Molecular Identification and Physiological Characterization of Halophilic and Alkaliphilic Bacteria Belonging to the Genus *Halomonas*

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Abstract

Alkaline saline lakes are unusual extreme environments formed in closed drainage basins. Qabar - oun and Um - Alma lakes are alkaline saline lakes in the Libyan Sahara. There were only a few reports (Ajali et al., 1984) on their microbial diversity before the current work was undertaken. Five Gram-negative bacterial strains, belonging to the family of *Halomonadaceae,* were isolated from the lakes by subjecting the isolates to high salinity medium, and identified using 16S rRNA gene sequencing as *Halomonas pacifica*, *Halomonas sp, Halomonas salifodinae, Halomonas elongata and Halomonas campisalis.* Two of the *Halomonas* species isolated (*H. pacifica* and *H. campisalis*) were chosen for further study on the basis of novelty (*H. pacifica*) and on dual stress tolerance (high pH and high salinity) shown by *H. campisalis*. Both species showed optimum growth at 0.5 M NaCl, but *H. campisalis* alone was able to grow in the absence of NaCl. *H. pacifica* grew better than *H. campisalis* at high salinities in excess of 1 M NaCl and was clearly a moderately halophile. *H. pacifica* showed optimum growth at pH 7 to 8, but in contrast *H. campisalis* could grow well at pH values up to 10. ^{13}C - NMR spectroscopy was used to determine and identify the compatible solutes accumulated *by H.* pacifica *and H. campisalis* grown in rich and minimal media at different concentrations of NaCl. *H. pacifica* and *H. campisalis* accumulated betaine in rich (LB) medium with ectoine only appearing at the highest salinity tested (2.5 M NaCl). In contrast, in M9 minimal medium, no betaine was detected and ectoine and hydroxyectoine were accumulated at high salinities. *H. campisalis* was able to grow well with urea or nitrate as the sole source of nitrogen and was shown to be capable of efficiently removing nitrate from the medium under aerobic assimilatory conditions, where it is incorporated into biomass.

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Contents

Abbreviations

a_i	Concentration inside the cell
a_o	Concentration outside the cell
ATP	Adenosine triphosphate
bp	base pair (s)
BSA	Bovine serum albumin
^0C	Centigrade
CAPS	3-(cyclohexylamine)-I-propanesulfonic acid
CFE	Crude cell free
CTAB	Cetyltrimethylammonium bromide
dpm	Disintegration per minute
dH_2O	distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
D2O	Deuterium Oxide
EB	Ethidium bromide
ECV	Extracellular volume
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin adenine dinucleotide
g	Gram (s)
h	Hour (s)
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
ICV	Intracellular volume
Kb	Kilobase (s)
LB	Luria-Bertani medium
M	Molar
MES	2-(N-morpholino) ethanesulfonic acid
mg	Milligram (s)
min	Minute (s)
ml	Millilitre (s)

mM	Millimole (s)
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide (oxidise form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
OAA	Oxaloacetic acid
OD	Optical density
PCR	Polymerase chain reaction
PEP	Phospho-enol-pyrouvate
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
$\Delta\Psi$	Transmembrane electrical potential
µg	Microgram
µl	Microlitre
%	Percentage

CHAPTER 1

General Introduction

1 General Introduction

1.1 Hypersaline Environments

Hypersaline environments can be found on all continents and in most countries around the world (Gillevet, 2002) and have been defined as environments with total salt concentrations exceeding that of seawater (Grant, 2004). Most hypersaline aquatic ecosystems are classified into thalassic waters that describe marine waters with high concentrations of NaCl and athalassic waters that refer to non-marine waters with an appreciable salt content (Maturrano et al., 2006). A typical example of a thalassohaline environments are solar salterns, where sea salt is produced as a result of the evaporation of seawater (Grant, 2004). Table 1.1 illustrates the different ionic compositions found in thalassic and athalassic brines.

Athalassohaline hypersaline environments contain ionic compositions markedly different from that of thalassohaline environments. For example, athalassic waters (non-marine waters) are dominated by anions other than chloride and/or cations other than sodium (Maturrano et al., 2006). Potassium, magnesium, or sodium are the most dominant in these systems and are frequently the sources of potash, magnesium metal, soda and borax if the waters also contain high concentration of boron (Gillevet, 2002). However, the chemical composition of seawater brines (thalassohaline) is chiefly characterized by having NaCl as its major anion (Galinski and Trüper, 1994). In addition to ionic compositions, pH is important in determining the nature of microbial populations in hypersaline water (Grant, 2004). In the neutral saline lakes (pH 6 - 8.5), usually sodium and magnesium form the major cations and chloride and sulphate represent the major anions with low buffering capacity (Jones et al., 1998, Banciu et al., 2004).

