ATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCT
CTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGG
GATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAA
TTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGT
GAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACA
CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAA
TGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAA
CTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTA
ACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCG
TTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAA
GCCCACGG

Figures 4. 8: 16S rRNA gene sequence of aerobic laboratory strain of *Bacillus* (entry name NCSQ25435_BCA). Number of base pairs = 598

4.2.5. Phylogenetic Analysis

The 16S rRNA gene sequence similarity of aerobic environmental strain designated as MAK7 isolated from River Lathkill in the Derbyshire Peak District, did not give a genus level match in the MicroSeq database. Therefore the sequence was searched against the EMBL public database and the best match is to a sequence from an unclassified strain of *Bacillus* (with a 99.4% sequence similarity) associated with marine sponges (Table 4.1). There is evidence that demonstrates the involvement of associated microorganisms in the secondary metabolism of sponges, which was originally attributed to the sponge host (Hentschel *et al.,* 2001). A phylogenetic tree and sequence alignment of 16S rRNA gene sequences for aerobic environmental strain MAK7 were constructed and are shown in Figures 4.9 and 4.10 respectively.

Furthermore, the 16S rRNA gene sequence of aerobic laboratory strain of *Bacillus,* which was isolated as a contaminant in cultures of MAK7 strain at pH 10 in Horikoshi medium, shows that this strain is identical (with a 100% sequence similarity) to *Bacillus cereus* (Table 4.2). A phylogenetic tree and sequence alignment of 16S rRNA gene sequences for aerobic laboratory strain of *Bacillus* were constructed and are shown in Figures 4.11 and 4.12 respectively.

Match species/ strain	% Match	Match species/ strain	% Match
unclassified *Bacillus* strain	99.40	*Bacillus simplex*	92.98
Bacillus benzoevorans	95.73	*Bacillus oleronius*	92.83
Bacillus firmus	95.35	*Bacillus siralis*	92.58
Bacillus lentus	95.03	*Bacillus flexus*	91.92
Bacillus circulans	94.95	*Bacillus psychrosaccharolyticus*	91.88
Bacillus niacini	94.76	--	--

Table 4.1: Similarity between 16S rRNA gene sequence of aerobic environmental strain MAK7 and other related species / strains based on MicroSeq.

Match species/ strain	% Match	Match species/ strain	% Match
Bacillus cereus	100.0	*Bacillus thuringiensis*	99.60
Bacillus thuringiensis	99.91	*Bacillus thuringiensis*	99.33
Bacillus thuringiensis	99.81	*Bacillus pseudomycoides*	99.31
Bacillus thuringiensis	99.71	*Bacillus thuringiensis*	99.28
Bacillus thuringiensis	99.69	*Bacillus mycoides*	99.01

Table 4.2: Similarity between 16S rRNA gene sequence of aerobic laboratory strain of *Bacillus* and other related species / strains based on MicroSeq.

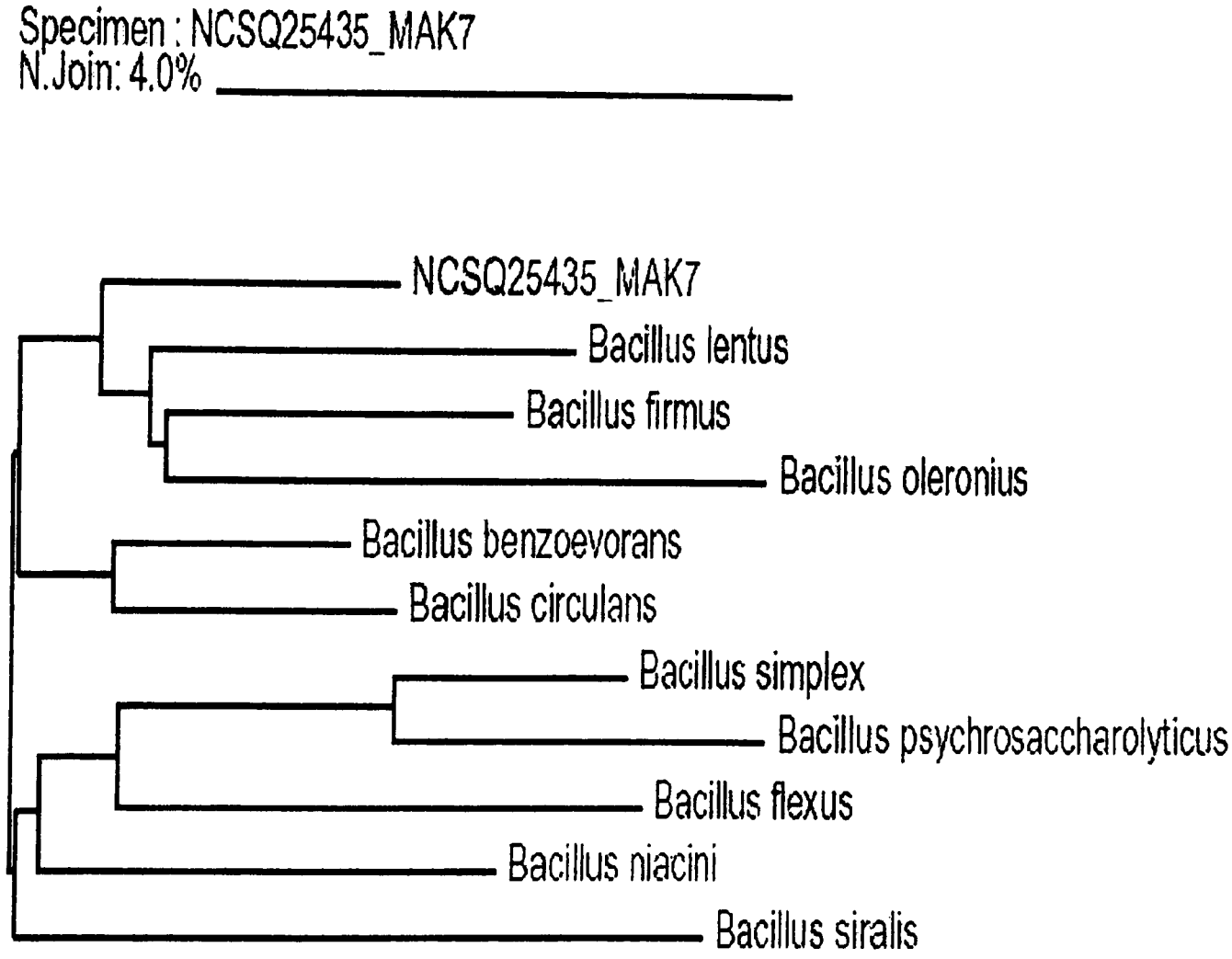


Figure 4.9: Phylogenetic tree for aerobic environmental strain of *Bacillus* MAK7 associated with other members of the genus *Bacillus* based on 16S rRNA gene sequences. The 16S rDNA sequences of MAK7 strain was determined and compared with those of related *Bacillus* sp.

```
EM_PRO:AF218239; AF218239 Bacillus sp. 16S ribosomal R  (1398 nt)
rev-comp initn: 2244 init1: 1240 opt: 2320  Z-score: 1761.2  bits: 336.8 E():
7.6e-90
banded Smith-Waterman score: 2320; 99.4% identity (99.4% similar) in 475 nt
overlap (474-1:905-1378)

                                        470       460       450
MAK7-                                 CTGGTTAGGTACCGTCAAGGTACCGGCAGT
                                      ::::::::::::::::::::::::::::::
EM_PRO GGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTACCGGCAGT
         880       890       900       910       920       930

         440       430       420       410       400       390
MAK7-  TACTCCGATACTTGTTCTTCCCTAACAACAGAGTTTTACGATCCGAAAACCTTCATCACT
       :::::::::::::::::::::::::: ::::::::::::::::::::::::::::::::
EM_PRO TACTCCGATACTTGTTCTTCCCTAACGACAGAGTTTTACGATCCGAAAACCTTCATCACT
          940       950       960       970       980       990

         380       370       360       350       340       330
MAK7-  CACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCC
         1000      1010      1020      1030      1040      1050

         320       310       300       290       280       270
MAK7-  CGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTA
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::: ::
EM_PRO CGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGG-TA
         1060      1070      1080      1090      1100      1110

         260       250       240       230       220       210
MAK7-  CGCATCGTGGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGCCCATC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CGCATCGTGGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGCCCATC
         1120      1130      1140      1150      1160      1170

         200       190       180       170       160       150
MAK7-  TGTAAGTGACAGCCGAAACCGTCTTTCAGCCTTTCCTCATGTGAGGAAAGGGATTATCCG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO TGTAAGTGACAGCCGAAACCGTCTTTCAGCCTTTCCTCATGTGAGGAAAGGGATTATCCG
         1180      1190      1200      1210      1220      1230

         140       130       120       110       100        90
MAK7-  GTATTAGCTCCGGTTTCCCGAAGTTATCCCAGTCTTACAGGCAGGTTGCCCACGTGTTAC
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GTATTAGCTCCGGTTTCCCGAAGTTATCCCAGTCTTACAGGCAGGTTGCCCACGTGTTAC
         1240      1250      1260      1270      1280      1290

          80        70        60        50        40        30
MAK7-  TCACCCGTCCGCCGCTAATCTTTGGGAGCAAGCTCCCTCAGATTCGCTCGACTTGCATGT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO TCACCCGTCCGCCGCTAATCTTTGGGAGCAAGCTCCCTCAGATTCGCTCGACTTGCATGT
         1300      1310      1320      1330      1340      1350

          20        10
MAK7-  ATTAGG-CACGCCGCCAGCGTTCGT
       :::::: ::::::::::::::::::
EM_PRO ATTAGGTCACGCCGCCAGCGTTCGTCCTGAGCCAGGATCAAACTC
         1360      1370      1380      1390
```

Figures 4.10: Sequence alignment of 16S rRNA gene sequences of aerobic environmental strain of *Bacillus* MAK7 versus the unclassified *Bacillus* strain, which is the nearest match (see Table 4.1).

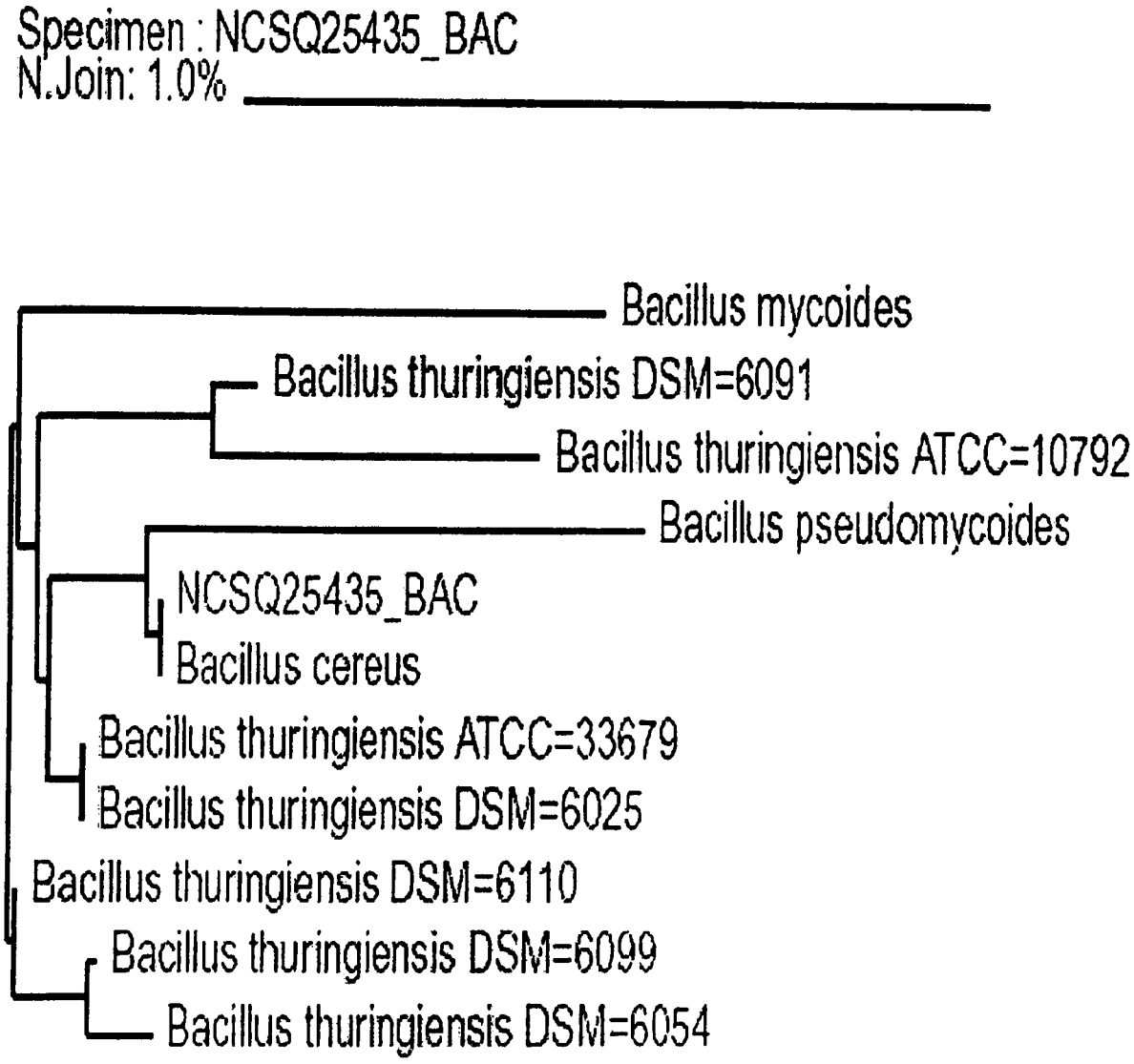


Figure 4. 11: Phylogenetic tree for aerobic laboratory strain of *Bacillus* associated with other members of the genus *Bacillus* based on 16S rRNA gene sequences. The 16S rDNA sequences of the laboratory strain of *Bacillus* was determined and compared with those of related *Bacillus* sp.

ref|NZ_ACMQ01000314.1| *Bacillus cereus* AH676 contig00559, whole genome shotgun sequence

gb|ACMQ01000314.1| *Bacillus cereus* AH676 contig00559, whole genome shotgun sequence

Length = 1626

Score = 1105 bits (598), Expect = 0.0

Identities = 598/598 (100%), Gaps = 0/598 (0%)