Well studied ecosystems such as Mono Lake (US), the Dead Sea, the alkaline soda lakes in Egypt (e.g. Wadi Natrun) and Lake Magadi in Kenya are typical examples of athalassohaline hypersaline systems (Gillevet, 2002). All these ecosystems are populated by microorganisms, which must be very well adapted to the conditions (Oren, 2002b).

Table 1-1 Concentration of Ions in Thalssaohaline and Athalassohaline Brines (Grant, 2004).

	concentration ($g\ l^{-1}$)						
ion	seawater	seawater at onset of NaCl precipitation	seawater at onset of KCl precipitation	Great Salt Lake (North America)	Dead Sea	Lake Magadi	Wadi Natrun Lake Zugm
Na^+	10.8	98.4	61.4	105.0	39.7	161.0	142.0
Mg^{2+}	1.3	14.5	39.3	11.1	42.4	0	0
Ca^{2+}	0.4	0.4	0.2	0.3	17.2	0	0
K^+	0.4	4.9	12.8	6.7	7.6	2.3	2.3
Cl^-	19.4	187.0	189.0	181.0	219.0	111.8	154.6
SO_4^{2-}	2.7	19.3	51.2	27.0	0.4	16.8	22.6
CO_3^{2-}/HCO_3^-	0.3	0.1	0.1	0.7	0.2	23.4	67.2
pH	8.2	7.3	6.8	7.7	6.3	11.0	11.0

1.1.1 The Dead Sea

The Dead Sea (Figure 1.1) is a hypersaline lake belonging to the athalassohaline category, and occupies the lowest place on the surface of the earth (Khlaifat et al., 2010). It has earned its name due to the very high salt concentration values of its water body, with a salinity value of 30% (Khlaifat et al., 2010). The maximum length and mean width of the Dead Sea is about 55 km x 11 km, with a volume of 132 km^3 , a surface area of 625 km^2, with maximum depth of ~ 30 m (Lensky et al., 2005). The Dead Sea lies some 422 m below sea level when it was last measured at the end of November 2009 (Talafeha, 2009). Its water components has proportions of salts very different from seawater, and the salt concentration of the water is about ten times higher than that of sea water (Ventosa et al., 1982, Lensky et al., 2005). The ionic composition of its water, with its high concentrations of divalent cations such as magnesium and calcium, is highly inhibitory even to those microorganisms highly adapted to life at high salinity (Oren, 1999). The dominant cations of the surface water body are Mg^{2+} , Na^{2+}, Ca^{2+} and K^{2+} (Nissenbaum, 1975).

The Jordan River is the major source of freshwater entering its northern end (around 250 million cubic meters per year (MCM/year) , but there is no outlet (Khlaifat et al., 2010). The temperature of the upper water body ranges between 21 and 36°C with pH values between 5.5 to 6.4 (Nissenbaum, 1975). The major difference between the Dead Sea and the Great Salt Lake is that the Dead Sea is principally a magnesium chloride Lake whereas the Great Salt Lake is a sodium chloride lake with a high level of sodium sulphate (Post, 1977).

Figure 1-1 The Dead Sea in the Middle East. Figure adapted from: Dead Sea Nail: www.deadseanail.com

1.1.2 The Great Salt Lake

The Great Salt Lake, Utah (Figure 1.2) is the largest hypersaline thalassohaline lake located in the Great Basin of North America (Jones et al., 2009, Oren, 2002a). The lake is bordered by desert ecosystems to the south and west, and mountains to the east and north (Lucas and MacGregor, 2006). The Great Salt Lake has a maximum length of about 122 km and width of 50km, with a maximum depth of 10m, and its temperature ranges from 5 to 35 °C (up to approximately 45°C in the shallow margins) (Post, 1977).

Inflow to the lake is from three major rivers that drain mountain ranges to the east and empty into the southern arm of the lake, from precipitation directly on the lake, and from minor groundwater inflow (Jones et al., 2009). In the northern arm, there is a massive accumulation of organic matter resulting from more than 100,000 years of productivity, cycling from a freshwater to a saline lake, plus the influence of human industry and agriculture in more recent times (Post, 1977).

There is no a natural outlet to the sea, thus, the only outflow is by evaporation (Post, 1977, Jones et al., 2009). Salt concentration in the lake varies, and it ranges between 9% (in the southern arm) to 18% (in the northern arm) (Lucas and MacGregor, 2006).