Strand = Plus/Plus

```
Query  1    ATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTC  60
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Query  421  GTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  481  GCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGA  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  551  GCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGA  610

Query  541  ATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGG  598
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  611  ATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGG  668
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Figure 4. 12: Sequence alignment of 16S rRNA gene sequences of aerobic laboratory strain of *Bacillus* versus *Bacillus cereus* AH676, which is the nearest match (see Table 4.2).

4.3. Conclusion

Genomic DNA of both environmental strain MAK7 and laboratory strain of *Bacillus* was extracted. The 16S rRNA gene from total genomic DNA of these strains was amplified using PCR assay and purified to complete removal of contaminating compounds. The amplified 16S rRNA genes were inserted into plasmid (vector) to obtain a longer and higher quality sequence for comparison with other organisms in the MicroSeq database. On the basis of 16S rRNA gene sequence similarity and phylogenetic analysis, laboratory strain of *Bacillus* was confirmed as belonging to the genus *Bacillus,* with highest sequence identity to *Bacillus cereus* (100%).

On the other hand the 16S rRNA gene sequence similarity of environmental strain of MAK7 using the MicroSeq database did not give a species level match and therefore the sequence obtained for this isolate was searched against the EMBL public database. However, the best match is to a sequence from an unclassified strain of *Bacillus* (99.4% similarity), associated with marine sponges.

Marine macroorganisms of which sponges predominate are a potential source for new bioactive compounds (Anand *et al.,* 2006). Numerous marine microorganisms especially bacteria living associated with sponges are actually producing the bioactive substances (Siebert *et al.,* 2004). Various natural products from sponges show striking structural similarities to metabolites of microorganisms, suggesting that these microorganisms may be involved in their biosynthesis or are the true source of these metabolites (Proksch *et al.,* 2002). These compounds have been shown to have a wide variety of

biotechnological activities (pharmacological activities) including antimicrobial, antiviral or generally cytotoxic properties, and antitumour (cancer treatment) (Hentschel *et al.*, 2001). Other products are currently being developed as analgesics to treat inflammation (Proksch *et al.*, 2002). The association between some marine bacterial strains and four species of sponges (*Echinodictyum* sp., *Spongia* sp., *Sigmadocia fibulatus* and *Mycale mannarensis*) was used for antibiotic production which was effective against four bacteria (*Bacillus subtilis, Escherichia coli, Vibrio parahaemolyticus, Vibrio harveyi*) and the fungal pathogen *Candida albicans* (Anand *et al.*, 2006).

Bacillus cereus is a Gram-positive, rod-shaped, facultative aerobe, which produces protective endospores and is beta hemolytic. It is commonly present in nature, frequently isolated from soil environments (Luksiene *et al.*, 2009). *Bacillus cereus* has the ability to grow on plants and adapt for growth in the intestinal tract of insects and mammals (Arnesen *et al.*, 2008). From these habitats it is easily spread to foods, therefore, it can be isolated from a wide range of foods and also food ingredients, including rice, spices, dried foods, dairy products (milk powder, milk substitute, and dairy desserts), fresh vegetables and meat products (Reyes *et al.*, 2007; Arnesen *et al.*, 2008). The bacterium is responsible for gastrointestinal disease and non-gastrointestinal disease (wound and eye infections plus systemic infections) (Ehling-Schulz *et al.*, 2004). It is a major cause of two different forms of food poisoning (*B. cereus* foodborne disease), the emetic and the diarrhoeal syndromes (Finlay *et al.*, 2002a and 2002b; Ehling-Schulz *et al.*, 2004; Clavel *et al.*, 2007). The emetic type of food poisoning is probably the most dangerous as it has been associated with life-threatening acute conditions such as fulminant liver failure. It is caused by ingestion of a preformed toxin in the food, while the diarrhoeal

poisoning is caused by variety of toxins that can be formed in the food but also in the small intestine (Reyes *et al.,* 2007). The emetic toxin that causes vomiting is an extremely stable (heat and acid), small ring-form dodecadepsipeptide (peptide) which is resistant to proteolytic degradation, pH extremes and high temperature (Finlay *et al.,* 2001a and 2001b; Ehling-Schulz *et al.,* 2004; Arnesen *et al.,* 2008).Whereas the diarrhoeal disease is caused by one or more heat-labile protein enterotoxins produced during vegetative growth of *B. cereus* in the small intestine (Ehling-Schulz *et al.,* 2004; Arnesen *et al.,* 2008). Interestingly, some bacterial spore formers of *B. cereus* can be beneficial as probiotics for humans (Hong *et al.,* 2005). Probiotics are generally defined as "living micro-organisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition" (Ouwehand *et al.,* 2002; Reid *et al.*, 2003).

In the next chapter, the bioenergetics of growth at high pH values will be investigated using *Bacillus* MAK7 and *Bacillus cereus*.

Bioenergetics of Growth of both Environmental Alkaliphilic Strain *Bacillus* MAK7 and Laboratory Isolate of *Bacillus cereus*

Chapter Five

5.1. Introduction

Microorganisms exhibit a great variety of lifestyles and can adapt to a wide range of physical and chemical factors to allow growth in extreme environments. Therefore, microorganisms are widely distributed in nature, each organism being defined and characterized according to specific parameters which are essential for its development e.g. thermophiles, halophiles, acidophiles and alkaliphiles (Cook, 2000; Goto *et al.*, 2005).

Microorganisms which grow in environments of extreme pH (high or low) have to maintain a relatively constant cytoplasmic pH that is compatible with the best function and structural integrity of the intracellular proteins, and which supports growth during fluctuations in external pH (Albers *et al.,* 2001; Sydow *et al.,* 2002; Padan *et al.,* 2005). The range of cytoplasmic pH is strongly dependent on the external milieu (Padan *et al.,* 2005). For example, neutrophilic bacteria that grow optimally at neutral pH have intracellular pH (pH_i) values from 7.5 to 8.0 (Booth, 1985). Acidophiles which grow optimally under acidic conditions exhibit intracellular pH values in the range of 6.5 to 7.0 (Cook, 2000). However, under alkaline conditions, alkaliphilic bacteria must maintain their intracellular pH up to approximately 2 pH units more acidic than the external pH (i.e. inverted pH gradient) (Krulwich *et al.,* 1998; Olsson *et al.,* 2003; Albers *et al.,* 2001; Sydow *et al.,* 2002; Satyanarayana *et al.,* 2005; Liu *et al.,* 2008). This means alkaliphilic bacteria that grow well over an external pH range from 9 to 11.5 maintain their cytoplasmic pH values between 8.4 and 9.0 (pH_i is 0.5 to 2.5 pH units

lower than external pH_o) (Booth, 1985; Krulwich *et al.*, 1997; Peddie *et al.*, 1999; Cook, 2000; Cook *et al.*, 2003).

It has been accepted that cation/proton antiporters (both Na^+/H^+ and K^+/H^+ antiporters) in cells of alkaliphiles play a dominant role in pH homeostasis in alkaline environments by exchanging cytoplasmic cations for external protons to achieve a cytoplasmic pH considerably less alkaline than the external pH (Kitada *et al.*, 1994; Padan *et al.*, 2005; Wei *et al.*, 2007). Therefore, extreme alkaliphiles face several bioenergetic challenges (problems) in chemiosmotic energy generation and solute transport driven via the proton motive force across their membranes (Peddie *et al.*, 1999; Konings *et al.*, 2002). Since the total proton motive force (PMF or Δp) is the sum of the membrane electrical potential ($\Delta\Psi$; negative inside) and the pH gradient (ΔpH, acid out), then a large ΔpH generated by alkaliphilic bacteria in the opposite direction results in suboptimal Δp values for ATP synthesis (Cook *et al.*, 2003).

In contrast, if alkaliphilic bacteria used sodium as a coupling ion for solute transport, motility and ATP synthesis, this will help circumvent the bioenergetic problem (i.e. low proton motive force), because both ΔpNa^+ and $\Delta\Psi$ are generally oriented in the same direction and therefore add driving force to one another (Peddie *et al.*, 1999; Cook *et al.*, 2003). It was suggested that alkaliphiles might use Na^+ as the coupling ion for ATP synthesis (Guffanti *et al.*, 1981). However, Na^+-driven synthesis of ATP has only been demonstrated in halotolerant marine bacteria (Sakai-Tomita *et al.*, 1991). Furthermore, alkaliphiles (non-marine bacteria) exhibit a respiratory chain that pumps only protons,

with F_1/F_0–ATPases located in the cytoplasmic membrane (Krulwich and Guffanti, 1989b).

Therefore, in the present chapter the bioenergetics of cells adapted to grow at different pH values in Horikoshi medium of both *Bacillus* MAK7 and the laboratory strain of *Bacillus cereus* were examined using silicone oil technique. In section 5.2.1 the intracellular volume of both strains were investigated. Furthermore, membrane potentials ($\Delta\Psi$) at different external pH values of pH 7, 8.5 and 10 were also measured for both strains (section 5.2.2). Internal pH was also investigated for both *Bacillus* MAK7 and the laboratory strain of *Bacillus cereus* cells grown in different pH media (section 5.2.3).

5.2. Results and Discussion

5.2.1. Determination of Intracellular Volume of *Bacillus* MAK7 and *Bacillus cereus*

The intracellular volume (ICV) must be determined to calculate the concentration ratios of probes across the cytoplasmic membrane and hence the membrane potential ($\Delta\psi$) and internal pH (pH_i). The intracellular volume of bacterial cells was measured by centrifugation through silicone oil as described in section 2.17.1 based on the method described by Gimmler and Schirling (1978). Determination of both pellet volume (PV) using tritiated water (^3H_2O) and extracellular volume (ECV) using ^{14}C-dextran for all cells grown over a wide pH range of culture media enables us to calculate the intracellular volume by the equations given in Hard and Gilmour (1996).

^{14}C-dextran is not taken into cells due to the complex structure of this high molecular weight polymer preventing diffusion of ^{14}C-dextran across the cell membrane. In contrast, the tritiated water ($^{3}H_2O$) can diffuse into cell. The ratio of ^{3}H between the pellet and supernatant fractions corresponds to the total volume of the pellet (see Figure 2.4). A percentage of this volume is due to fluid packed between the cells. The ratio of ^{14}C (also between pellet and supernatant) corresponds to this volume and thus the subtraction of the ^{14}C volume or extracellular volume from the ^{3}H total pellet volume (including excess liquid trapped between the cells) results in the estimation of the intracellular volume alone.