Figure 1-2 The Great Salt Lake, Utah, USA. Figure Adapted from: Great Salt Lake of Utah: www.destination360.com

1.1.3 Soda lakes

Hypersaline alkaline environments (Soda lakes) are universally distributed, naturally occurring highly alkaline environments where pH values commonly exceed pH 10 (Rees et al., 2004). They are often formed in areas with a dry climate as a result of complex interactions of the geological, climatic, and biogeochemical conditions that allows accumulation of salt in brine (Sorokin and Kuenen, 2005, Banciu et al., 2004). These ecosystems provide the most stable, high-to-extremely high pH habitats on Earth (usually around 9.5 - 10.5) because of the high buffering capacity of sodium carbonate (Sorokin and Kuenen, 2005, Jones et al., 1998).

The Soda Lakes are characterized by containing high alkalinity and salinity environments that form in closed drainage basins exposed to high temperature, high light intensities and high evaporation rates (Jones et al., 1998). Water chemistry of soda lakes shows low amounts of Mg^{2+} and Ca^{2+}. Hence, the lakes become enriched in CO_3^{2-} and Cl^-, with pH values ranging between 8 to >12 (Jones et al., 1998). Ionic composition and pH of the brine are considered to be the main differences between the soda lakes and other hypersaline environments (Kristjánsson and Hreggvidsson, 1995).

The formation of a soda lake has similarities with the generation of an athalassohaline salt lake, but with the main difference that a soda lake (pH 9 - 11) is characterized by high concentrations of sodium carbonate (Na_2CO_3+$NaHCO_3$) or carbonate complexes as the major anion in brine and low amounts of both magnesium (Mg^{2+}) and calcium (Ca^{2+}) because of the limited solubility of those cations at high pH (Jones et al., 1998, Galinski and Trüper, 1994, Duckworth et al., 1996).

Hence, the organisms that inhabit these environments usually require very low concentrations of both Mg^{2+} and Ca^{2+} in their medium (Tindall et al., 1980). Microorganisms which thrive in alkaline saline lakes possess special mechanisms to adapt to both high pH and high salt concentration that make them attractive for fundamental research and for applications in industrial biotechnology (Margesin and Schinner, 2001b)**.** Table 1.2 shows the location of a number of hypersaline Soda Lakes including the Rift Valley of Kenya and Tanzania (Lakes Bogoria, Magadi and Natron), in the Libyan Desert, in Egypt (Wadi Natrun), and in the western mountain deserts of the USA (California and Nevada) (Ventosa et al., 1998a, Sorokin and Kuenen, 2005).

Table 1-2 Distribution of Soda Lakes and Soda Deserts in Africa, US and Europe (Grant, 2006). (Qabar-oun and Um-Alma lakes are not recorded).

Africa	**The Americas USA**	**Europe**
Egypt: Wadi Natrun East Africa: Lake Magadi, Lake Natron, Lake Bogoria, etc. Libya: Fezzan (Qabar – oun and Um -Alma Central Africa: Lake Chad	California: Owens Lake (Inyo County), Searles Lake, Trona (San Bernardino County), Borax Lake (Lake County) Nevada: Ragtown Soda Lakes (near Carson Sink) Wyoming: Union Pacific Lakes (near Green River and along Union Pacific Railroad) Oregon: Albert Mexico: Lake Texcoco Venezuela: Lagunilla Chile: Antofagasta	Russia: Caspian Sea region Hungary: Szegedin district

1.1.3.1 Wadi Natrun Lakes

The Wadi Natrun alkaline saline lakes occupy an elongated depression about 90 km northwest of Cairo (Taher, 1999). They are considered to be eutrophic ecosystems that contain high levels of inorganic and organic nutrients, with phosphate concentrations ranging between 116 - 6,830 µM, nitrate between 53 - 237 µM, ammonia between 2 - 461 µM, and dissolved organic carbon in the range 136 - 1552 mg l^{-1} (Oren, 2002b).

The area has average length and width of about 60km × 10km, and its depth ranges from 0 - 30 cm and decreases in the the lakes where groundwater discharges occur (Taher, 1999). The lakes are highly alkaline; with pH 9.0 - 9.5 found for most of the lakes.

The Wadi Natrun depression is surrounded by a series of evaporitic lakes (marine and non-marine), which are different in size depending on their position in the wadi and the time of the year (Shortland, 2004). All these lakes contain the highest levels of water in winter and some are dry completely in summer with differing amounts of salts during the hottest months of the year (Taher, 1999, Shortland, 2004).