In order to measure intracellular volume based on the distribution of isotopes, the time course of uptake for ^{14}C-dextran probe was determined (using *B. cereus* cells) in order to ensure there was no active uptake or efflux of the isotope after the initial distribution between cells and medium, and that the uptake was rapid and levelled off after a short time. The results indicated that 5 minute incubation periods were suitable for pellet volume (PV), extracellular volume (ECV) and the subsequent intracellular volume determination (ICV) (Figures 5.1 and 5.2).

Table 5.1 illustrates the measurements of intracellular volume of both environmental *Bacillus* MAK7 strain and laboratory strain of *Bacillus cereus* cells adapted to grow at different pH values (7, 8.5 and 10) in Horikoshi medium. It was found that cells of both strains grown over a range of pH media showed different volumes. This was concluded to be a direct result of external pH stress and is particularly clear for *Bacillus cereus* strain.

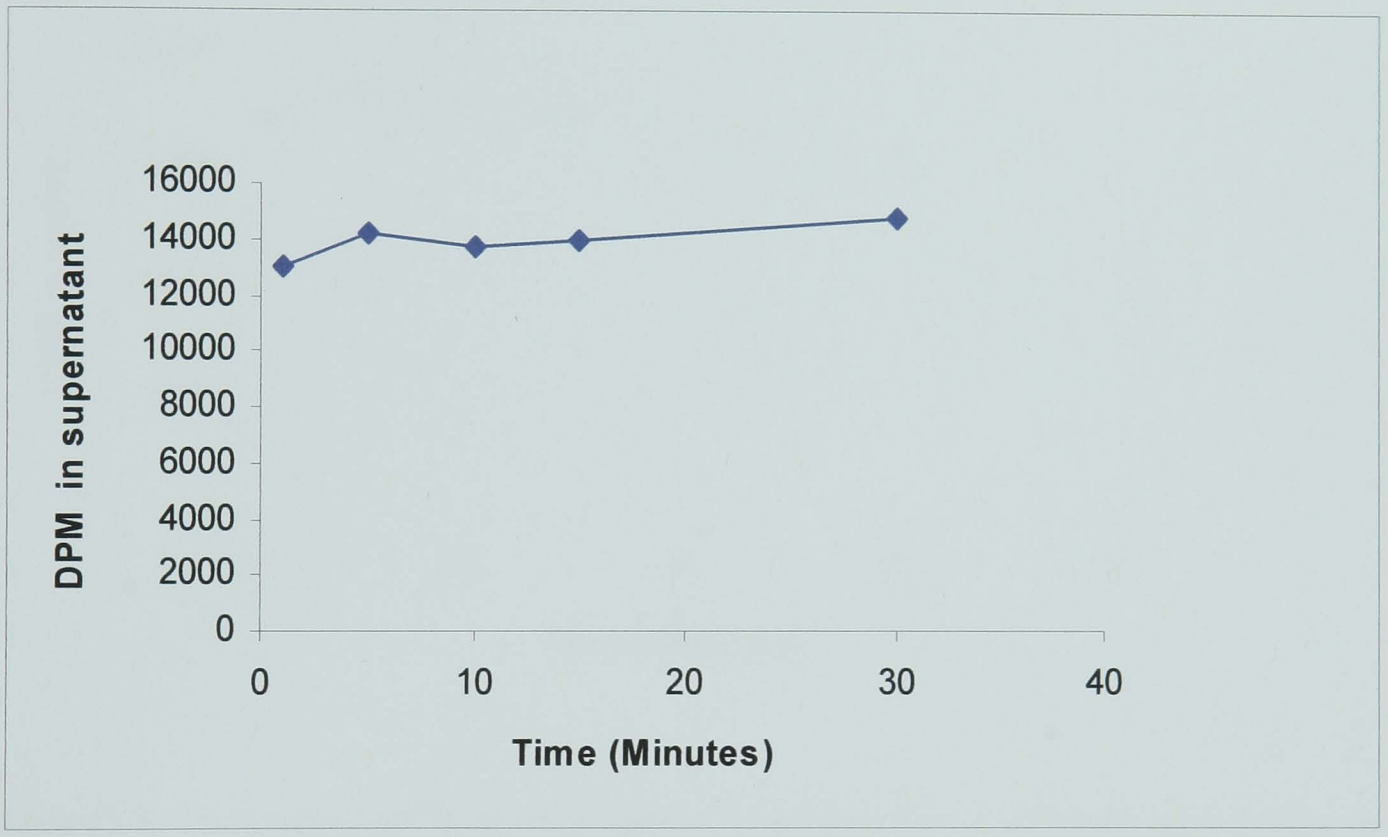

Figure 5.1: Time course of ^{14}C-dextran uptake by *Bacillus cereus*. DPM in supernatant during the 30 minute incubation period. Cells were grown at pH 10 in Horikoshi media overnight at 25°C. Means of three replicates were used.

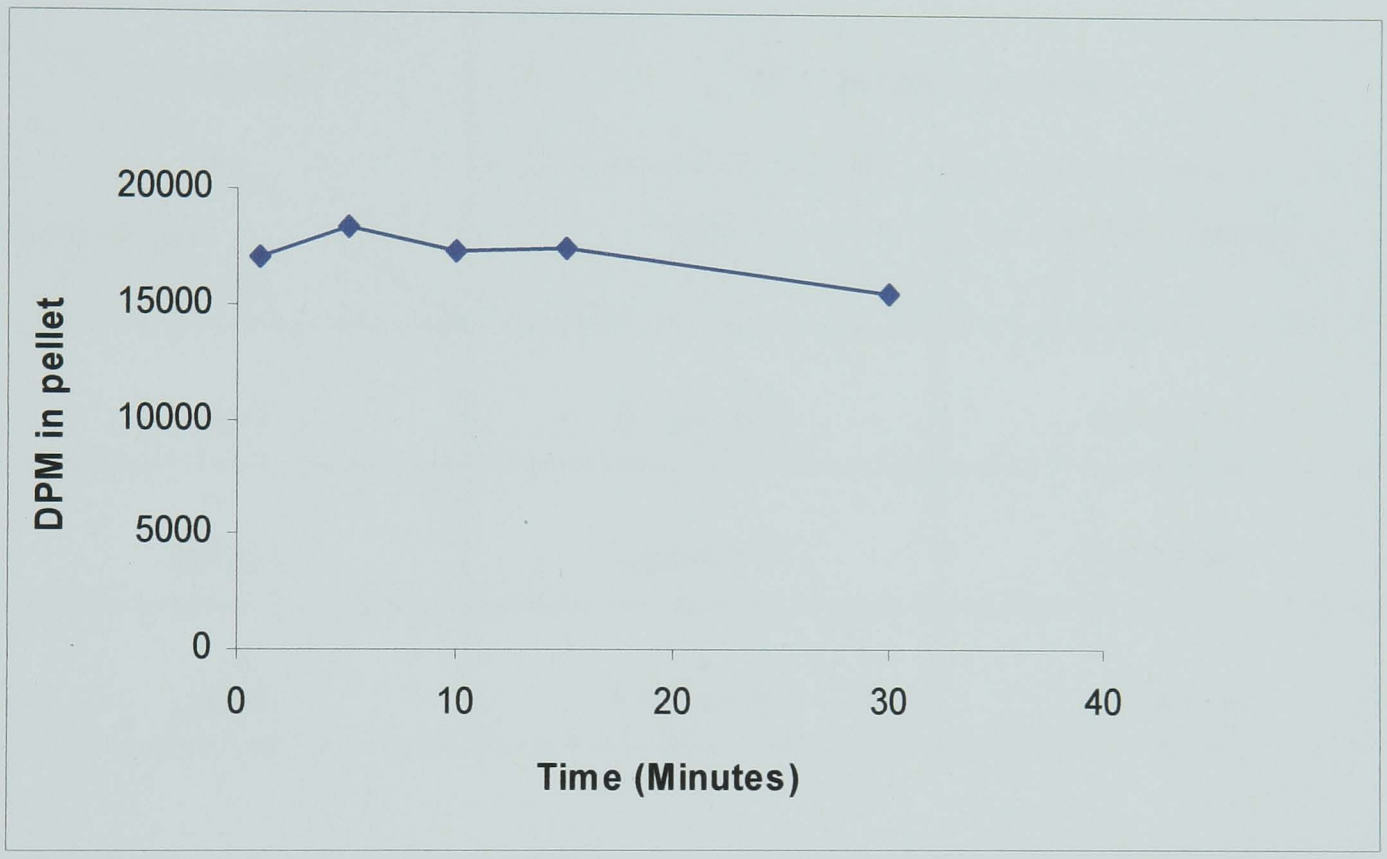

Figure 5.2: Time course of ^{14}C-dextran uptake by *Bacillus cereus*. DPM in pellet during the 30 minute incubation period. Cells were grown at pH 10 in Horikoshi media overnight at 25°C. Means of three replicates were used.

Samples / Growth pH ⇩ ⇨	ICV (µl mg^{-1} protein)	
	MAK7	***Bacillus cereus***
pH 7	7.26 ± 0.98	3.4 ± 0.50
pH 8.5	6.86 ± 0.32	4.4 ± 0.96
pH10	7.53 ± 0.57	7.2 ± 1.60

Table 5.1: Intracellular volume (ICV) of both environmental strain *Bacillus* MAK7 and laboratory strain of *Bacillus cereus* as a function of external pH.

20 ml of cells adapted to grow at different pH values in Horikoshi medium overnight at 25°C were transferred to 50 ml Falcon tubes and harvested and then the pellet was resuspended in 8 ml of fresh Horikoshi medium of the same pH. Means and standard error for three replicates are shown.

The intracellular volume of *Bacillus cereus* strain varied over a wide range from 3.4 µl mg^{-1} soluble protein at external pH 7 to 7.2 µl mg^{-1} soluble protein at external pH 10 when compared to the intracellular volume of MAK7 strain which was 6.86 µl mg^{-1} soluble protein to 7.53 µl mg^{-1} soluble protein over the same external pH range. The increase in cell volume of *Bacillus cereus* strain may be considered to be a self defence mechanism of this bacterium to the extreme pH of the culture medium.

The cells of environmental MAK7 strain have a higher intracellular volume than *Bacillus cereus* strain at external pH 7 and 8.5. On the other hand the intracellular volumes of both strains were similar in value at external pH 10. The similarity in these larger cell volumes may be a response of the cells to external pH stress. The stressed cells may not have separated after dividing, resulting in clumps of cells being measured and not single cells, or the onset of cell division may have been delayed by the unfavourable pH conditions. It is certainly true that published values for intracellular volumes tend to be lower than found in the present study. For example, *Streptococcus cremoris* grown at pH 7 had an ICV of 3.8 ± 0.5 µl mg^{-1} protein (Brink and Konings, 1982), and the thermoalkaliphilic *Bacillus* sp. strain TA2.A1, which was able to grow from pH 7.5 to 10 with no significant change in its specific growth rate, exhibited an ICV of 3.9 ± 0.40 µl mg^{-1} protein (Olsson *et al.*, 2003). A higher ICV was reported for *Clostridium paradoxum*, which was 5 ± 1.8 µl mg^{-1} protein at pH 9.3 (Cook *et al.*, 1996), but the ICV measured for *Thermoanaerobacter wiegelii* bacterium grown over the pH range 5.1-7.7 was only 2.20 µl mg^{-1} protein (Cook, 2000).

5.2.2. Determination of Membrane Potential ($\Delta\Psi$) of *Bacillus* MAK7 and *Bacillus cereus*

It is essential for bacterial cells to maintain a stable internal balance when exposed to an external stress. Hence is very important to study the membrane potential and internal pH of cells in relation to change in external pH. Membrane potential is defined as the electrical potential difference that would be measured between two identical reference electrodes positioned on opposite sides of a cell membrane. It is a property of the whole system and is independent of the spatial location of the electrodes (Rottenberg, 1979).

Membrane potential ($\Delta\Psi$) that is dependent on the Na^+/H^+ antiporter plays a crucial role in pH homeostasis at high external pH values in alkaliphilic *Bacillus sp.* (Kitada *et al,* 1997). Determination of both $\Delta\Psi$ and pH gradient (ΔpH) across a membrane enables us to calculate the proton electrochemical potential difference or proton motive force (Δp) by the equation given in section 2.17.3. Measurements of Δp for cells growing or surviving at different external pH values are very important for an experimental evaluation of the chemiosmotic hypothesis and are also an indicator of the coupling in energy conversion membrane systems (Rottenberg, 1979).

The most reliable method to measure membrane potential is the use of microelectrodes across the membrane, but bacterial cells are too small. Therefore, an indirect method involving ^3H-TPP^+ (tetraphenylphosphonium) as a probe was used for $\Delta\Psi$ determination, using the silicone oil centrifugation technique as described by Rottenberg

(1979 and 1989). TPP^+ a positively charged ion, is distributed across the membrane according to the membrane potential.

The time course of ^3H-TPP^+ uptake was determined using *B. cereus* cells; the uptake was rapid and levelled off after a short time (Figures 5.3 and 5.4). This indicated that this probe was suitable for membrane potential determination, if an incubation period of 5 minutes was used.

Table 5.2 shows the measurements of $\Delta\Psi$ as a function of the external pH for both environmental strain MAK7 and laboratory strain *B. cereus* cells grown over a range of external pH values from 7 to 10 in Horikoshi media. It was found that both strains showed similar results, except at pH 8.5, where MAK7 strain showed a larger membrane potential than *B. cereus*. Both strains (MAK7 and *B. cereus*) generated substantial $\Delta\Psi$ values over the entire external pH range with a gradual increase from -135.6 mV at external pH 7 to -172.0 mV at external pH 10 and from -139 mV at external pH 7 to -171 mV at external pH 10 respectively. It is clear that the membrane potential is increasing with increasing external pH (Table 5.2).

Similar results were found by others (Krulwich and Guffanti, 1992; Cook *et al.,* 1996; Olsson *et al.,* 2003; Chen *et al.,* 2007). For example, the obligately alkalophilic strains *Bacillus alcalophilus* and *B. firmus* RAB, showed an increase in $\Delta\Psi$ from -80 mV at external pH 8.0 to -150 mV at external pH 11.5 (Krulwich, 1986).

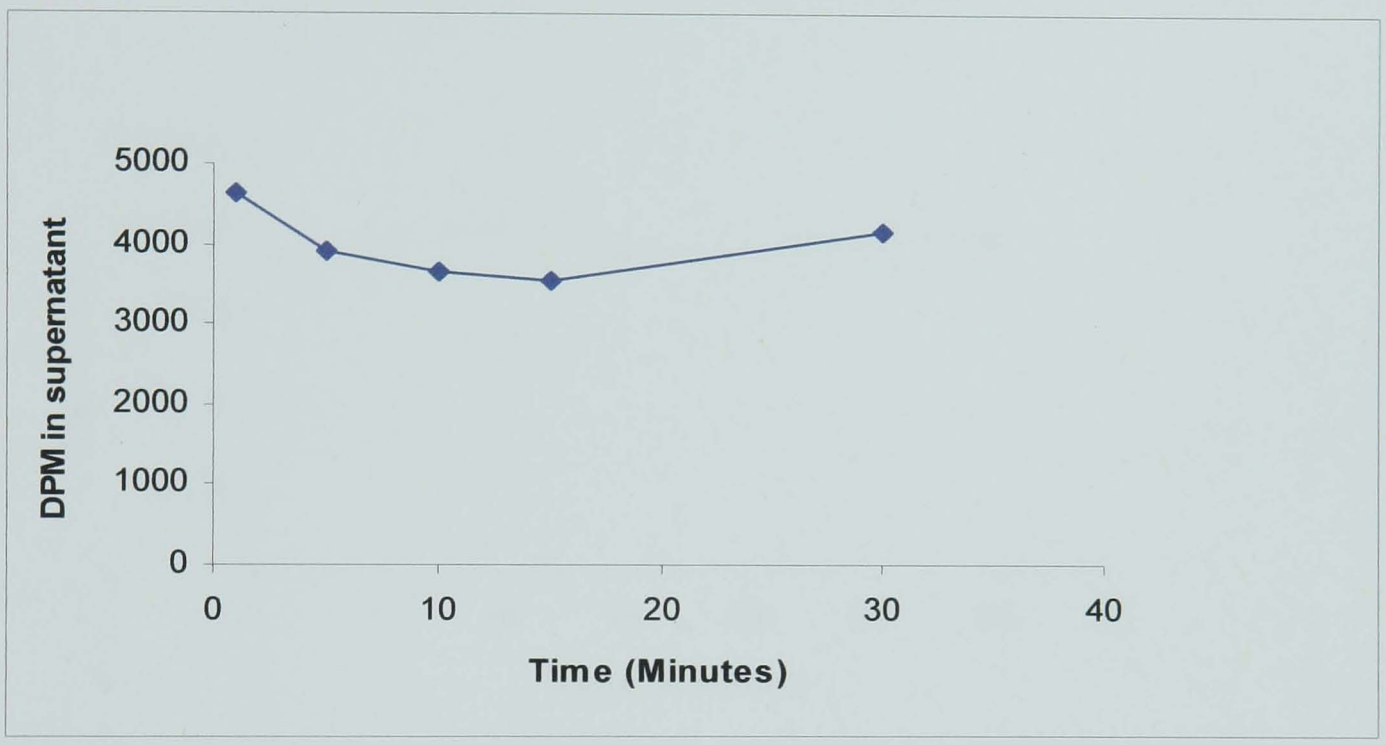

Figure 5.3: Time course of ^{3}H-TPP^{+} uptake by *Bacillus cereus*. DPM in supernatant during the 30 minute incubation period. Cells were grown at pH 10 in Horikoshi media overnight at 25°C. Means of three replicates were used.

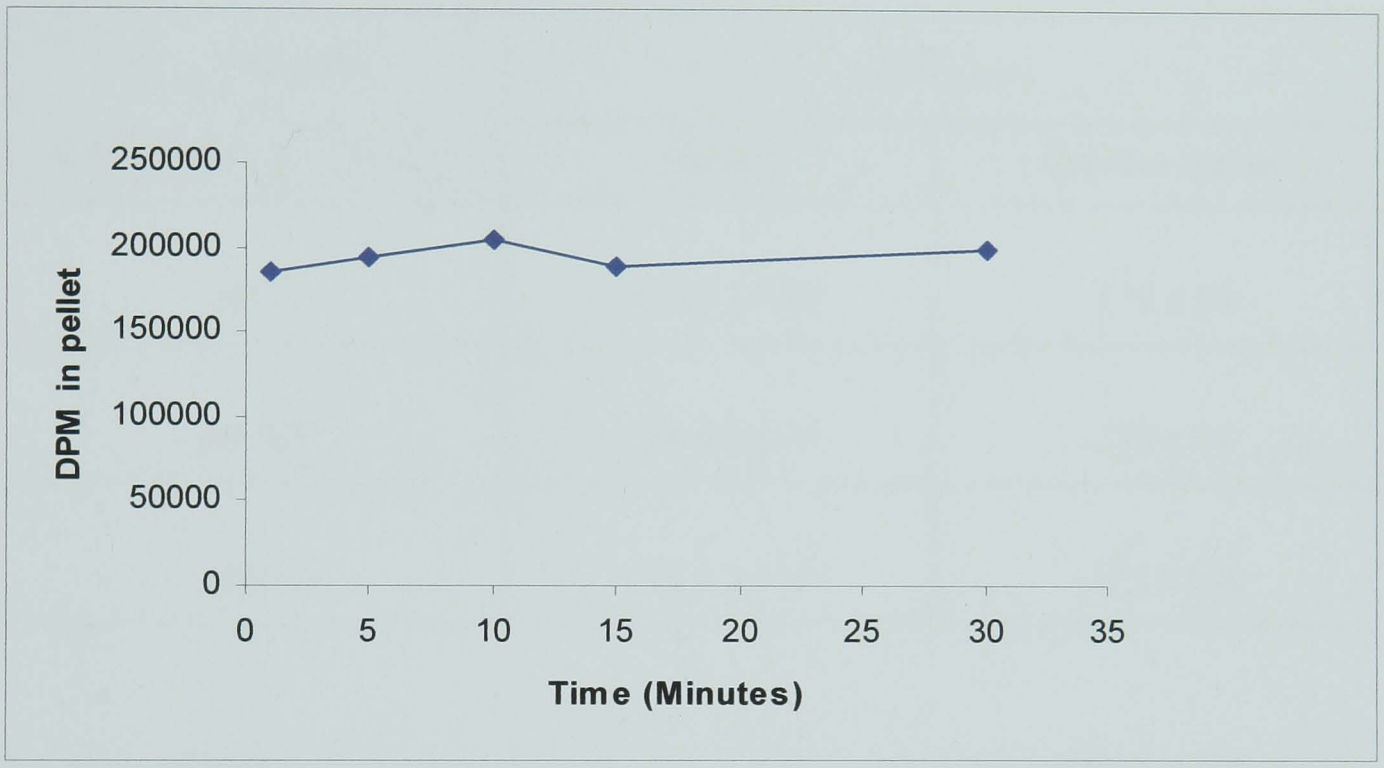

Figure 5.4: Time course of ^{3}H-TPP^{+} uptake by *Bacillus cereus*. DPM in pellet during 30 minute incubation period. Cells were grown at pH 10 in Horikoshi media overnight at 25°C. Means of three replicates were used.

Samples / Growth pH ⇩	$\Delta\Psi$ (mV)	
	MAK7	***Bacillus cereus***
pH 7	-135.6 ± 3.20	-139 ± 10
pH 8.5	-166.6 ± 2.60	-154 ± 17
pH10	-172.0 ± 4.96	-171 ± 9.8

Table 5.2: Membrane potential ($\Delta\Psi$) of both environmental strain MAK7 and laboratory strain of *Bacillus cereus* as a function of external pH.

20 ml of cells adapted to grow at different pH values in Horikoshi medium overnight at 25°C were transferred to a 50 ml Falcon tube and harvested and then the pellet was resuspended in 8 ml of fresh Horikoshi medium of the same pH. Means and standard error for three replicates are shown.

The increasing $\Delta\Psi$ of both strains at high external pH suggested that the membrane potential is the driving force for the activity of Na^+/H^+ antiporter (Grant and Horikoshi, 1992), which is the key to overcoming the bioenergetic problems regarding energy generation by the chemiosmotic process due to the abnormal pH gradient across the cell membrane. Therefore, maintaining an acidic internal pH by exchanging H^+ for Na^+ requires more activity of Na^+/H^+ antiporter in order for bacterial cells to maintain a stable internal pH.

This association was furthermore supported by measurements of membrane potential of cells of an alkaliphile that were suspended in sodium free medium, since membrane potential measured in sodium carbonate buffer at pH 10.5 exhibited higher values (-123 mV) than membrane potential for cells resuspended in potassium carbonate buffer under the same conditions (-50 mV) (Al- Saidi, 1995).

5.2.3. Determination of Internal pH (pH_i) and Δ pH of *Bacillus* MAK7 and *Bacillus cereus*

The measurement of cytoplasmic pH of cells adapted to grow and survive at different external pH values is very important for an experimental evaluation of the limits of alkaline pH homeostasis. The cytoplasmic pH of bacteria has usually been calculated from controlled pH (pre-set external pH) and the pH gradient (ΔpH) across the cytoplasmic membrane of the cell (Padan *et al.*, 2005).

Determination of cytoplasmic pH was carried out using the silicone oil centrifugation method as described by Rottenberg, (1979) (section 2.17) to separate cell pellets from their external media allowing the measurement of both intracellular and extracellular volumes of cells. The probe for ΔpH is the distribution of a weak base across the cell membrane according to the pH gradient (ΔpH, acid inside relative to outside). Therefore, the probe accumulates within the more acidic cytoplasm that bacteria maintain when the external pH is high (section 2.17.3). In the current study the labelled radioisotope probe ^{14}C-methylamine, with a pKa of 10.6, was used to measure the cytoplasmic pH of cells of both environmental strain MAK7 and laboratory strain of *Bacillus cereus* grown over a range of external pH values from 7 to 10 in Horikoshi media.

The time course of uptake for ^{14}C-methylamine probe was carried out using *B. cereus* cells in order to ensure there was no active uptake or efflux of the isotope after the initial distribution between cells and medium. The uptake was rapid and levelled off after a short time (Figures 5.5 and 5.6). The results indicated that a 5 minute incubation period was suitable for cytoplasmic pH determination.

Table 5.3 shows the measurements of internal pH, ΔpH and Δp as a function of external pH for both environmental strain MAK7 and laboratory strain of *Bacillus cereus* cells grown over a range of external pH values. The results very clearly indicate that the cells of both MAK7 and *Bacillus cereus* strains exhibited similar internal pH values at external pH 7 and 8.5.

Figure 5.5: Time course of ^{14}C-methylamine uptake by *Bacillus cereus*. DPM in supernatant during 30 minute incubation period. Cells were grown at pH 10 in Horikoshi media overnight at 25°C. Means of three replicates were used.

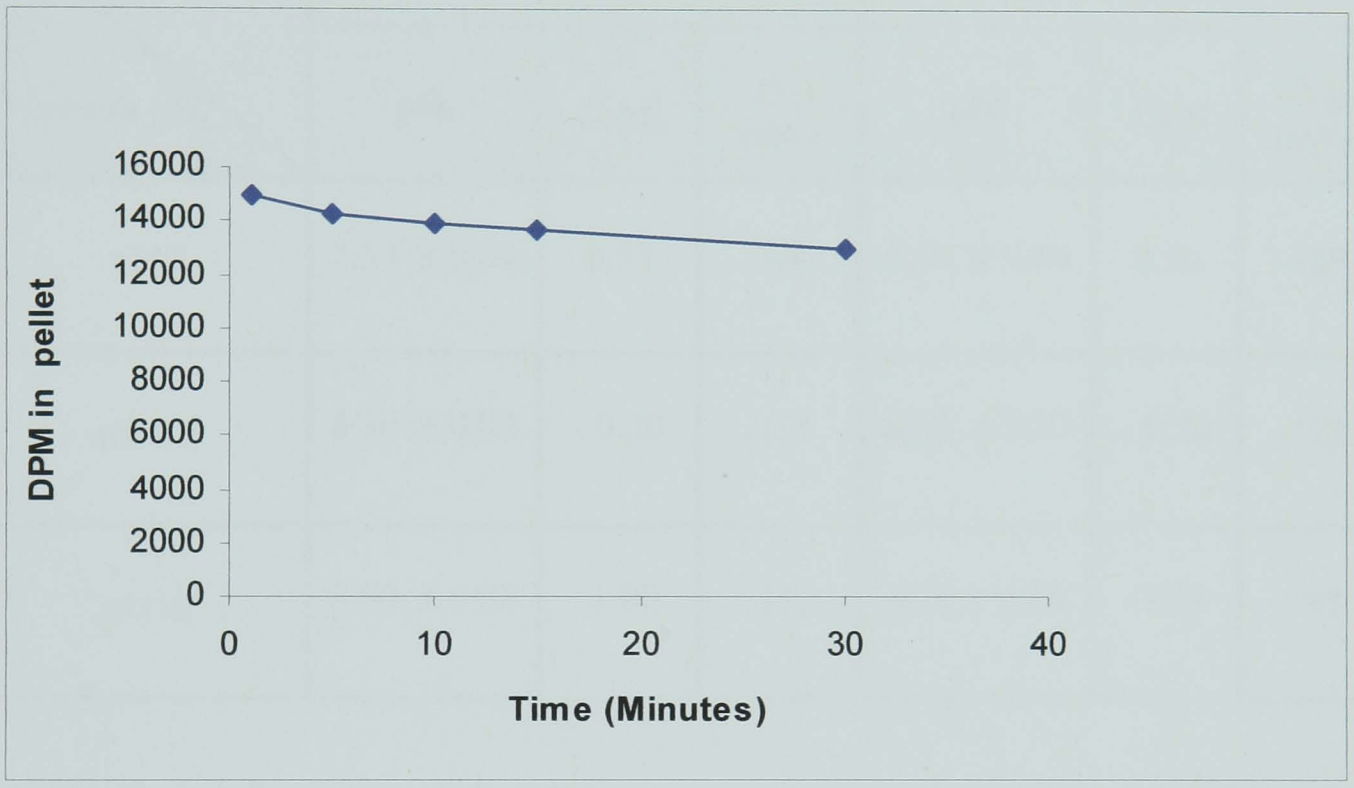

Figure 5.6: Time course of ^{14}C-methylamine uptake by *Bacillus cereus*. DPM in pellet during 30 minute incubation period. Cells were grown at pH 10 in Horikoshi media overnight at 25°C. Means of three replicates were used.

Samples ⇨ / Growth pH ⇩	MAK7			*Bacillus cereus*		
	pH_i	ΔpH	Δ p (mV)	pH_i	ΔpH	Δ p (mV)
pH 7	7.53 ± 0.04	0.53	-168.	7.50 ± 0.04	0.50	-169