The area is characterized as an arid climate region, with a long period of dry summer (temperature ranges from 14.2°C in January to 29.5°C in August), low humidity, low and inconsistent rainfall (Taher, 1999). Consequently, the occasional rain over the area contributes little to subsurface water. Furthermore, salt concentrations tend to be highest at the surface of the brine (Taher, 1999).

Table 1-3 Salt Concentrations (g/l) and pH of the Wadi Natrun Lakes (data for August 1976) (Oren, 2002b, Imhoff et al., 1979) *NR* = not reported.

Lake	Salinity	Na^{2+}	K^{2+}	Cl^{-}	CO_3^{2-}	SO_4^{2-}	pH
Rizunia	91.9	31.9	0.21	20.9	19.8	17.3	10.9
Gabara	237.9	79.9	1.0	86.9	23.4	28.8	11
Hamra	393.9	142.0	2.3	154.6	67.2	22.6	11
Zugm	374.2	137.0	1.40	73.7	6.60	48.0	10.9
Gaar	159.2	54.0	0.60	66.3	4.4	18.1	NR
Muluk	388.6	135.5	2.5	156.0	49.2	31.7	11.2

Figure 1-3 Lake Zug, Wadi Natrun showing development and modern exploitation of evaporates, adapted from (http://antiquity.ac.uk/projgall/shortland/shortland.html).

1.1.3.2 Lake Magadi

The Kenyan-Tanzanian Rift Valley contains a number of alkaline saline lakes, which have total dissolved salts in the range from 5% w/v to 35% w/v (saturation) and pH values in the range 8.5 to >11.5 (Tindall et al., 1980, Rees et al., 2004). Lake Magadi which is located in southern part of the Rift valley of Kenya, about 100 km southwest of Nairobi, is one of this type (Atmaoui and Hollnack, 2003). The area represents a flat plain, where the lake itself stretches in a N–S direction and has the lowest altitude (around 575 m) of the district (Atmaoui and Hollnack, 2003).

Lake Magadi (Figure 1.4) is solid sodium sesquicarbonate (trona, Na,CO, NaHCO,$2H_20$) for most of the year (Tindall et al., 1980). During dry periods, Lake Magadi is covered by trona deposits about 1 - 2 cm thick, which leads to the presence of a large number of purple photosynthetic bacteria (Zhilina and Zavarzin, 1994). Due to the solar effect and high temperature (55°C), the pH of 10.2 is buffered by trona (Zhilina and Zavarzin, 1994). The lakes of the Rift valley vary in salinity, from almost fresh water to salt-saturated (Oren, 2002b). Flooding of Lake Magadi often takes place in spring season (March and April), dries again by June and July, and there is no outlet source connected with the lake. Hence, the lake is fed by runoff, groundwater and saline hot springs (Oren, 2002b).

The genesis of alkaline and neutral hypersaline lakes is shown in Figure 1.5.

Figure 1-4 Red colour due to Haloalkaliphilic Archaea (Natronobacteria) on Trona crusts at Lake Magadi, Kenya. Adapted from (Grant, 2006).