pH 8.5	8.30 ± 0.02	- 0.20	-155	8.00 ± 0.33	- 0.50	-123
pH10	9.00 ± 0.08	-1.00	-112	8.76 ± 0.28	-1.24	- 97

Table 5.3: Internal pH (pH_i), Δ pH generation and proton motive force (Δp) measurements from cells of both environmental strain MAK7 and laboratory strain of *Bacillus cereus* as a function of external pH.

20 ml of cells adapted to grow at different pH values in Horikoshi medium overnight at 25°C were transferred to a 50 ml Falcon tube and harvested and then the pellet was resuspended in 8 ml of fresh Horikoshi medium of the same pH. Means and standard error for three replicates are shown.

However, there was a slight (but statistically insignificant) difference between the strains at pH 10, where the pH_i was 9.00 and 8.76 respectively. At pH 7 only, the internal pH values were found to be higher than external pH, i.e., cytoplasmic pH more alkaline for both strains (pH_i is 7.53 and 7.50 respectively). Total proton motive force (Δp) values for cells of both strains at external pH 7 were calculated to be -168 and -169 mV, respectively.

In the same series of experiments, the ΔpH was shown to be reversed at external pH 8.5 and pH 10 i.e. the internal pH values were found to be lower than external pH (internal pH is more acidic than external pH). The maximum internal pH measured at the upper limit of external pH (pH 10) was 9.0 (for MAK7 strain) which is 1 unit less than the external pH. Δp was found to decrease when the external pH was increased from 8.5 to 10, from approximately -155 to -112 mV for MAK7 strain and from -123 to -97 for *Bacillus cereus* strain. However, the Δp generation is in the correct direction for driving ATP synthesis. These measurements of internal pH and ΔpH are compatible with the findings of other workers such as Horikoshi and Akiba, (1982); Krulwich and Guffanti, (1983); Krulwich, (1986) and Horikoshi (2008).

The results suggest that both strains were able to grow at high external pH levels despite their pH_i becoming more alkaline. Nevertheless, the pH_i levels are always lower than the external pH, when the external pH is set at 8.5 or above (Table 5.3).

5.3. Conclusion

Moderate environments are normally important for life to continue. The essential problem for microorganisms which are adapted to grow and survive at alkaline pH values of 9 to 10 is the maintenance of a relatively acidified cytoplasm and therefore. to maintain a large chemical proton gradient via proton motive force or sodium motive force across the membrane. The maintenance of a more neutral internal pH allows the cells to function normally.

To investigate the effect of the pH of the culture medium on the bioenergetics of growth of both environmental MAK7 strain and laboratory strain of *Bacillus cereus,* the ICV, $\Delta\psi$ and cytoplasmic pH (pHi) of cells were all examined. The use of radiolabelled isotopes in the silicon oil experiments described in the present study produced plausible results. This method allows the calculation of a volume for a pellet of cells through the ratio of two isotope species; tritiated water ($^{3}H_2O$) and ^{14}C-dextran.

The cell volume of environmental MAK7 strain and the laboratory strain of *Bacillus cereus* was determined in cells adapted to grow at different external pH values of 7, 8.5 and10. It can be seen from this chapter that there was correlation between cell volume and changes in external pH stress, i.e., the intracellular volume of both strains was increased with increasing external pH. This increase in cell volume may be considered to be a self defence mechanism of these bacteria to the extreme pH of the culture medium. On the other hand, the intracellular volume of both strains was similar in value

to each other at external pH 10. The similarity in these larger cell volumes may suggest that this is a response of the cells to external pH stress.

The intracellular pH (pH_i) of cells was determined by the distribution of ^{14}C-methylamine between the cell and the surrounding medium at different external pH values. The internal pH for environmental MAK7 strain and *Bacillus cereus* cells studied was lower than the external pH value suggesting that the cells actively tried to maintain a near natural internal pH (Table 5.3). However, at external pH 10, the pH_i for both strains was around pH 9. The maintenance of a more acidic pH_i at extremes of external pH may be the result of the cell membrane being largely impermeable to H^+ or the bacterial cells may be capable of pumping H^+ into the cells. The maintenance of a more acidic internal pH at extremes of external pH is also dependent on the presence of sodium, which is exchanged from cytoplasm into the medium by Na^+/H^+ antiporters (section 1.7.2). The intracellular pH measurements for *Bacillus cereus* cells were slightly closer to neutral than those from environmental MAK7 strain. This indicates that either *Bacillus cereus* is better able to maintain a more acidic internal pH or that environmental MAK7 strain is better able to function over a range of internal pH values.

Additionally, ^3H-TPP^+ (tetraphenylphosphonium) was used for membrane potential determination using the silicone oil centrifugation technique at a range of external pH values. At high external pH the membrane potential of the cells was found to be highest and at low external pH the membrane potential was lowest for both strains. As a consequence, the proton motive force (Δp) was decreased, in the opposite direction

resulting in suboptimal Δp values for ATP synthesis at high external pH. Therefore, the magnitude of membrane potential is dependent on the external pH (Kitada *et al.*, 1997) and it plays a key role in allowing cells to grow at extremely alkaline pH values.

In the next chapter, the physiology of growth of these two alkaliphilic strains will be examined in more detail.

Physiology of Growth of Alkaliphilic Strain *Bacillus* MAK7 and Laboratory Isolate of *Bacillus cereus*

Chapter Six

6.1. Introduction

Extremophilic microorganisms have the ability to grow and survive under extreme environmental conditions that would be uncomfortable for the normal functioning of humans (Gomes and Steiner, 2004). Several of these extremophiles are able to withstand multiple extremes (Irwin and Baird, 2004). For example, bacteria that grow in soda lakes have to adapt to both high pH and high concentrations of Na^+, and therefore, adapt to low water activity and scarcity of some micronutrients, as well as the alkaline pH (Wei *et al.,* 2007). To grow and survive in hypersaline habitats, some halophiles (mainly Archaea) accumulate high concentrations of salts, such as KCl, to maintain osmotic balance (van den Burg, 2003). Their soluble enzymes are, therefore, themselves adapted to a high salt environment (Madern and Zaccai, 2004). It has been accepted that the Na^+/ H^+ and K^+/ H^+ antiporters in these organisms play important roles in pH homeostasis at alkaline pH by exchanging cytoplasmic cations for external protons to reach an intracellular pH considerably less alkaline than the external pH (Kitada *et al.,* 1994; Wei *et al.,* 2007).

In the present chapter, both *Bacillus* MAK7 and *B. cereus* cells will be grown at pH 10 and the role played by Na^+ or K^+ in growth at pH 10 will be examined using measurements of growth and respiration rates.

The capacity of bacteria to adapt to grow and survive at both high salinity and high alkaline pH values (over pH 9) is of great importance due to the biotechnological potential of alkali-tolerant enzymes and polymers. It has been suggested that

haloalkaliphilic microorganisms can be used for waste water treatment due to their ability to grow successfully under harsh conditions (Detkova and Pusheva, 2006). It has been shown in the previous chapter that the internal pH of both environmental strain *Bacillus* MAK7 and laboratory strain *B. cereus* cells is more acidic than the external pH of the medium, when the external pH is 8.5 or above. Nevertheless, the pH_i for *Bacillus* MAK7 and *B. cereus* cells at external pH 10 is around pH 8.8 to 9.0 (Table 5.3). Therefore, in this chapter, the effect of pH_i on intracellular enzyme activities (malate dehydrogenase, fumarase and hexokinase) was determined using crude cell-free extracts from both strains.

6.2. Results and Discussion

6.2.1. Growth Rate and Growth Yield of *Bacillus* MAK7 and *Bacillus cereus* Strains in Comparison with *Bacillus subtilis*

The generation time is the period of time that is required for a cell parameter to double in size e.g. cell number of dry weight (Gerday *et al.,* 2000). The generation time is increased at high alkaline pH values, this association is strongly correlated with an apparent decrease in the ability to tightly regulate cytoplasmic pH and with the appearance of chains of cells (Sturr *et al.,* 1994). Therefor, the value of the generation time of cells growing or surviving at different external pH values is very important for experimental evaluation of the cellular stress. However, the growth yield of the alkalophiles under alkaline conditions is comparable with that of the neutrophilic species, this implies the alkaliphiles must possess alternative effective mechanisms of energy transduction and accumulation (Muntyan *et al.,* 2005). Thus, this experiment was

carried out to determine specific growth rates and doubling times of *Bacillus* MAK7 and *B. cereus* cells grown at pH 10 in comparison with *Bacillus subtilis* cells grown at pH 7. This experiment will test whether alkaliphilic bacteria may be more efficient growing at pH 10 than neutrophilic bacteria growing at pH 7 in Horikoshi medium. The optical density (O.D) at 600 nm was measured against distilled water blank immediately after inoculation and then every hour over an incubation period on a rotary shaker at 250 rpm for all three strains (environmental strain *Bacillus* MAK7 and laboratory strain of *Bacillus cereus* cells grown in pH 10 Horikoshi medium at 25°C as alkaliphilic bacteria and *Bacillus subtilis* cells grown at pH 7 in the same medium at 37°C as neutrophilic bacteria). The growth rate of three strains was plotted against time of incubation to calculate the generation time (Figure 6.1).

Cells of both alkaliphilic strains (MAK7 and *B. cereus*) grown at pH 10 in Horikoshi medium showed shorter generation times than the neutrophilic *B. subtilis* cells grown at pH 7 in the same medium. The generation time of these strains was 55, 40 and 74 minutes respectively (Appendix D). The results suggest that both alkaliphilic bacteria, MAK7 and *B. cereus,* may be more efficient growing at external pH 10 than neutrophlic bacteria growing at pH 7. However, this is almost certainly not the case, the results reflect the poor growth of *B. subtilis* cells on Horikoshi medium, which is clearly sub-optimal for growth of this strain.

Alkaliphilic *Bacillus firmus* OF4, when grown at pH 10.6, exhibited a similar generation time to that found in this study for *B. cereus,* it was approximately 38 minutes (Sturr *et al.,* 1994). A similar generation time of 45 minutes was reported when *Alkaliphilus transvaalensis* gen. was grown at pH 10.5 and 40°C (Takai *et al.,* 2001).

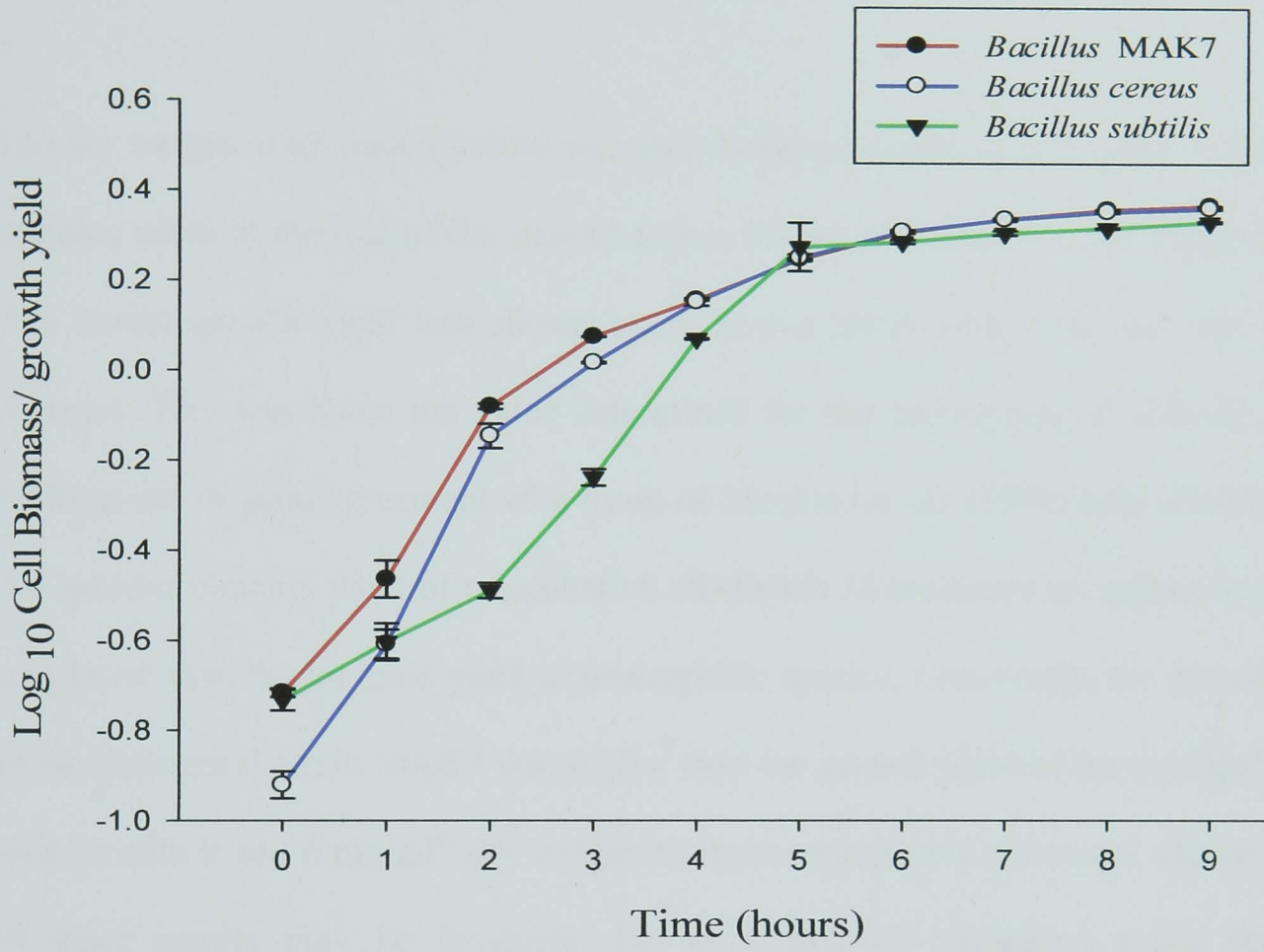

Figure 6.1: Growth rate of environmental strain *Bacillus* MAK7 and the laboratory strain of *Bacillus cereus* in comparison with *Bacillus subtilis.* Cells of MAK7 and *B. cereus* were grown in Horikoshi medium (pH 10) at 25°C. Whereas cells of *B. subtilis* were grown at pH 7 in the same medium at 37°C. Growth was determined by measuring the OD at 600 nm every hour. Each point represents the average from triplicate samples plus or minus standard error.