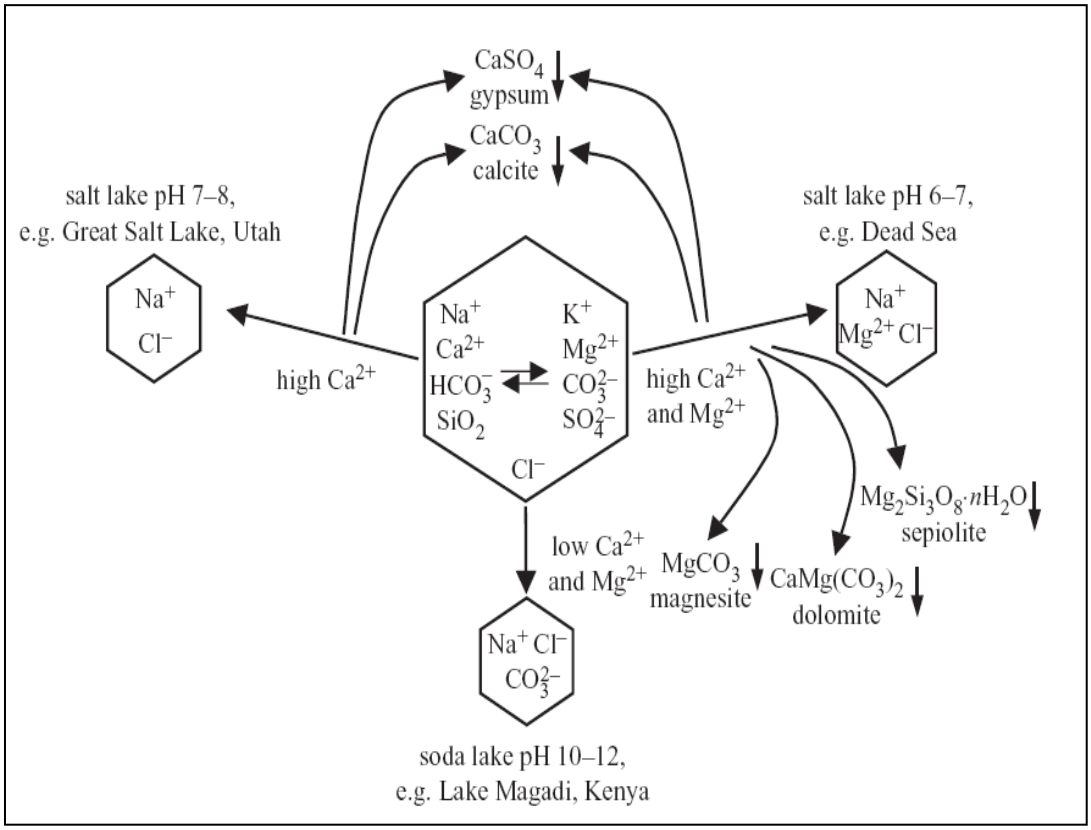


Figure 1-5 Schematic representation of the genesis of hypersaline brines. Alkaline lake development is dependent on low levels of Ca^{2+} and Mg^{2+}. Neutral lakes develop where Ca^{2+} and Mg^{2+} levels are high. High Mg^{2+} lakes are more acidic due to reactions. (Grant, 2004).

1.2 Biodiversity of Halophilic and Halotolerant Microorganisms

Biological diversity is usually defined as the total variability of life on Earth (Seckbach, 2000). It can also be defined as the quantity of prokaryotic species and their relatives present in a community, or the distribution of information in a community, which can be expressed in different ways: as phylogenetic trees, physiology, metabolism and genomics (Torsvik et al., 2002). Traditionally, the unit of biodiversity is the species (Øvreås, 2000).

Prokaryotic organisms have inhabited the Earth for about 3.8 billion years, which is 3 billion years longer than that of the animal and plant kingdoms. However, there are at least a dozen groups of microbial life forms on Earth (bacteria, fungi, algae and protozoa) that are even more diverse than higher eukaryotic organisms such as animals and plants (Øvreås, 2000). In addition, techniques used over the past 20 years have shown that so far only an extremely small fraction of the microbial diversity has been cultivated from all environments investigated (Keller and Zengler, 2004)

Microbes are the most diversity group of organisms that exist on Earth, constituting 60% of the total biomass (Singh, 2010). Bacteria make up a major fraction of the biological diversity on Earth because they play essential roles in quite diverse environments (Pontes et al., 2007). Therefore, diversity analysis is important for many reasons e.g. microorganisms are responsible for essential geochemical cycling, which maintains the biosphere (Singh, 2010). Furthermore, it is important to understand the regulation of biodiversity and its functional role, and to increase the knowledge of the diverse genetic resources in microbial communities (Øvreås, 2000).

1.2.1 Halophilic and Halotolerant Microorganisms

Halophilic microorganisms are defined as organisms that require salt for growth, whereas halotolerant microorganisms are able to grow in the absence of salt and tolerate varying concentrations of salt (Margesin and Schinner, 2001b, Ventosa and Nieto, 1995). Microorganisms capable of growth in the presence of salt are distributed among diverse groups in the domains *Archaea*, *Bacteria*, and *Eukarya* (Oren, 2002a, Ma et al., 2010, Ventosa et al., 1998b, Margesin and Schinner, 2001b). Halophilic microorganisms are defined as those organisms that show good growth at salt concentrations exceeding 100 g 1^{-1} (Oren, 2002b).

Many authors have classified microorganisms according to their response to the salt into several categories (Russell, 1989, Kushner, 1978, Reed, 1986). One original set of definitions of halophilic and halotolerant microorganisms by Kushner, is still relevant today i.e. (1) slight halophiles (marine bacteria) grow best from 0.2 - 0.5 M NaCl, (2) moderate halophiles grow best from 0.5 - 2.5 M NaCl, and (3) extreme halophiles; these organisms grow best at 2.5 - 5.2 M NaCl. Halotolerant microorganisms are those that grow best in media containing < 0.2 M NaCl, and tolerate higher concentrations (Kushner, 1978, Margesin and Schinner, 2001b).

The organisms found in saline habitats were classified as *moderately halophilic* if they grow optimally between 0.2 and 2.0 M NaCl, with a growth range from 0.1 - 4.5 M NaCl. *Extreme halophiles* show optimum growth between 3.0 – 5.0 M NaCl and have a growth range from 1.5 - 5.5 M NaCl, whereas *halotolerant* microorganisms grow optimally between 0 and 0.3 M NaCl with a growth range from 0 - 1 M NaCl (Gilmour, 1990). Moderately halophilic bacteria constitute a heterogeneous group of microorganisms

which require NaCl for growth and grow over a rather wide range of external salt concentrations, however, this range depends on growth conditions (temperature and nutrients) (Ventosa et al., 1982, Ventosa et al., 1998b) Many halophilic (salt-loving) microorganisms that live in large numbers in some ecosystems, can be recognized without the need for a microscope (Ma et al., 2010). These organisms give a pink-red colour to the brines due to the present of halophilic Archaea of the family *Halobacteriaceae* and other representatives of the Halobacteriales, Bacteria (*Salinibacter*), and Eucarya (*Dunaliella salina*) also contribute to this ecosystem (Ma et al., 2010, Oren and Rodríguez-Valera, 2001). Most of these organisms have a high content of carotenoid, especially C-50 carotenoid pigments (Oren, 2002a).

The previous studies that have been made into hypersaline environments, permitted the isolation and taxonomic characterization of a large number of halotolerant, moderately and extremely halophilic species (Ghozlan et al., 2006, Ventosa et al., 1998b, Ozcan et al., 2006, McKay, 2007, Tang et al., 2011). Most moderately halophilic species are represented by methanogenic Archaea, Gram-negative or Gram-positive aerobic or facultatively anaerobic moderately halophilic bacteria (Ghozlan et al., 2006). Gram-negative species considered to be at least moderately halophilic include *Halomonas*, *Deleya*, *Volcaniella*, *Flavobacterium*, *Paracoccus*, *Pseudomonas*, *Halovibrio* and *Chromobacterium*, The family of *Halomonadaceae*, which includes two genera of moderately halophilic bacteria: *Halomonas* and *Chromohalobacter* (Ventosa et al., 1998b, Mata et al., 2002) is considered one of the most widespread groups in high salinity environments. In general, saline lakes, salted foods and saline soils provide a

wide set of ecological niches for large numbers of moderate and extreme halophiles (Baati et al., 2008, Madigan and Orent, 1999, Oren, 2002b).

1.2.2 Haloalkaliphilic Microorganisms

Haloalkaliphiles require both pH > 9 and high concentrations of salinity (NaCl) up to 33% wt/vol (Horikoshi, 1999, Fujinami and Fujisawa, 2010). Furthermore, halotolerant and alkaliphilic bacteria also exist e.g. a Gram-negative bacterium was isolated from a salt pool in southern Italy, which grows aerobically at the optimum temperature of 37°C, and at pH 7.5 - 10 (Optimum 9.0), and was able to tolerate NaCl concentrations up to 20 %, (Romano et al., 2006).

Thus, soda lakes (naturally occurring alkaline and highly saline environments) are an ideal natural environments for developing and growing obligate haloalkaliphilic microorganisms at optimal pH values around 10 (Sorokin and Kuenen, 2005, Rees et al., 2004)**.** Soda lakes create a unique habitat for haloalkaliphilic species (Banciu et al., 2004) and eukaryotic microorganisms and higher organisms such as the brine shrimp and the brine fly are also present (Ventosa and Nieto, 1995).

Microbial communities are widespread in alkaline lakes with high salinities, and most soda lakes that show green or red colour are regarded as naturally eutrophic reservoirs for considerable microbial diversity (Mwirichia et al., 2010). In those ecosystems such as Lake Magadi (Rift Valley of Kenya), some of the Wadi Natrun lakes (Egypt) and similar lakes in a few other places on Earth, halophilic and alkaliphilic often impart a green, red and purple colour to the brines due to present of *Archaea* of the order *Halobacteriales*, photosynthetic purple bacteria, cyanobacteria and green algae (Oren, 2002b, Kristjánsson

and Hreggvidsson, 1995). In addition, the most abundant microorganisms observed are archaeal halophilic heterotrophs, halophilic sulfur bacteria of the genus *Halorhodospira*, *Halomonas* spp. and alkaliphilic *Bacillus* species (Oren, 2002b).