The similarities in generation times may indicate that these alkaliphilic strains are all stressed at higher pH values. Conversely, these findings do not agree with the results reported for *Alkaliphilus metalliredigens* cells grown at pH 9.5, where a generation time of approximately 4 h at 22°C was observed (Ye, *et al.,* 2004).

The dry weight of all three bacteria was also measured as described in section 2.9 using samples taken at the end of the growth curves shown in Figure 6.1 (see Appendix D). The lowest growth yield was shown by *B. cereus* strain with 3 mg ml^{-1} dry weight biomass. This was halve the value determined for the neutrophilic *B. subtilis*. These findings are in good agreement with those of Sorokin, *et al.,* (1998) who observed that the specific biomass yield of a facultative alkaliphile (*Nitrobacter* sp. grown at pH 10) was lower than the biomass yield of neutrophilic species. Conversely, the growth yield of environmental strain MAK7 was higher than the growth yield of the neutrophilic *B. subtilis* with 9 and 6 mg ml^{-1} dry weight biomass respectively. However, the reliability of these results may be in doubt due to a lack of replication (only duplicate measurements were made) and the use of a weighing balance that was only effective to 0.01 g.

6.2.2. Sodium Requirement for Growth of *Bacillus* MAK7 and *Bacillus cereus*.

Due to energetic considerations, major metabolic activities of cells in hypersaline environments are limited, because the salt lowers water activity (Jiang *et al.,* 2006 and 2007). It is well known that Na^+ is required for their growth of alkaliphilic bacteria at

high external pH values (Horikoshi 1999; Kitada *et al.,* 2000; Krulwich *et al.,* 2001; Ma, *et al.,* 2004a; Liew *et al.,* 2007). The requirement for sodium relates to both pH regulation and uptake of various solutes (Krulwich *et al.*, 1997). To determine if this was true for *Bacillus* MAK7 *B. cereus*, cells grown overnight at pH 10 in Horikoshi medium were washed three times with sodium free Horikoshi medium (section 2.12). This minimised the extracellular Na^+ concentration and then 1 ml of washed cells was added into 250 ml conical flasks containing 50 ml of Horikoshi medium with different concentrations of sodium chloride.

Figure 6.2 shows the effect of different concentrations of NaCl on the growth of both *Bacillus* MAK7 and *B. cereus* at pH 10 in Horikoshi medium. It was found that cells of environmental strain MAK7 were able to grow with optimum growth within the salinity range of 0 – 100 mM NaCl. This high growth was similar to the growth of cells in normal Horikoshi medium, which contains 100 mM NaCl. Increasing the NaCl concentration up to 200 mM did not significantly inhibit growth, only when the concentration of NaCl reached 400 mM did it start to show an inhibitory effect and the growth was significantly slower at 600 mM NaCl. *Bacillus* MAK7 showed poor growth in salinities of 600 – 800 mM NaCl, but no growth was evident at 1000 mM NaCl.

The laboratory strain of *B. cereus* was able to grow optimally over the salinity range of 0 – 400 mM Na^+. Growth was similar to growth in control Horikoshi medium, which contains 100 mM NaCl. The presence of 600 mM NaCl in the medium significantly reduced the growth rate of *B. cereus* and there was no growth above 600 mM NaCl (Figure 6.2).

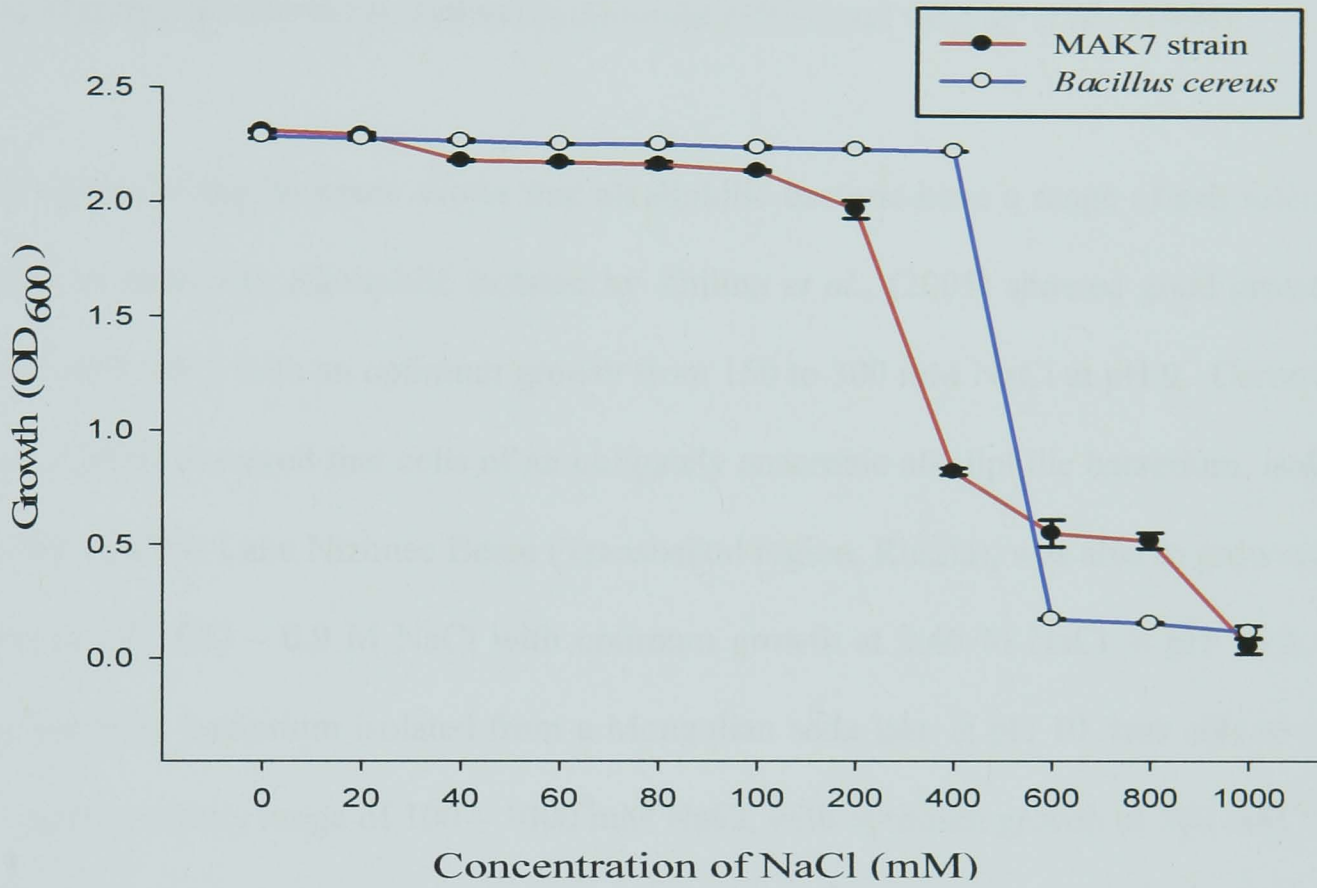

Figure 6.2: Na^+ requirement for growth of both *Bacillus* MAK7 and of *Bacillus cereus* at pH 10. Cells were grown in Horikoshi medium at pH 10 using 200 mM potassium carbonate buffer with different NaCl concentrations (0 – 1000 mM). The OD was measured at 600 nm after overnight incubation at 25°C with shaking at 250 rpm. Data points are the mean of three replicates plus or minus standard error.

It was concluded that the maximum salt tolerance for both MAK7 and *B. cereus* is 400 – 600 mM NaCl. Therefore, MAK7 and *B. cereus* are not halophiles because high growth was also observed in the absence of salt. The two organisms would be classified as slightly halotolerant as defined by Gilmour (1990) and Ollivier *et al.,* (1994).

A survey of the literature shows that alkaliphilic bacteria have a range of salt tolerance e.g. an anaerobic alkaliphile isolated by Zhilina *et al.*, (2005) showed good growth at 400 mM NaCl with an optimum growth from 150 to 300 mM NaCl at pH 9. Garnova *et al.,* (2004) observed that cells of an obligately anaerobic alkaliphilic bacterium, isolated from alkaline Lake Nizhnee Beloe (Transbaikal region, Russia), was able to grow over a range of 0.023 – 0.9 M NaCl with optimum growth at 0.46 M NaCl at pH 10.2. An alkaliphilic bacterium isolated from a Mongolian soda lake at pH 10, was able to grow over the salinity range of 100 – 1000 mM NaCl, with optimum growth at 300 mM NaCl (Sorokin, *et al.,* 2001b). Two related novel alkaliphiles (*Alkalimonas amylolytica* and *A. delamerensis*), isolated from soda lakes in China and East Africa respectively at pH 10 - 10.5, required 0.34 – 0.51 M NaCl and were described as slightly halophilic bacteria (Ma *et al.,* 2004a). In contrast, a very high requirement for sodium was demonstrated in *Thioalkalibacter halophilus* cells (a facultative alkaliphile), which was isolated from a hypersaline soda lake in Siberia. It was able to grow over a broad range of salinity (0.5 – 3.5 M of NaCl) with optimum growth at around 1 M NaCl over a pH range from 7.2 – 10 (Banciu *et al.,* 2008).

Very importantly, the ability of *Bacillus* MAK7 and *B. cereus* to grow well in the absence of Na^+ suggests that they do not require Na^+ for growth at pH 10. The lack of a

Na^+ requirement for growth at pH 10 is highly unusual since this cation normally plays a key role for pH homeostasis (Krulwich *et al.*, 1990 and 1997; Brett *et al.*, 2005; Padan *et al.*, 2005). Oh *et al.*, (1991) reported that some marine bacteria that possessed the primary Na^+ pump did not show apparent requirement of Na^+ for growth.

6.2.3. Potassium Requirement for Growth of *Bacillus* MAK7 and *Bacillus cereus*

K^+ may play an important role in pH homeostasis by being exchanged for Na^+ (Beck and Rosen, 1979). K^+/ H^+ antiporters have also been reported to play a major role in the cycling of protons in neutrophilic microorganisms such as *Bacillus subtilis* (Krulwich *et al.*, 1994 and1997), *Escherichia coli* (Krulwich *et al.*, 1979) and alkaliphilic *Bacillus* species (Padan *et al.*, 2005).

Therefore, it was decided to investigate the K^+ requirements for growth of *Bacillus* MAK7 and *B. cereus* at external pH 10 using Horikoshi medium with 200 mM sodium carbonate and NaH_2PO_4 instead of KH_2PO_4. It was necessary to wash cells three times with potassium free Horikoshi medium (section 2.13), to minimise the extracellular K^+ concentration.

Figure 6.3 shows the influence of different concentrations of KCl (0, 0.05, 0.1, 0.5, 1, 5 and 10 mM) on the growth of both MAK7 and *B. cereus* at pH 10. It can be seen from this figure that growth of MAK7 and *B. cereus* was unaffected by changing the K^+ concentration from 0 to 10 mM KCl.

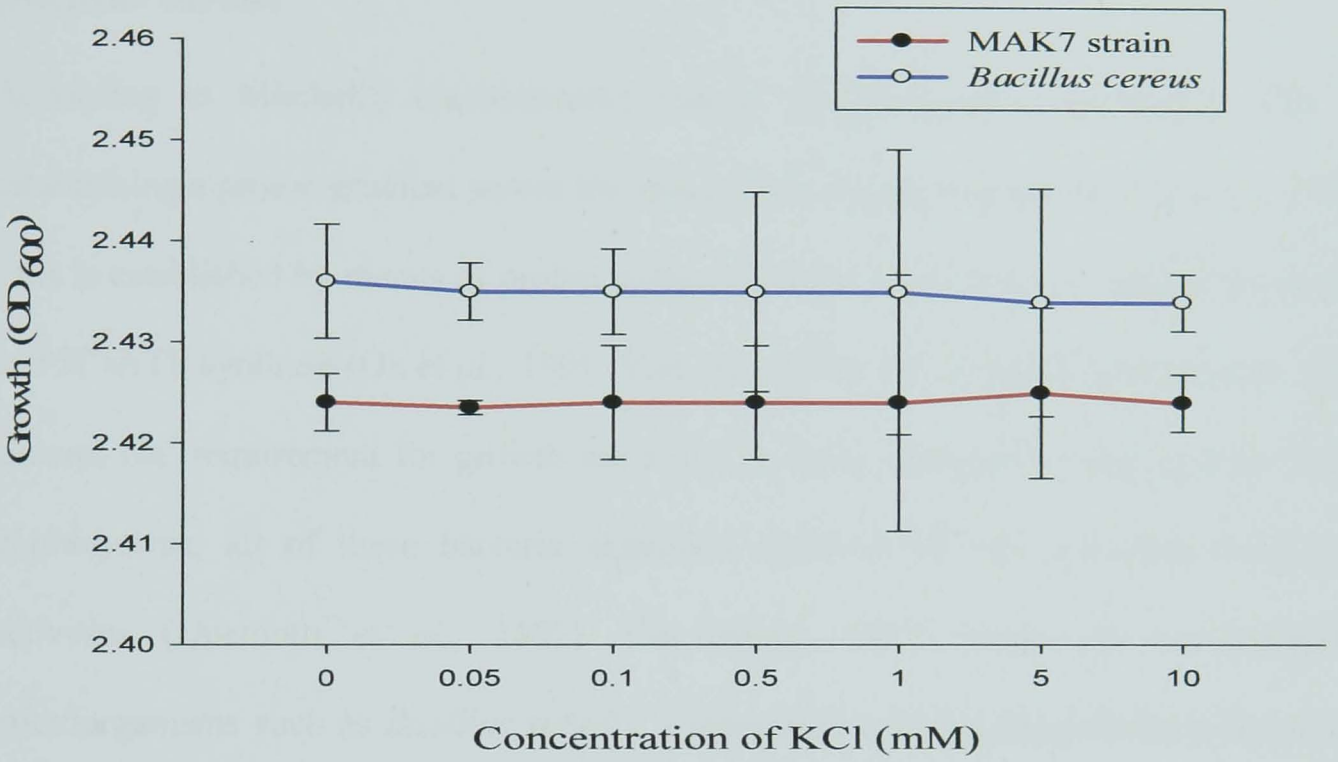

Figure 6.3: K^+ requirement for growth of both *Bacillus* MAK7 and *Bacillus cereus* at pH 10. Cells were grown in Horikoshi medium at pH 10 using 200 mM sodium carbonate buffer with different KCl concentrations (0, 0.05, 0.1, 0.5, 1, 5 and 10 mM). The OD was measured at 600 nm after overnight incubation at 25°C with shaking at 250 rpm. Data points are the mean of three replicates plus or minus standard error.

These data indicate that both environmental strain MAK7 and the laboratory strain of *B. cereus* do not show a requirement for K^+ ions for growth at pH 10.

6.2.4. Sodium Requirement for Respiration in *Bacillus* MAK7 and *Bacillus cereus*

According to Mitchell's chemiosmotic theory, microorganisms synthesise ATP by establishing a proton gradient across the energy transducing membrane (Oh *et al.,* 1990). This is established by means of proton pump, which is coupled to the respiratory system and H^+-ATP synthase (Oh *et al.,* 1991; Yumoto, 2002). Na^+-dependent respiration and a general Na^+ requirement for growth are found in many marine bacteria such as *Vibrio alginolyticus*, all of these bacteria examined required Na^+ for optimum respiratory activities (Unemoto *et al.,* 1992). Conversely, NaC1 stress on non-halophilic microorganisms such as *Bacillus subtilis, Escherichia coli* and *Salmonella oranienburg* led to reduced respiration rates (Nagata *et al.,* 2002).

It was necessary, therefore, to investigate the requirement of Na^+ for respiration of both environmental strain MAK7 and the laboratory strain of *B. cereus.* Cells were grown overnight at pH 10 and then resuspended in Horikoshi medium with different concentrations of NaCl. A Clarke type oxygen electrode was used to measure respiration rates as described in section 2.10. The difference in respiration rate shown by cells of both organisms resuspended in the presence of different amounts of NaCl may be due to a specific requirement for Na^+ ions. To investigate this possibility, the respiration rate of cells of both organisms was measured over a range of different concentrations of NaCl

(0, 25, 50, 100, 200 and 400 mM) at high external pH (pH 10) to find out the minimum requirement that these organisms have for NaCl. The cells of both strains grown at pH 10 were concentrated and protein was determined using the Bradford method (see section 2.11) to calculate the respiration rate in µmoles O_2 taken up mg^{-1} protein h^{-1}.

Figure 6.4 shows that for both organisms when the NaCl concentration was increased from 0 to 400 mM the rate of respiration decreased. The lowest levels of respiration rate were found at 400 mM NaCl suggesting that these strains are not particularly halotolerant and are certainly not halophilic. In fact, they behave like *E. coli,* where respiration is also inhibited by high NaCl concentrations (Nagata *et al.,* 2002; Natesan *et al.,* 2000). The observation that significant levels of respiratory activity for both strains continued in the absence of Na^+ (Figure 6.4) indicates that MAK7 and *B. cereus* do not require Na^+ for their respiratory chain to function. This is in contrast to some marine bacteria (*Marinococcus* species), which showed low respiration rates in the absence of Na^+ (Unemoto *et al.*, 1992).

6.2.5. Potassium Requirement for Respiration in *Bacillus* MAK7 and *Bacillus cereus*

To study the effect of different KCl concentrations on the respiration rate of both environmental strain MAK7 and the laboratory strain of *B. cereus*, cells grown in Horikoshi medium overnight at pH 10 were resuspended in Horikoshi medium containing different concentrations of KCl. Respiration rate was measured using a Clarke type oxygen electrode.

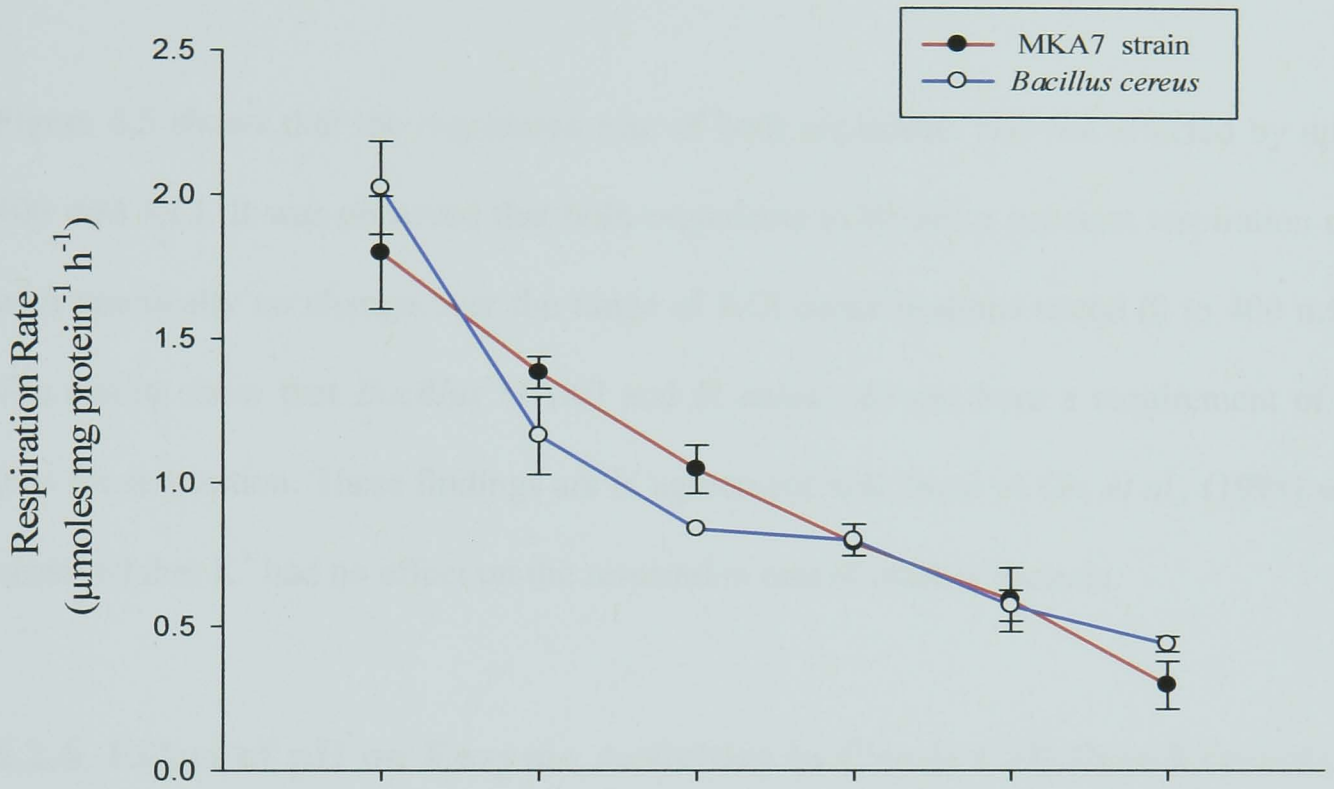

Figure 6. 4: Effect of increasing salinity on the respiration rate of both *Bacillus* MAK7 and *Bacillus cereus* at pH 10. Respiration rate of the cells was measured in Horikoshi medium using 200 mM potassium carbonate buffer with different concentrations of sodium chloride (0, 25, 50, 100, 200 and 400 mM). Cells grown in Horikoshi medium at pH 10 overnight at 25°C with shaking at 250 rpm. Data points are the mean of three replicates plus or minus standard error.

The cells of both strains grown at pH 10 were concentrated and protein was determined using the Bradford method (see section 2.11) to calculate the respiration rate in μmoles O_2 taken up mg^{-1} protein h^{-1}.