Recently, the isolation of several anaerobic *halo-alkaliphiles* was possible from sediments in the soda lakes (Kristjánsson and Hreggvidsson, 1995). Extensive researches on salt lakes and other saline alkaline lakes and hypersaline environments in different Countries, has led to the characterization and taxonomic description of many novel species within the family of *Halobacteriaceae* and the *Halomonadaceae* (Oren, 2008)

Several studies have been conducted on isolation and characterization of novel species belonging to family *Halomonadaceae* of the genus *Halomonas* from lakes of the East African Rift Valley and different (hyper) saline environments around the world (Sánchez-Porro et al., 2003, Ghozlan et al., 2006, Duckworth et al., 2000). Deep understanding on phylogenetic and physiological diversity as well as their biotechnological applications within the *Halobacteriaceae* and the *Halomonadaceae* has increased tremendously in past years (García et al., 2004, Duckworth et al., 2000, Skerratt et al., 1991, Ghozlan et al., 2006, Arahal et al., 2002, Mata et al., 2002, Mwirichia et al., 2010, Oren, 2008)

In addition, a new groups of haloalkaliphilic sulfer-oxidising bacteria, that play an important role in element cycling in natural environments was isolated from different soda lakes (Sorokin and Kuenen, 2005).

Figure 1-6 Hypersaline Zeebhstein Sea. The pink colour caused by microbes with an extreme preference for salt. Adapted from Dr. Karsten Kotte/Universiy at Heidelberg: http://www.rsc.org/chemistryworld/News/2009/April/06040901.asp.

1.3 Diversity of Compatible Solute

Compatible solutes are defined as small organic molecules responsible for providing osmotic balance without interfering with cell metabolism (Pflüger and Müller, 2004, Brown, 1976). They can be divided into different classes of chemical compounds (Figure 1.7, Tables 1.4 and 1.5), including sugars, or sugar alcohols, polyols and derivatives , α and β amino acids and their derivatives such as glycine betaine and ectoine (E.A, 1995, Roberts, 2005) and are mainly used for osmotic balance in cells (Roberts, 2005). Most compatible solutes are either uncharged or zwitterionic (Oren, 2008, Roberts, 2005). Within the domain Bacteria, ectoine and glycine betaine appear to be the most widely used osmotic solutes (Oren, 2008). The presence of high concentrations of salt lowers environmental water activity and causes many organisms difficulties in regulating and maintaining cell turgor (Cummings and Gilmour, 1995, Ono et al., 1998).

Microorganisms accumulate compatible solutes, either by uptake from the medium or by *de novo* synthesis in order to respond to osmotic stress (Empadinhas and da Costa, 2008). It is enables bacteria to reduce the difference between osmotic potentials of the cell cytoplasm and the extracellular environment (Lai et al., 1991). Bacteria and eukaryotes usually accumulate neutral compatible solutes, whereas archaea prefer negatively charged solutes (Martin et al., 1999, Roberts, 2004). Accumulation of compatible solutes plays an important role not only in balancing the external osmotic pressure, but also in the maintenance of protein structure and stability, and increased solubility of proteins (Pflüger and Müller, 2004). ^{13}C nuclear magnetic resonance (NMR) is a useful method for the identification of organic compatible solutes of the *Halomonadaceae* (Wohlfarth et al., 1990).