Figure 6.5 shows that the respiration rate of both organisms was not affected by up to 400 mM KCl. It was observed that both organisms exhibited a constant respiration rate with practically no change over the range of KCl concentrations tested (0 to 400 mM). The results show that *Bacillus* MAK7 and *B. cereus* do not have a requirement of K^+ ions for respiration. These findings are in agreement with those of Oh, *et al.*, (1991) who observed that K^+ had no effect on the respiration rate of marine bacteria.

6.2.6. Effect of pH on Enzyme Activities in Crude Cell-Free Extracts of *Bacillus* MAK7 and *Bacillus cereus*

Although many extracellular enzymes from alkaliphiles, such as proteases and amylases were isolated and studied in detail because of their commercial importance (section 1.8), investigation of the properties of intracellular enzymes is still very limited. To investigate the relationship between the growth pH and the pH profile for the activity of intracellular enzymes, a number of enzymes were extracted from cell suspensions of both environmental strain MAK7 and the laboratory strain of *B. cereus* grown at pH 7, 8.5 and 10. The activity of the enzymes was then measured at different assay pH values. The protein concentration of the cell free extract was determined by the Bradford method as described in section 2.11, in order to calculate the specific activity of each enzyme.

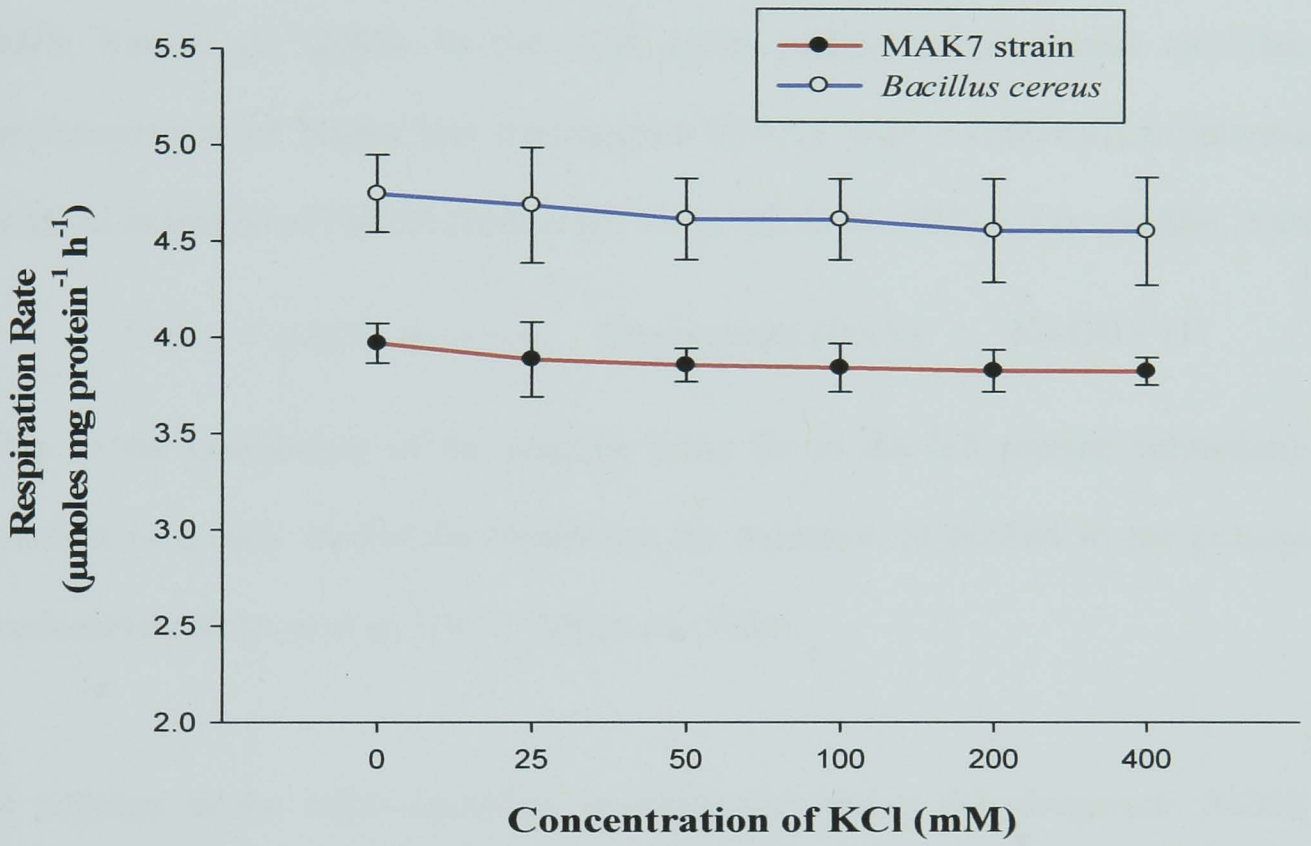

Figure 6.5: Effect of different concentrations of KCl on the respiration rate of both *Bacillus* MAK7 and *B. cereus* at pH 10. Respiration rate of the cells was measured in Horikoshi medium using 200 mM sodium carbonate buffer with different concentrations of potassium chloride (0, 25, 50, 100, 200 and 400 mM). Cells grown in Horikoshi medium at pH 10 overnight at 25°C with shaking at 250 rpm. Data points are the mean of three replicates plus or minus standard error.

6.2.6.1. Malate dehydrogenase (EC 1.1.1.37)

Malate dehydrogenase (MDH) is an essential enzyme in the tricarboxylic acid cycle (TCA cycle, which is also called the citric acid cycle or Krebs cycle) as well as the noncyclic anaplerotic pathway of prokaryotes and eukaryotes (animals and plants) (Falk *et al.,* 1980; Park *et al.,* 1995; Molenaar, *et al.,* 2000; Eprintsev *et al.,* 2003; Maki *et al.,* 2006; Yao *et al.,* 2008). In the TCA cycle, malate dehydrogenase catalyses the interconversion of malate and oxaloacetate (OAA), with a concomitant reduction of NAD or oxidation of NADH (Park *et al.,* 1995; Oh, *et al.,* 2002; Chan and Sim, 2004).

$$\text{Malate} + NAD^+ \longleftrightarrow \text{Oxaloacetate (OAA)} + NADH + H^+$$

Due to the equilibrium of the reaction lying far to the left (malate formation), the reaction is usually studied by measuring the oxidation of NADH in the presence of oxaloacetate (Reeves *et al.,* 1971; Falk *et al.,* 1980).

In addition to the NAD-dependent (cytoplasmic) malate dehydrogenase (MDH; EC 1.1.1.37) a new type of malate dehydrogenase called malate:quinone oxidoreductase (MQO; EC 1.1.99.16) was found and described as an essential enzyme in the TCA cycle of both *Escherichia coli* (van der Rest *et al.,* 2000) and *Corynebacterium glutamicum* (Molenaar, *et al.,* 1998 and 2000). However, in the current study the activity of the NAD-dependent malate dehydrogenase (L-malate: NAD^+ oxidoreductase; EC 1.1.1.37; MDH) was measured in crude cell-free extracts prepared from cells of both environmental strain MAK7 and the laboratory strain of *B. cereus.* The activity of malate dehydrogenase was determined spectrophotometrically at 340 nm and expressed as μmoles min^{-1} mg $protein^{-1}$ according to the methods of Reeves *et al.,* (1971).

Figures 6.6 A, B and C show the measurements of the NAD-dependent malate dehydrogenase specific activity in cell-free extracts from both isolates grown at different external pH values of 7, 8.5 and 10, and assayed over the same range of pH values. The results indicated that there was no significant difference in both the minimum and maximum specific activity of malate dehydrogenase in crude cell-free extracts from cells of both MAK7 and *B. cereus* grown overnight at pH values examined (7, 8.5 and 10). Conversely, these results very clearly indicated that there was a significant difference in the responses of the malate dehydrogenase activity for different assay pH values and the specific activity was increased significantly as the assay pH was increased from 7 to 10. Good rates of enzymes activity were observed over an assay pH range from 8.5 to 10 with optimum enzymatic activity at pH 10, where the enzyme is stable and able to catalyse the reaction at alkaline pH. Cells of both strains grown at different external pH values tested and assayed at pH 7 had the lowest levels of malate dehydrogenase activity. The activity of malate dehydrogenase from MAK7 tended to be slightly higher than the *B. cereus* malate dehydrogenase activity over the range of pH values tested (Figures 6.6 A, B and C).

These findings are in good agreement with those of Shah and Andrews (1994) who observed that the NAD-dependent malate dehydrogenase (MDH; EC 1.1.1.37) from *Actinobacillus actinomycetemcomitans*, showed high activity at pH 10. Irwin *et al.*, (2001) found the same result with malate dehydrogenase from a psychrophilic bacterium, which showed high activity at pH 10 – 10.2. In contrast to the findings of this study, the activity of malate dehydrogenase from *Corynebacterium glutamicum* was reported to be optimum at pH 6.5 (Genda *et al.*, 2003).

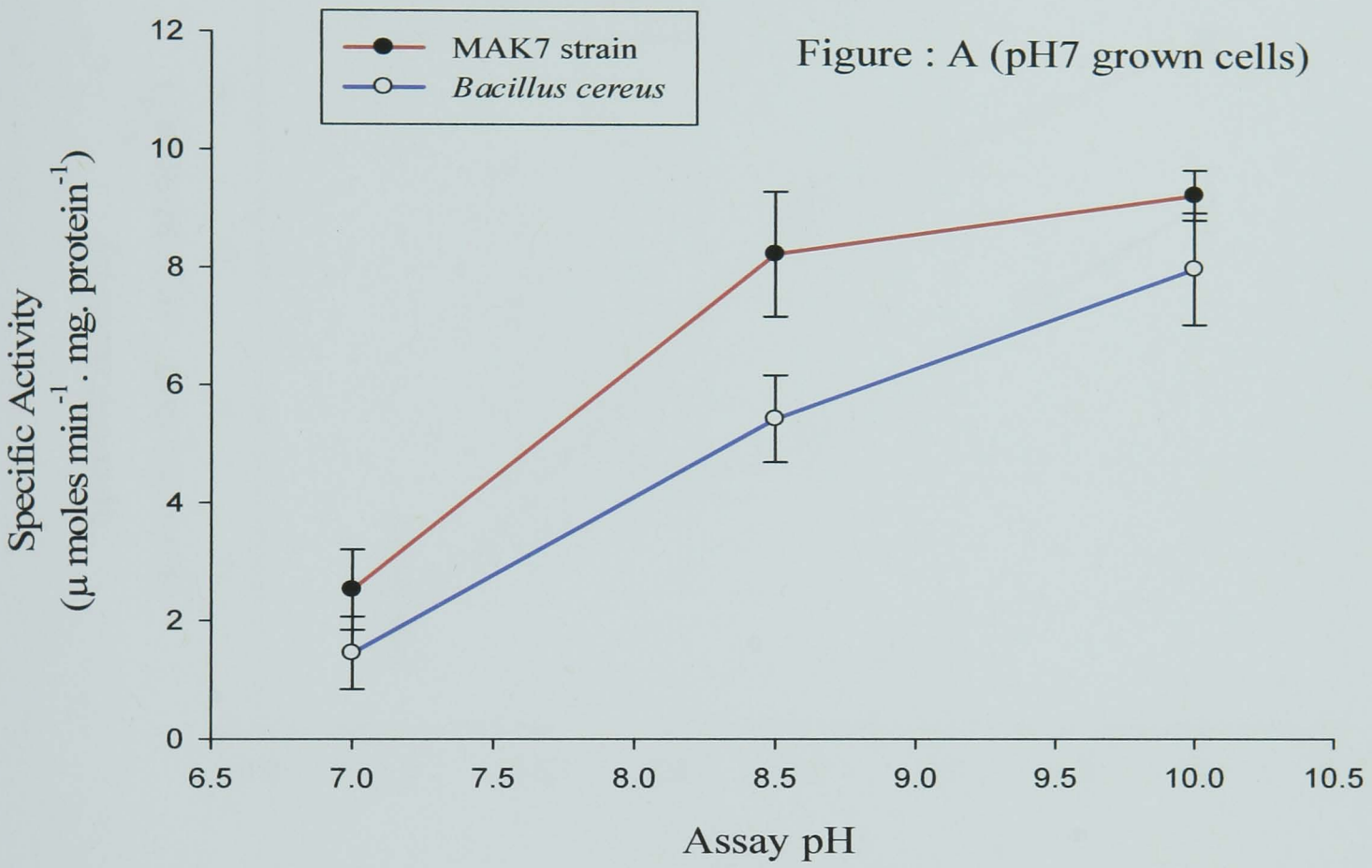

Figure : A (pH7 grown cells)

Figure 6.6A: Effect of pH on malate dehydrogenase specific activity in crude cell-free extracts prepared from cells of both *Bacillus* MAK7 and *Bacillus cereus*. Cells were grown in Horikoshi medium at pH 7 overnight at 25°C with shaking at 250 rpm. The enzyme extraction and assay methods were described in sections 2.18.1 and 2.18.3. Enzyme activity is expressed as μmoles NADH oxidised min^{-1} mg $protein^{-1}$. Data points are the mean of three replicates plus or minus standard error.

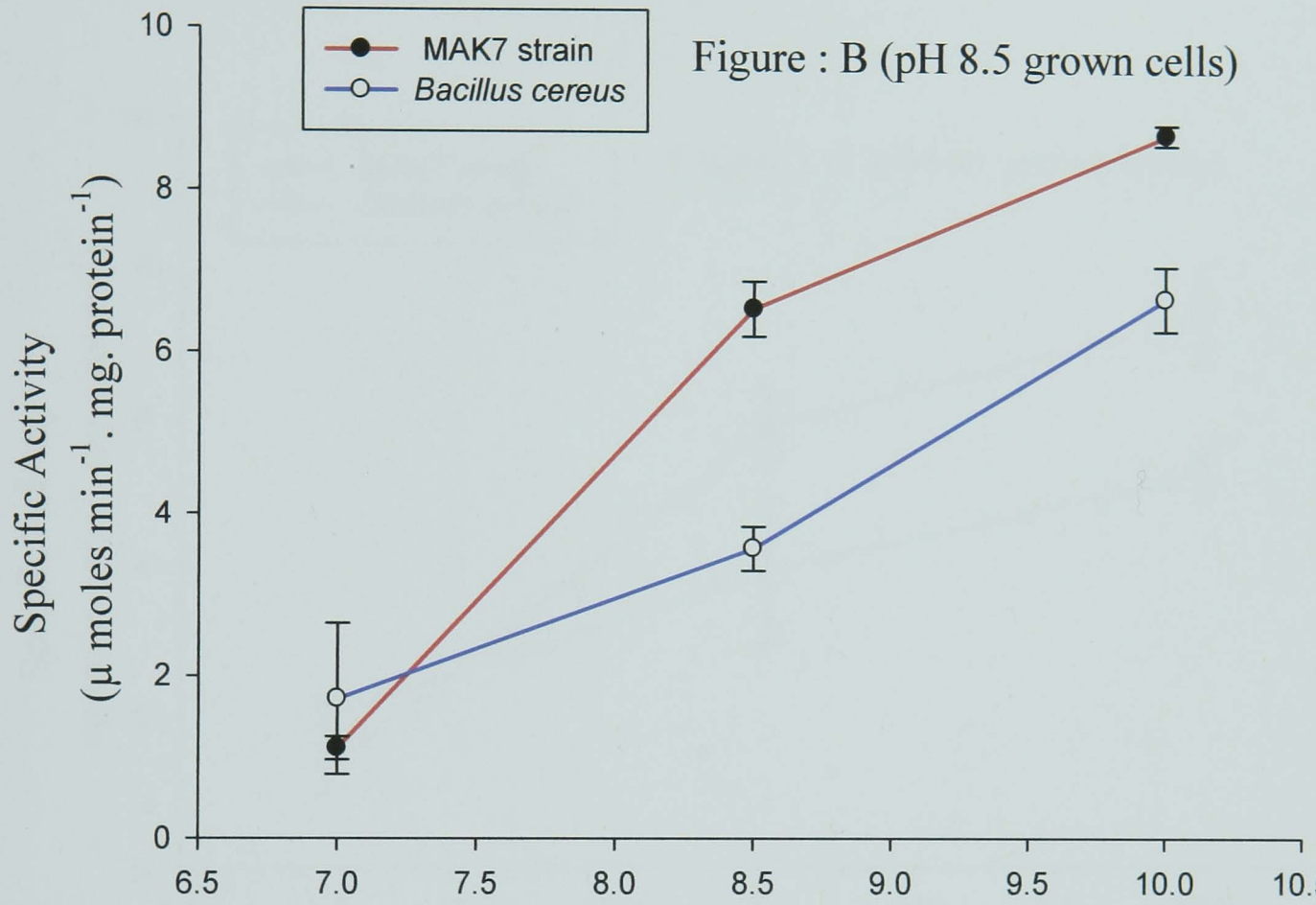

Figure : B (pH 8.5 grown cells)

Figure 6.6B: Effect of pH on malate dehydrogenase specific activity in crude cell-free extracts prepared from cells of both *Bacillus* MAK7 and *Bacillus cereus*. Cells were grown in Horikoshi medium at pH 8.5 overnight at 25°C with shaking at 250 rpm. The enzyme extraction and assay methods were described in sections 2.18.1 and 2.18.3. Enzyme activity is expressed as μmoles NADH oxidised min^{-1} mg $protein^{-1}$. Data points are the mean of three replicates plus or minus standard error.

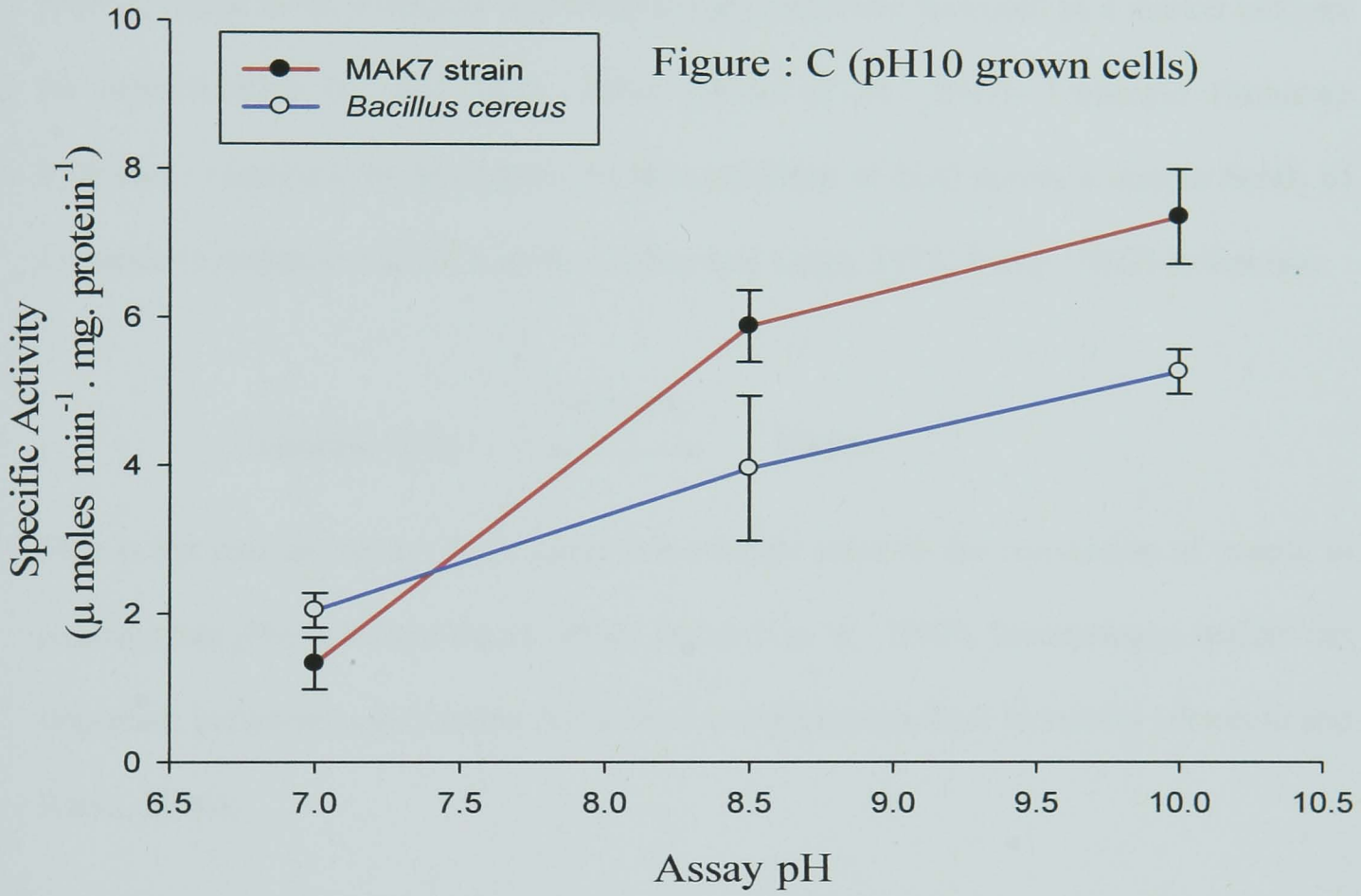

Figure : C (pH10 grown cells)

Figure 6.6C: Effect of pH on malate dehydrogenase specific activity in crude cell-free extracts prepared from cells of both *Bacillus* MAK7 and *Bacillus cereus*. Cells were grown in Horikoshi medium at pH 10 overnight at 25°C with shaking at 250 rpm. The enzyme extraction and assay method were described in sections 2.18.1 and 2.18.3. Enzyme activity is expressed as μmoles NADH oxidised min^{-1} mg $protein^{-1}$. Data points are the mean of three replicates plus or minus standard error.

6.2.6.2. Fumarase (EC 4.2.1.2)

Fumarase is an enzyme widely distributed in a variety of organisms (animals, plants and bacteria) because of its central role in the TCA cycle during aerobic cell metabolism (Park and Gunsalus, 1995; Goh *et al.,* 2005). In eukaryotes, it is a nuclear DNA-encoded protein, found in the matrix of mitochondria and therefore was used as a marker enzyme for mitochondria (Li and Shah, 2002; Bowes *et al.,* 2007). Fumarase (fumarate hydratase) catalyses the hydration reaction (addition of H_2O across a double bond) of fumarate to malate in the TCA cycle (Tolley and Craig, 1975; Tseng, 1997) as follows:

$$\text{Fumarate} + H_2O \xleftrightarrow{\text{Fumarase}} \text{Malate}$$

This is the reaction in the TCA cycle immediately prior to the conversion of malate to oxaloacetate (Presecki and Racki, 2006; Presecki *et al.,* 2007). Interestingly, malate has important industrial applications in the food and pharmaceutical industries (Presecki and Racki, 2006).

The activity of fumarase was measured in crude cell-free extracts prepared from cells of both MAK7 and *B. cereus* grown at pH 7, 8.5 and 10 overnight. The activity was assayed over a range of pH values (7, 8.5, 9, 9.5 and 10) to investigate the effect of pH on the enzymatic activity. Fumarase activity was determined spectrophotometrically at 240 nm and expressed as μmoles min^{-1} mg $protein^{-1}$ according to the methods of Hill and Bradshaw (1969).

Figures 6.7 A, B and C show that the highest (optimal) fumarase activity was always at assay pH 7 for both MAK7 and *B. cereus*, with a much reduced activity at pH 8.5. There was a very low level or no fumarase activity recorded when the assay was conducted at pH 9 or above. There was little evidence for adaptation in cells grown at pH 8.5 or 10, and it appears that both strains would need to maintain a near neutral intracellular pH to allow significant levels of fumarase activity.

6.2.6.3. Hexokinase (EC 2.7.1.2)

Hexokinase (HK, Glucokinase) is an enzyme found in every organism. It catalyses the phosphorylation of glucose to glucose-6-phosphate (G6P) in the cell using the gamma phosphate of ATP as a phosphoryl donor (van Wijk *et al.,* 2003). Hexokinase also facilitates the phosphorylation of other hexoses such as fructose (Dorr *et al.,* 2003). This phosphorylation reaction is important because it provides the activation energy required for glycolysis.

The specific activity of hexokinase enzyme was measured in crude cell-free extracts prepared from cells of both environmental strain MAK7 and laboratory strain of *B. cereus* grown at external pH values of 7, 8.5 and 10 overnight. It was measured spectrophotometrically at 340 nm according to the malate dehydrogenase assay (section 2.18.3) and expressed as μmoles NADH oxidised min^{-1} mg $protein^{-1}$. Due to technical problems, all assays were carried out at pH 7.

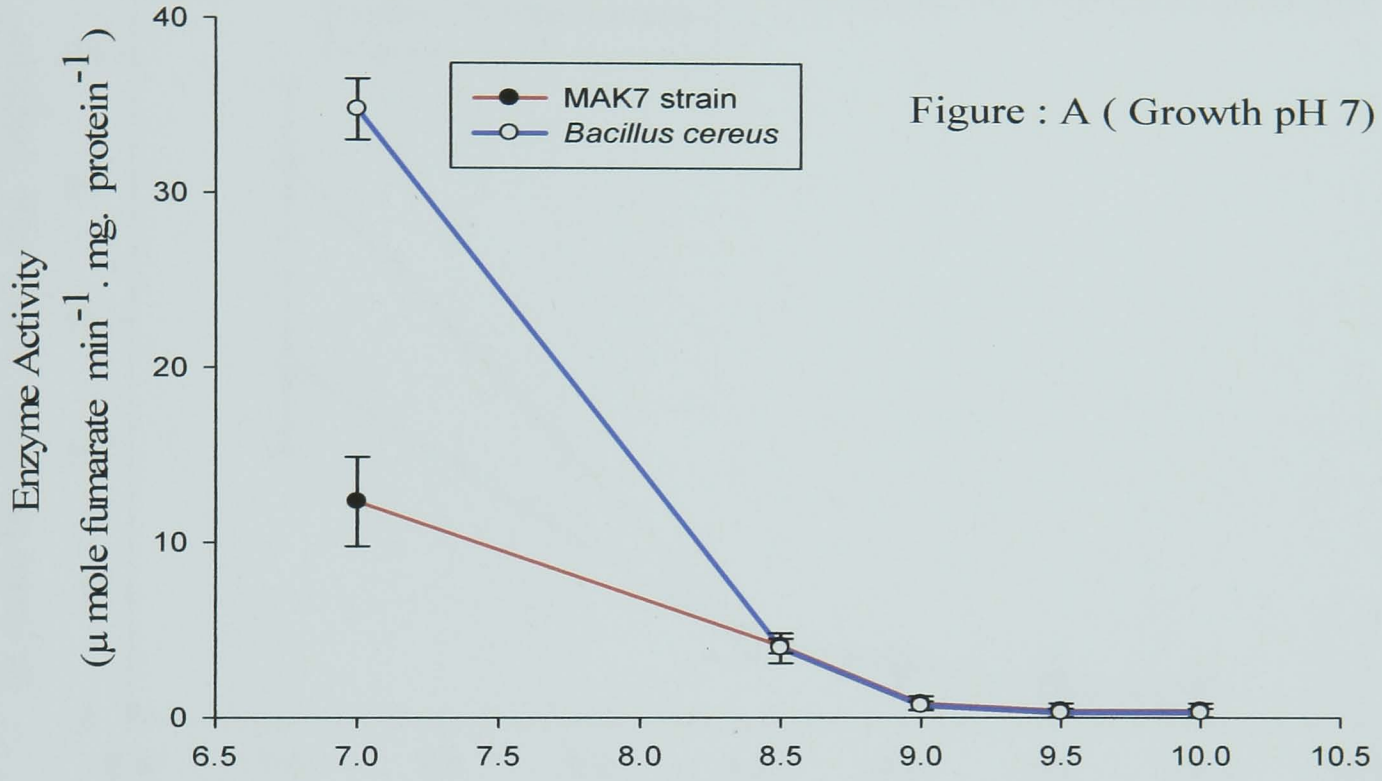

Figure : A (Growth pH 7)

Figure 6.7A: Effect of pH on fumarase specific activity in crude cell-free extracts prepared from cells of both *Bacillus* MAK7 and *Bacillus cereus*. Cells were grown in Horikoshi medium at pH 7 overnight at 25°C with shaking at 250 rpm. The enzyme extraction and assay methods were described in sections 2.18.1 and 2.18.4. Enzyme activity is expressed as µmoles fumarate produced min^{-1} mg $protein^{-1}$. Data points are the mean of three replicates plus or minus standard error.

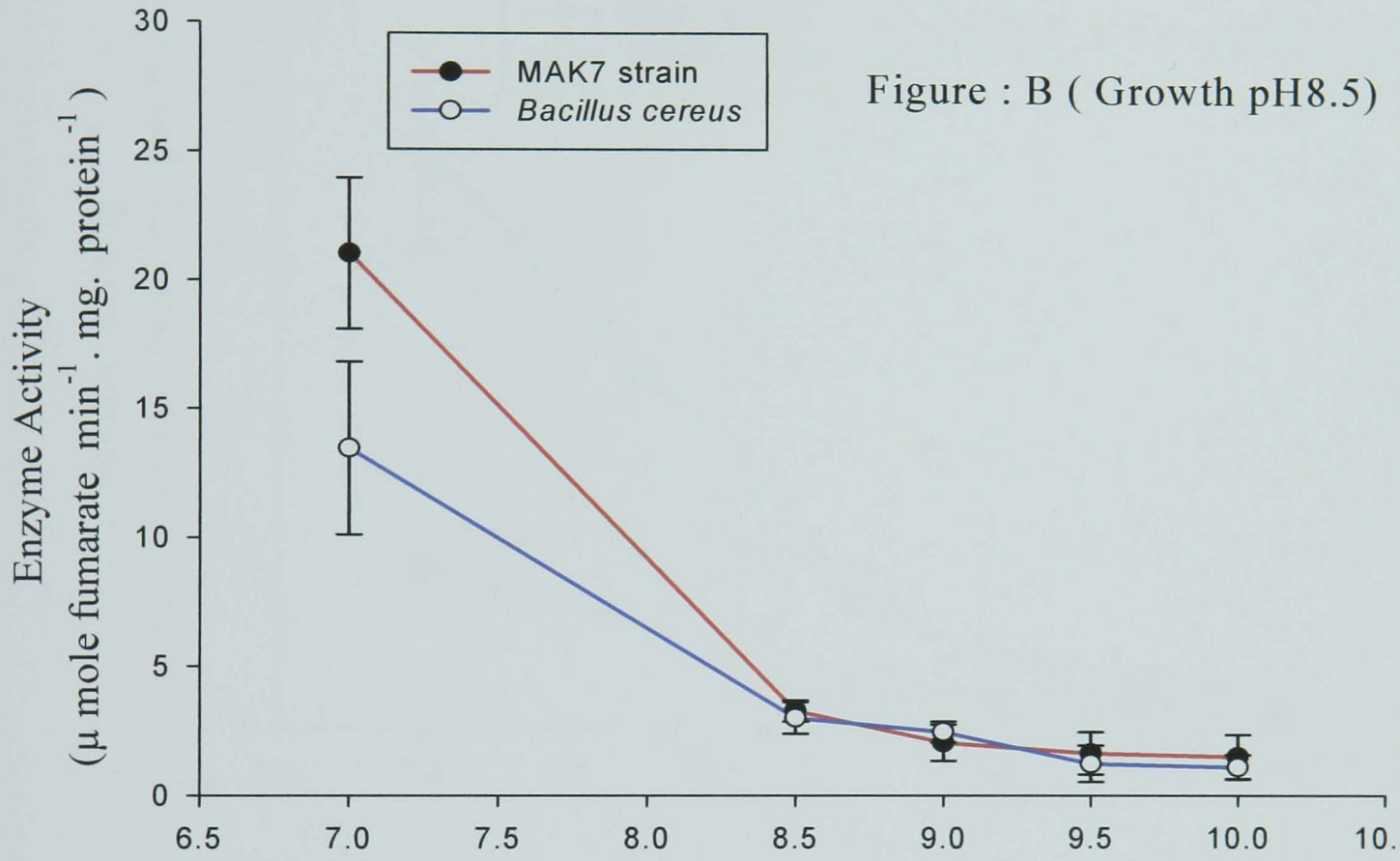

Figure : B (Growth pH8.5)

Figure 6.7B: Effect of pH on fumarase specific activity in crude cell-free extracts prepared from cells of both *Bacillus* MAK7 and *B. cereus*. Cells were grown in Horikoshi medium at pH 8.5 overnight at 25°C with shaking at 250 rpm. The enzyme extraction and assay methods were described in sections 2.18.1 and 2.18.4. Enzyme activity is expressed as µmoles fumarate produced min^{-1} mg $protein^{-1}$. Data points are the mean of three replicates plus or minus standard error.

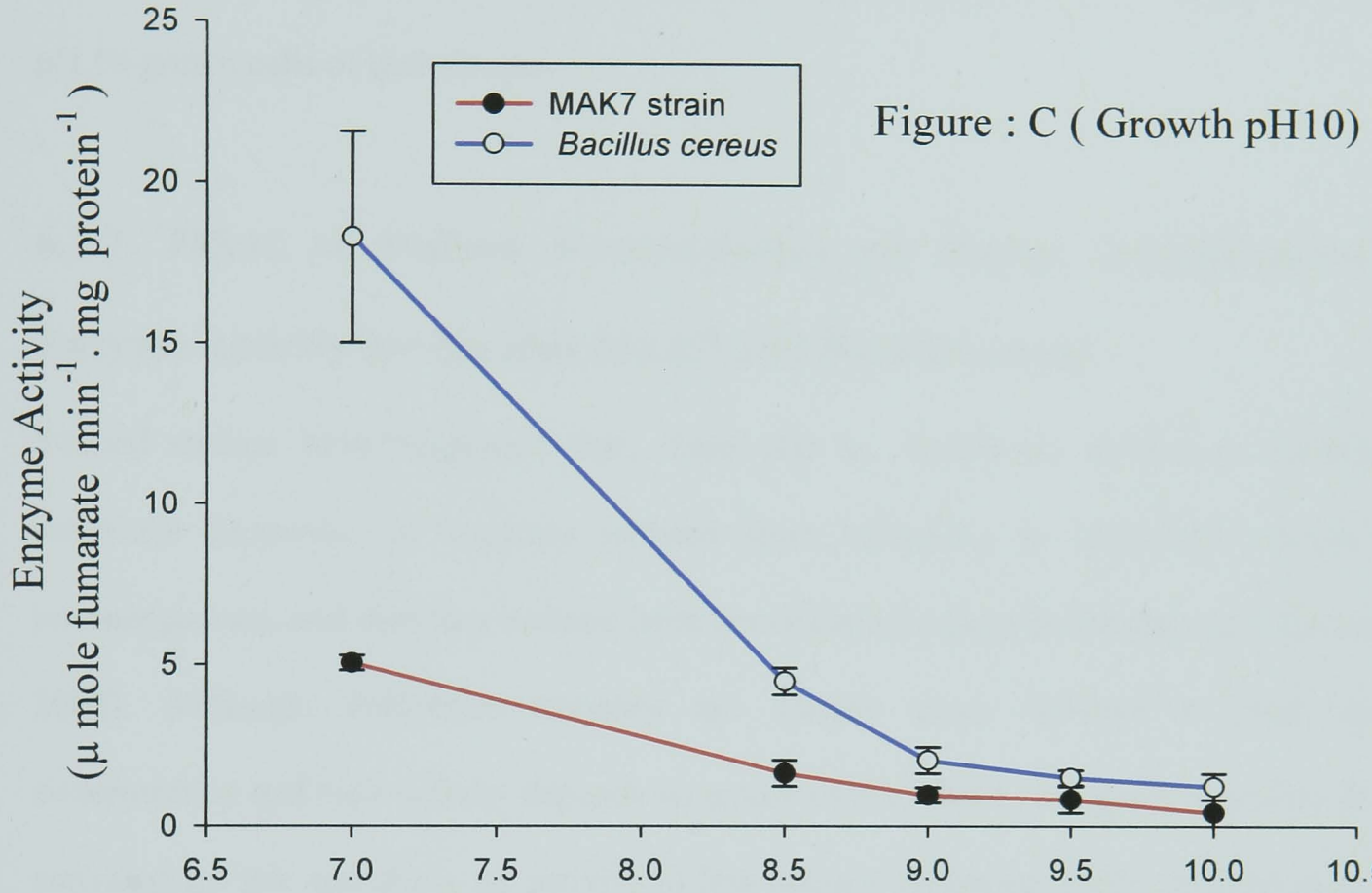

Figure : C (Growth pH10)

Figure 6.7C: Effect of pH on fumarase specific activity in crude cell-free extracts prepared from cells of both *Bacillus* MAK7 and *Bacillus cereus*. Cells were grown in Horikoshi medium at pH 10 overnight at 25 °C with shaking at 250 rpm. The enzyme extraction and assay methods were described in sections 2.18.1 and 2.18.4. Enzyme activity is expressed as μmoles fumarate produced min^{-1} mg $protein^{-1}$. Data points are the mean of three replicates plus or minus standard error.

Figure 6.8 shows that the optimal activity of the hexokinase enzyme was found in crude cell-free extracts from pH 7 grown cells for both MAK7 and *B. cereus*. Similar much reduced rates of hexokinase activity were observed in cell-free extracts from pH 8.5 and pH 10 grown cells of both strains.

6.2.7. Effect of Sodium Concentration on Malate Dehydrogenase Enzyme Activity for *Bacillus* MAK7 and *Bacillus cereus*

Several studies have suggested that, there was no significant difference between molecular properties of enzymes isolated from halophilic or halotolerant aerobic microorganisms and enzymes isolated from non-halophilic bacteria (Madern and Zaccai, 2004). Although, halophilic enzymes are usually more resistant to high salt concentration and their activity depends on nature of the salt i.e., halophilic enzymes are activated by salt and show no activity in low salinity (Gilmour, 1990; Madern *et al.*, 2000). In fact, for some halophilic enzymes, activity in KCl is significantly higher than in NaCl (Madern *et al.*, 2000). For example, NAD-dependent malate dehydrogenase (MDH; EC 1.1.1.37) from *Haloarcula marismortui* (halophilic archaeon) was shown to require KCl concentrations higher than 2 M to function and maintain its native state (Madern *et al.,* 2000; Mevarech *et al.,* 2000). In contrast to most other halophilic enzymes, malate dehydrogenase enzyme from the extreme halophilic bacterium. *Salinibacter ruber* is completely stable in absence of salt and its activity is reduced by high salt concentration (Madern and Zaccai, 2004).

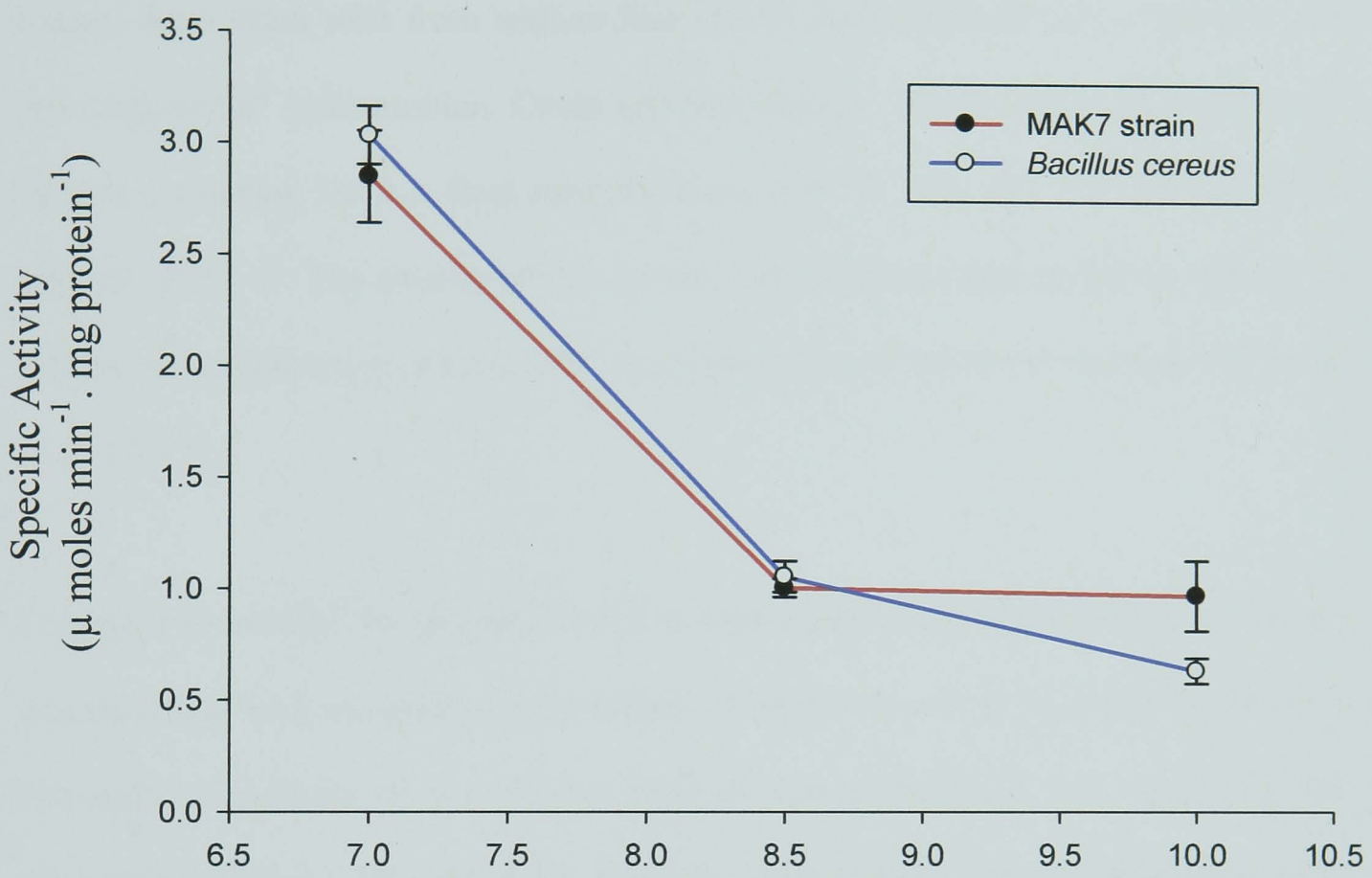

Figure 6.8: Effect of growth pH on hexokinase specific activity in crude cell-free extracts prepared from cells of both *Bacillus* MAK7 and *B. cereus*. Cells were grown in Horikoshi medium at pH 7, 8.5 or 10 overnight at 25°C with shaking at 250 rpm. The enzyme extraction and assay methods were described in sections 2.18.1 and 2.18.5. All assays were carried out at pH 7. Enzyme activity is expressed as μmoles NADH oxidised min^{-1} mg $protein^{-1}$. Data points are the mean of three replicates plus or minus standard error.

This study was intended to determine the effect of different salts on the malate dehydrogenase enzyme activity in both environmental strain MAK7 and the laboratory strain of *B. cereus*. Cells were grown overnight at pH 10 in Horikoshi medium then washed three times with fresh sodium free pH 10 Horikoshi medium to minimise the extracellular Na^+ concentration. Crude cell-free extracts were resuspended in the assay mixture containing NaCl at final concentrations of 0, 50, 100, 200, 400, 800 and 1000 mM, all at pH 10. The activity of this enzyme was measured spectrophotometrically at 340 nm and expressed as μmoles min^{-1} mg $protein^{-1}$ according to the methods of Reeves *et al.* (1971).

Figure 6.9 shows that the specific activity of malate dehydrogenase enzyme from MAK7 was increased with increasing concentration of NaCl to reach a maximum at 400 mM. However, the activity of the enzyme from *B. cereus* increased with increasing salt concentrations up to 200 mM NaCl, although increasing the NaCl concentration up to 400 mM showed no significant difference on the activity of malate dehydrogenase enzyme. At higher concentrations than 400 mM NaCl, the activity of malate dehydrogenase enzyme from both microorganisms was reduced and much lower rates were detected in cell-free extracts resuspended in 1000 mM NaCl.

Malate dehydrogenase from both MAK7 and *Bacillus cereus* is not a halophilic enzyme, but it is of interest that the optimal malate dehydrogenase activity for both organisms was at 400 mM. This suggests that moderate levels of NaCl enhance malate dehydrogenase activity, but higher concentrations of NaCl quickly become inhibitory (Figure 6.9).

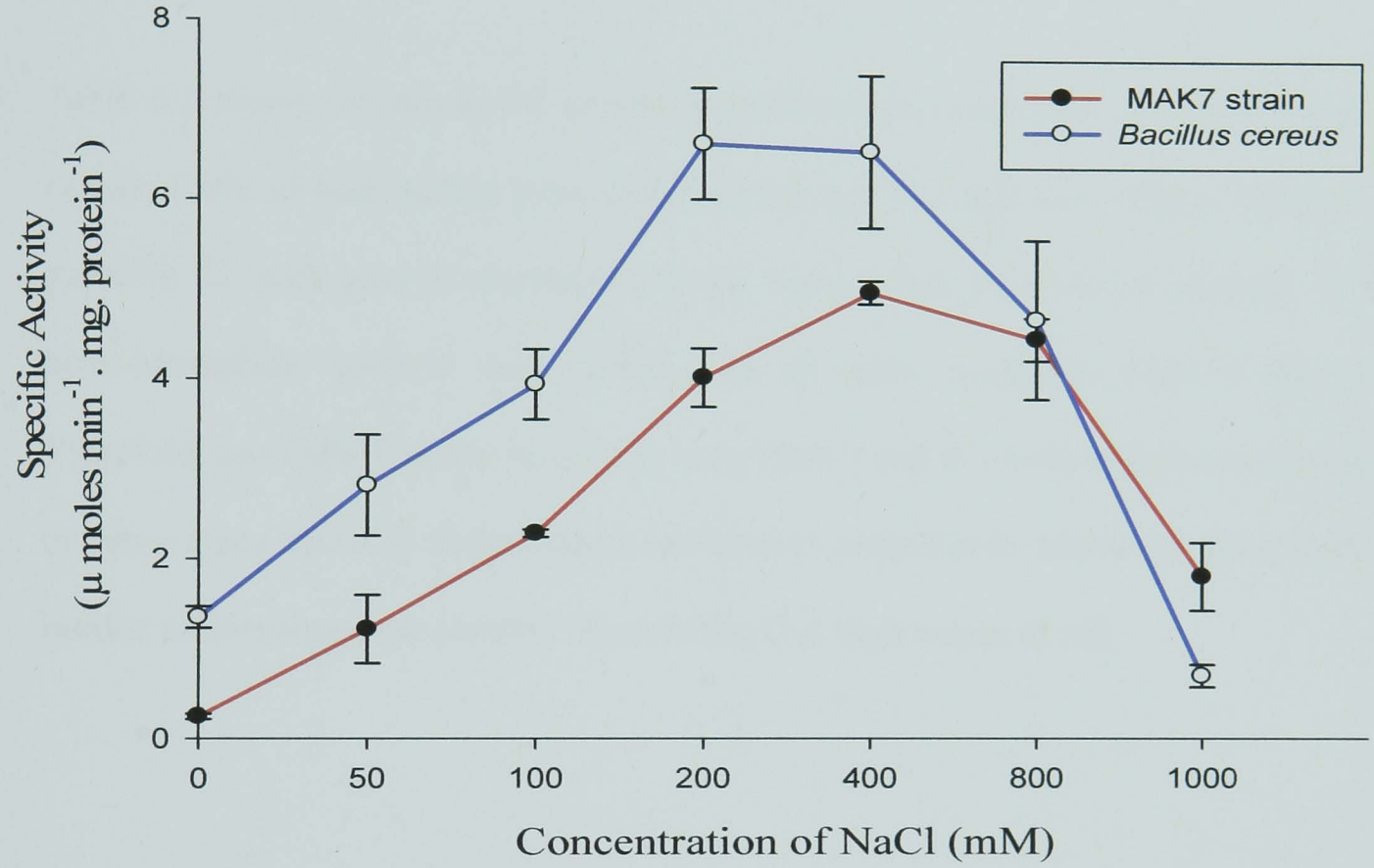

Figure 6.9 : Effect of increasing salinity on malate dehydrogenase specific activity in crude cell-free extracts prepared from cells of both environmental strain MAK7 and laboratory strain of *Bacillus cereus*. Cells grown in Horikoshi medium at pH10 overnight at 25°C with shaker at 250 rpm were washed in sodium free Horikoshi medium (pH 10) and resuspended in final concentrations of odium chloride of 0, 50, 100, 200, 400, 800 and 1000 mM. Enzyme activity is expressed as μmoles NADH oxidised min^{-1} mg $protein^{-1}$. Data points are the mean of three replicates plus or minus standard error.

6.2.8. Sensitivity to Antibiotics

Antibiotic resistance profiles were performed by culturing bacteria on agar plates of Horikoshi medium at pH 10 and placing small circular disks on the plates containing different antibiotics (Oxoid).

Table 6.1 shows the results of sensitivity tests to four antibiotics for MAK7 and *B. cereus*. Cells of both strains produced a similar result. They were highly resistant to penicillin G, with growth comparable to the control (has no effect on growth). While both alkaliphilic bacteria were sensitive to all other antibiotics used in this test. Therefore, penicillin G could be used in both MAK7 and *B. cereus* cultures to minimize or remove any bacterial contaminants sensitive to penicillin G. However, more work is needed to investigate the stability of penicillin G at high values of pH.

Antibiotic disk	**Zone inhibition (mm)**	
	Environmental strain MAK7	**Laboratory strain of *Bacillus cereus***
Streptomycin 25 µg	0.29 ± 0.01	0.23 ± 0.015
Tetracycline 50 µg	0.25 ± 0.01	0.15 ± 0.006
Neomycin 30 µg	0.24 ± 0.01	0.18 ± 0.015
Penicillin G 10 units	Zero	Zero

Table 6.1: Growth response of both *Bacillus* MAK7 and *Bacillus cereus* to four antimicrobial susceptibility test disks. Mean and standard error for three replicates are shown

6.3. Conclusion

In this chapter, a comparison was made between growth rates, generation (doubling) time and biomass yield of both environmental strain *Bacillus* MAK7 and the laboratory strain of *Bacillus cereus* grown in pH 10 with *Bacillus subtilis* cells grown at pH 7. Although the alkaliphilic strains showed faster growth than *B. subtilis,*no clear conclusions can be reached because it is likely that Horikoshi medium did not support good growth of *B. subtilis*.

To better understand the effect of salt concentrations on the metabolic functions of cells, the Na^+ ion requirement for growth of both organisms was investigated using Na^+-free Horikoshi medium with 200 mM potassium carbonate and different concentrations of sodium chloride (Figure 6.2). The results of this study demonstrated that MAK7 and *B. cereus* were able to grow optimally up to salinities of 100 and 400 mM NaCl respectively. Elevated levels of NaCl were not required and were inhibitory at concentrations above 400 mM. Therefore, both strains do not appear to have a requirement for Na^+ for their growth, because optimal growth is possible in the absence of Na^+. The K^+ ion requirement for growth of MAK7 and *B. cereus* was also investigated using Horikoshi medium with different concentrations of KCl (Figure 6.3). Both strains grew well in all concentrations of KCl tested (up to 10 mM) with no significant difference. Therefore, both environmental strain MAK7 and the laboratory strain of *B. cereus* do not require K^+ for growth.

To further investigate the requirement of both Na^+ and K^+ ions for growth of both MAK7 and *B. cereus,* the effect of these cations on respiration rate was examined. It is clear that as the Na^+ concentration increased from 0 - 400 mM, the rate of respiration significantly decreased (Figure 6.4). Therefore, both organisms do not have a requirement for Na^+ for optimal respiration. In contrast to Na^+, K^+ had no specific effect on the respiration activity of both isolates within the range of KCl concentrations from 0 - 400 mM (Figure 6.5). It appears that K^+ is much less inhibitory to respiration rates than Na^+ for both MAK7 and *B. cereus*.

The study of enzyme activity in bacterial cells should help establish the optimum pH for enzyme activity and it can be concluded that the intracellular enzymes of the environmental strain MAK7 and the laboratory strain of *B. cereus* differ in their response to external pH. The NAD-dependent malate dehydrogenase, which is the main enzyme for malate oxidation in *Bacillus* strains was found to be more resisatant to alkaline pH than the other enzymes examined. Malate dehydrogenase showed an activity close to optimum at pH 10 and no significant difference was observed in the specific activity of malate dehydrogenase extracted from cells grown at different pH values of 7, 8.5 and 10 (Figures 6. 6 A, B and C). In contrast, the specific activity of the other enzymes examined was significantly reduced at pH 8.5 or above. Fumarase was the least tolerant to alkaline pH, showing only very small amount of activity at pH 8.5. Also with fumarase, the enzyme extracted from cells grown at pH 8.5 and 10 was slightly, but not significantly, more tolerant to high pH than fumarase extracted from cells grown at pH 7 (Figures 6. 7 A, B and C). For hexokinase, the highest activity was detected in cell-free extracts from cells grown at pH 7 and there was no significant difference in the activity

of hexokinase extracted from cells grown at pH 8.5 and 10 (Figure 6. 8). The influence of increasing salinity on malate dehydrogenase activity in crude cell-free extract from both environmental strain MAK7 and the laboratory strain of *Bacillus cereus* was examined. It was found that malate dehydrogenase from both strains did not require high intracellular salt for activation, but activity was increased by increasing the salinity up to 400 mM NaCl (Figure 6. 9).

Antimicrobial susceptibility test was used to indicate which antibiotic should be applied to minimize or remove bacterial contaminants from MAK7 and *B. cereus* cultures. Since both strains were resistant to penicillin G (Table 6.1), but sensitive to the other three antibiotics tested, penicillin G would be the first choice for treating contaminated cultures of *Bacillus* MAK7 and *B. cereus*.

General Conclusions and Future Work

Chapter Seven

7.1. Conclusions

Microorganisms capable of optimal growth at extreme environments are called extremophiles. Alkaliphiles are extremophiles which require high pH to grow and survive, usually between pH 9 and 10. Alkaliphiles have received a great deal of attention for two major reasons. Firstly, alkaliphiles have different adaptive strategies to cope with the extremely alkaline environments above pH 9 (section 1.7). Secondly, they have the ability to produce alkaline extracellular enzymes, which have been used in important industrial applications in pharmaceuticals, food, waste treatment and biological detergents (section 1.8). In this chapter, the main conclusions of this work will be summarised.

As stated in section 1.10, one of the main aims of this study was the isolation, identification and investigation of the bioenergetics and physiology of a newly isolated, alkaliphilic strain MAK7. The results for MAK7 were compared with the previous literature on alkaliphilic or alkalitolerant microorganisms and with a Gram-positive alkaliphilic species, which was isolated as a laboratory contaminant from a MAK7 culture in Horikoshi medium at pH 10. The contaminant bacterium was purified and tentatively identified as an alkaliphilic strain of the genus *Bacillus* using microscopy and morphological techniques.

Both alkaliphilic strains of environmental MAK7 and laboratory *Bacillus* species were identified to the genus level using 16S rRNA gene sequence. Phylogenetic trees were constructed for both isolates to predict their genetic relatedness to their closest matches. The 16S rRNA gene sequence similarity of environmental strain MAK7 did not initially

give a genus level match, as a result this sequence was searched against the EMBL public database. The best match is to a sequence from an unclassified strain of *Bacillus* associated with marine sponges, with 99.4% sequence similarity. 16S rDNA analysis of the laboratory *Bacillus* isolate confirmed that this bacterium belongs to the genus *Bacillus* with highest sequence identidy to *Bacillus cereus*, with a 100% sequence similarity. The 16S rRNA gene sequence of both microorganisms was deposited in the National Collection of Industrial, Marine and Food Bacteria (NCIMB), Aberdeen, UK under accession number NCIMB 25435.

Another main aim of this work was to isolate extremophilic bacteria from a non-extreme environment. Therefore, water and sediment samples were taken from two unpolluted rivers in the in the Derbyshire Peak District, where the pH ranged from 7.8 – 8.3 (Table 3.1). Enrichment cultures of Horikoshi medium were set up at pH 10 and the serial sub-culture technique were used for isolating alkaliphilic bacteria. Three strains of bacteria were isolated and showed good growth at pH 10. They were designated as MAB4, MAK7 and MAK10. When exposed to a range of pH values in Horikoshi medium, MAK7 strain showed the best growth at pH 10, poorest growth at pH 8.5 and no growth at pH 7 at early stage in the adaptation process. These results indicated that the Gram-positive MAK7 was the most alkaliphilic of the three strains. Moreover, the influence of external pH on the respiration rate of MAK7 at an early stage in the adaptation process was also examined. It was found that MAK7 had optimum respiration rate at pH 10. These findings are in agreement with the fact that the alkaliphilic microorganisms grow optimally or very well at pH values above 9 but cannot grow or grow only slowly at the near-neutral pH values (Horikoshi, 1999). Clearly, alkaliphilic and alkali-tolerant

bacteria were isolated from the slightly alkaline Derbyshire rivers (Bradford and Lathkill). MAK7 was chosen for further study and over a period of several weeks, cells of this strain were fully adapted to grow at pH 7, 8.5 and 10 by repeated sub-culturing. However, the highest respiration rate for MAK7 was still found at pH 10, even after the cells were fully adapted to the pH range 7 to 10 (Table 3.3).

The ability of MAK7 to grow using different carbon sources was investigated. It was found that M9 minimal medium (with a range of carbon sources added separately) failed to support the growth of MAK7 compared with good growth in rich Horikoshi medium. Furthermore, the best growth was found to be in Horikoshi medium containing both yeast extract and tryptone. If only one is added, better growth is found with yeast extract than with tryptone. This need for a mixture of carbon sources may be related the nutrient conditions in their river habitat or to the particular requirements for growth at pH 10.

One of the most fascinating aspects of alkaliphilic microorganisms is their ability to maintain a more acidic cytoplasmic pH when grown at pH 10. This results in a pH gradient across the cytoplasmic membrane that is reversed in the chemiosmotic sense and a low total proton motive force (Ivey and Krulwich, 1994). The same situation was found for both *Bacillus* MAK7 and *B. cereus* in the present work, where the internal pH was 9.00 and 8.76 respectively when grown at pH 10 (Table 5.3). To compensate for the reversed ΔpH, the membrane potential was increased to about -170 mV in both strains (Table 5.2). This partly offsets the reduction in the proton motive force, but it still decreases to -112 and -97 mV for MAK7 and *B. cereus* respectively. However,

given the good growth achieved at pH 10, this level of proton motive force is sufficient to drive ATP synthesis.

A requirement for significant amounts of cations (Na^+ and K^+) has been reported for growth of alkaliphilic bacteria at pH values of 9 and above (Wei *et al.*, 2007). In the present work, *Bacillus* MAK7 was found to grow optimally between 0 and 100 mM NaCl and *B. cereus* had an optimum salinity range from 0 - 400 mM NaCl (Figure 6.2). Therefore, both strains do not appear to have a requirement for Na^+ for their growth. Based on these results, both strains were classified as non-halophilic bacteria, because optimal growth was possible in the absence of NaCl. Moreover, both MAK7 and *B. cereus* also exhibited no requirement for K^+ ions (Figure 6.3). These findings were confirmed by the observations that neither strain required Na^+ or K^+ for optimum respiration rates (Figures 6.4 and 6.5).

The activity of three intracellular enzymes, malate dehydrogenase, fumarase and hexokinase, was investigated to determine their response to changes in pH. The enzymes extracted from MAK7 and *B. cereus* behaved in the same way. Malate dehydrogenase was found to be more resisatant to alkaline pH showing the highest specific activity at pH 10 (Figure 6.6). The activity of fumarase and hexokinase was significantly reduced at pH 8.5 and above (Figures 6.7 and 6.8).

The influence of increasing salinity on malate dehydrogenase activity in crude cell-free extracts from both environmental strain MAK7 and the laboratory strain of *B. cereus* was observed. It was found that the activity of malate dehydrogenase from both strains

increased with increasing salinity up to 400 mM NaCl, but then declined very significantly at salinities above 800 mM NaCl (Figure 6.9). The reason for the increased activity of malate dehydrogenase in the presence of NaCl is not known and does not reflect a general requirement for NaCl by *Bacillus* MAK7 and *B. cereus*.

7.2. Future Work

Attempts to measure the NADH oxidase specific activity from both environmental strain *Bacillus* MAK7 and *B. cereus* were ultimately unsuccessful (data not included). However, further investigation into the effect of pH on activity of intracellular enzymes such as NADH oxidase will provide further information about the uniformity of internal pH in cells of *Bacillus* MAK7 and *B. cereus*.

The amino acid analogue α-aminoisobutyric acid (AIB) has been widely used in the study of active transport of amino acids into cells of alkaliphilic *Bacillus* bacteria. Therefore, the study of AIB uptake into cells of *Bacillus* MAK7 and *B. cereus* would allow the investigation of the role played by the membrane potential and pH gradient in the uptake of amino acids at pH 10. Investigations into the effect of sodium and potassium concentration on amino acid (^{14}C-AIB) transport would also provide further insights into the bioenergetic properties of the both alkaliphilic *Bacillus*.

Furusawa and Koyama (2004) have suggested that unsaturated fatty acids added to the medium exhibit an inhibitory effect on the growth of various bacteria. Thus, the study of the effect of saturated and unsaturated fatty acids on the membrane potential of alkaliphilic *Bacillus* MAK7 and *B. cereus* may provide more information about the bioenergetic requirements of alkaliphilic bacteria.

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Chapter Eight

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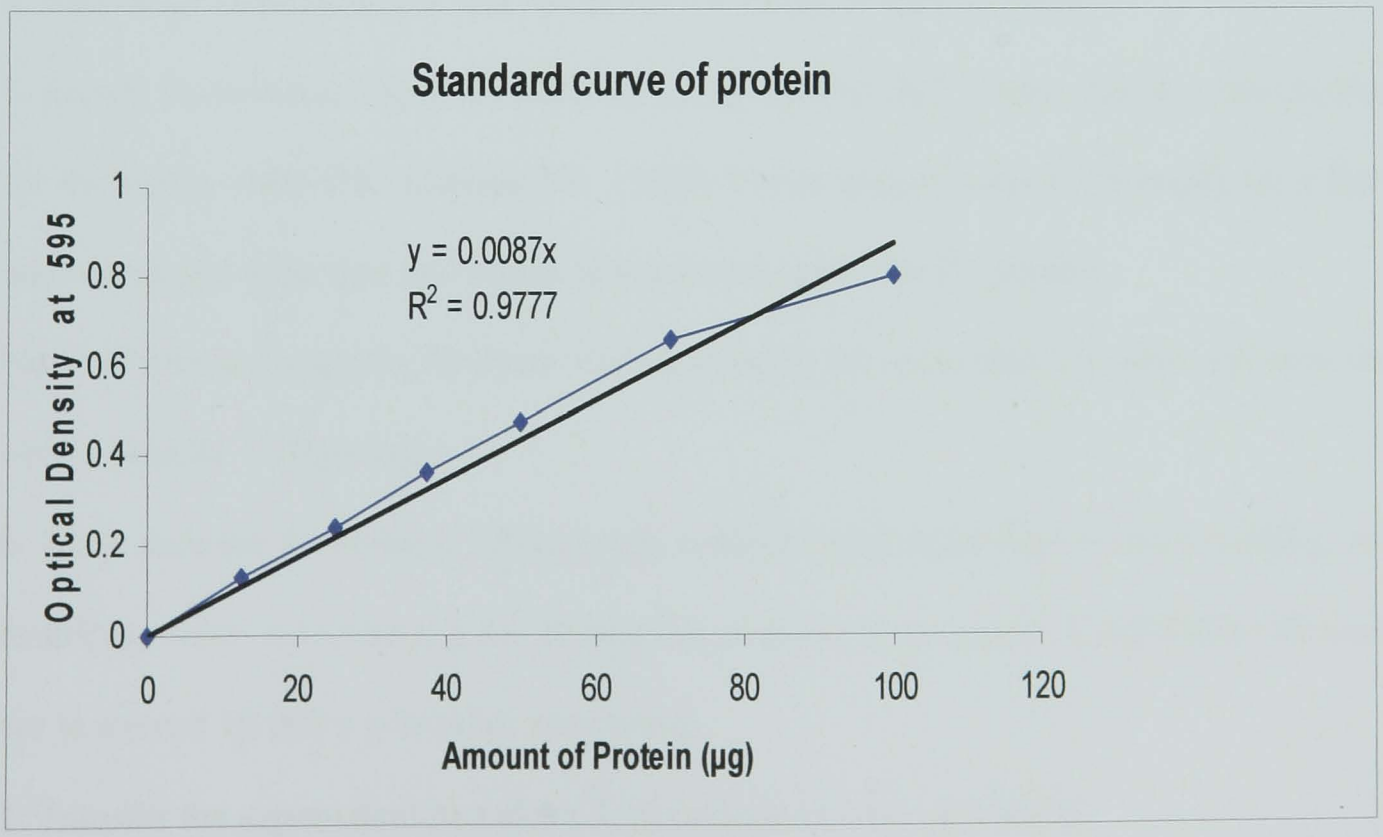
Appendices

Appendix A

Protein standard curve

Tube number	Volume of BSA Stock solution (µl)	Volume of distilled water (µl)	Total volume in each tube (µl)	Amount of Protein (µg)	O.D. 595
1	0	100	100	0 (Blank)	0
2	2.5	97.5	100	12.5	0.127
3	5	95	100	25	0.241
4	7.5	92.5	100	37.5	0.361
5	10	90	100	50	0.471
6	15	85	100	75	0.653
7	20	80	100	100	0.796



Appendix B

PowerSoil DNA kit (MoBio Laboratories Inc, California)

Experienced User Protocol

(Please wear gloves at all times)

1. To the PowerBead Tubes provided, add 0.25 grams of soil sample.

2. Gently vortex to mix.

3. Check Solution C1. If solution **C1** is precipitated, heat solution to 60°C until dissolved before use.

4. Add 60 µl of Solution **C1** and invert several times or vortex briefly.

5. Secure PowerBead Tubes horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog No. 13000-V1) or secure tubes horizontally on a flat-bed vortex pad with tape and vortex at maximum speed for 10 minutes.

Note: If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.

6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing and centrifuge tubes at10,000 x *g* for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.

7. Transfer the supernatant to a clean 2 ml collection tube (provided).

Note: Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.

8. Add 250 µl of solution **C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.

9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.

10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean 2 ml collection tube (provided).

11. Add 200 µl of solution **C3** and vortex briefly. Incubate at 4°C for 5 minutes.

12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.

13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean 2 ml collection tube (provided).

14. Shake to mix Solution **C4** before use. Add 1200 µl of solution **C4** to the supernatant and vortex for 5 seconds.

15. Load approximately 675 µl onto a Spin Filter and centrifuge at 10,000 x *g* for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the Spin Filter and centrifuge at 10,000 x *g* for 1 minute at room temperature. Load the remaining supernatant onto the Spin Filter and centrifuge at 10,000 x *g* for 1 minute at room temperature.

Note: A total of three loads for each sample processed are required.

16. Add 500 µl of solution **C5** and centrifuge at room temperature for 30 seconds at 10,000 x *g*.

17. Discard the flow through.

18. Centrifuge again at room temperature for 1 minute at 10,000 x *g*.

19. Carefully place spin filter in a clean 2 ml collection tube (provided). Avoid splashing any solution **C5** onto the Spin Filter.

20. Add 100 µl of solution **C6** to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR grade water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog No. 17000-10).

21. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.

22. Discard the Spin Filter. The DNA in the tube is now ready for any downstream application. No further steps are required.

Appendix C

List of solution used

50 X TAE

242 g Trizma base, 57.1 ml glacial acetic acid and 18.6 g EDTA are added to 900 ml dH_2O before adjusting the final volume to 1 litre with additional dH_2O. This solution is diluted 1 in 50 to produce 1X TAE suitable for use as an electrophoresis buffer.

SOC

The following reagents are added to 900 ml dH_2O:

20 g Tryptone, 5 g Yeast Extract 2 ml of 5 M NaCl, 2.5 ml of 1 M KCl, 10 ml of 1 M $MgCl_2$, 10 ml of 1 M $MgSO_4$ and 20 ml of 1 M glucose before adjusting the final volume to 1 litre. Adjust pH to 7 prior to autoclaving at 121°C (15 lbs in^{-1}) for 15 minutes.

Appendix D

1. Results of Growth Rate Calculations

Strains	Y	X	X1-X2	x 60
Bacillus MAK7	Y1= -0.30 Y2= -0.60	X1= 1.396 X2 = 0.471	0.925	55 min
Bacillus cereus	Y1= -0.30 Y2= -0.60	X1= 1.646 X2= 0.972	0.674	40 min
Bacillus subtilis	Y1 = +0.20 Y2 = - 0.10	X1= 4.643 X2= 3.417	1.226	74 min

2. Results for Biomass

Strains	Weight of aluminium cap (g)	Weight of aluminium cap after drying (g)	Difference in g / 5ml	Divide by 5 to get mg ml^{-1}	Average mg ml^{-1}
MAK7 strain	1.88 1.92	1.92 1.97	0.04 0.05	8 10	9
Bacillus cereus	1.39 1.39	1.41 1.40	0.02 0.01	4 2	3
Bacillus subtilis	1.40 1.95	1.43 1.98	0.03 0.03	6 6	6