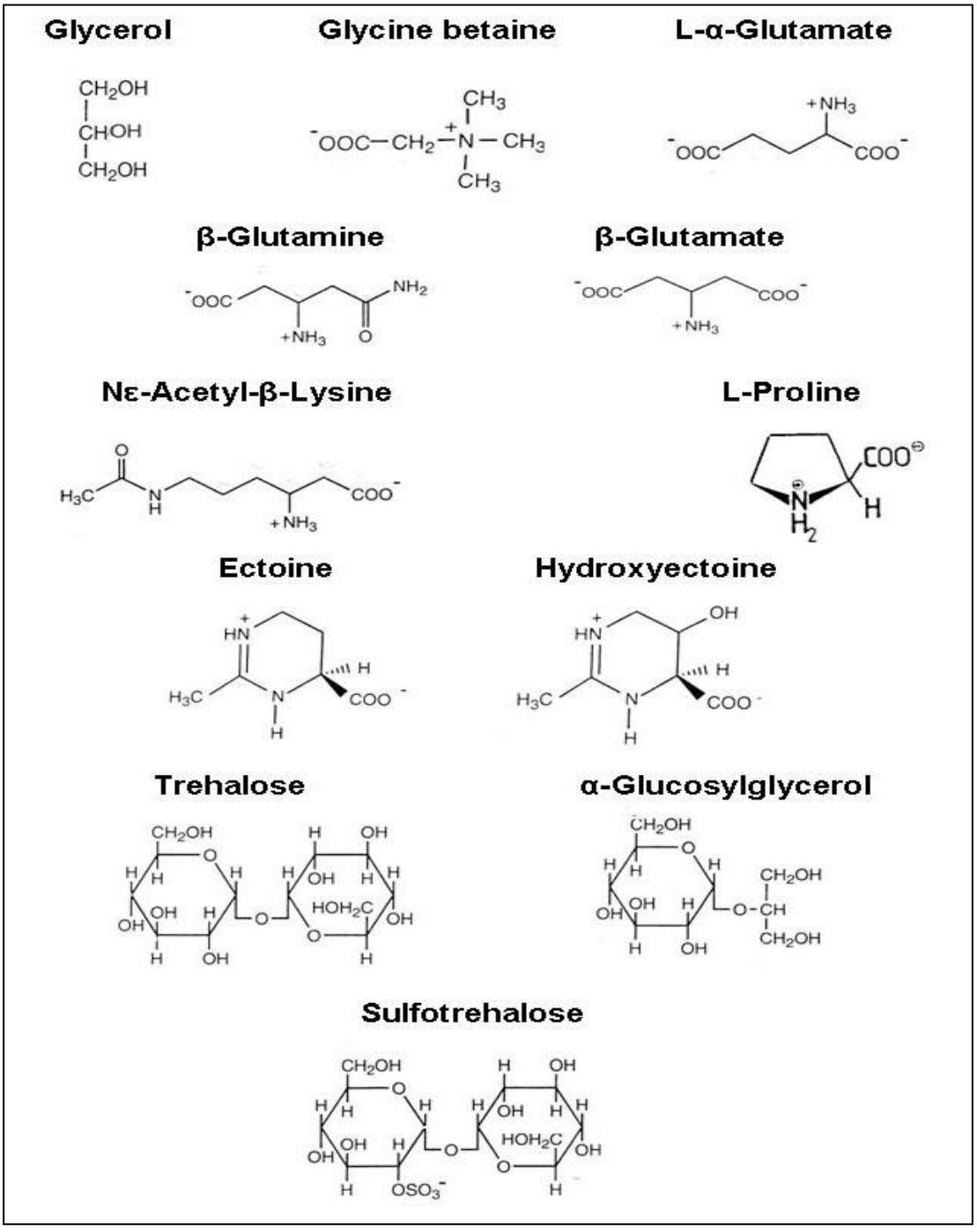


Figure 1-7 A selection of organic osmotic solutes found in halophilic and halotolerant prokaryotic and eukaryotic microorganisms (Roberts, 2005, Oren, 2008).

Table 1-4 Distribution of Compatible Solutes in Prokaryotes.

Compounds	Occurrence
Amino acids and derivatives	
N-Acetyl-ß-lysine	Unique to methanogenic Archaea
N-δ-Acetyl-ornithine	*Bacillus* spp., *Planococcocus citreus, Sporosarcina halophila*
Alanine	*Streptomyces*
Ectoine and hydroxyectoine	Halophilic/halotolerant *Bacillus* strains, *Ectothiorhodospira halochloris*, aerobic heterotrophic bacteria, most halophilic proteobacteria, *Micrococcus* spp., *Bacillus* spp., *Marinococcus* spp., *Halobacillus halophilus*
α-Glutamate	Some methanogenic archaea, marine bacteria, *Petrotoga miotherma, P. mobilis, Aquifex pyrophilus, Halobacillus halophilus*
Glutamine	*Streptomyces, Corynebacterium* sp., *Halobacillus halophilus*
β-Glutamine	Halophilic methanogens
Glycine betaine	*Bacteria, Archaea* (universal compatible solute)*a* [4]
Proline	*Streptomyces*, halophilic/halotolerant *Bacillus* strains, halophilic *Bacillus* strains, *B. subtilis* (non-halophilic)
Sugars	
Sucrose	*Anabaena, Synechocistis, Nitrosomonas europaea*
Trehalose	*Corynebacterium glutamicum, Mycobacterium tuberculosis,Thermus termophilus, Rubrobacter xylanophilus*

Tables **1.4 and 1.5** were adapted from Empadinhas and da Costa (2008).

Table 1-5 Distribution of Compatible Solutes in Prokaryotes (cont).

Compounds	Occurrence
Phosphodiesters	
Di-*myo*-inositol phosphate	*Pyrococcus woesei, Aeropyrum, Aquifex, Archaeoglobus, PyrodictiumPyrolobus, Stetteria, Thermococcus, Thermotoga, Rubrobacter xylanophilus, Persephonella marina*
Di-mannosyl-di-myo-inositol phosphate	*Thermotoga* spp.
Glyceryl-*myo*-inosityl	*Aquifex pyrophilus, Archaeoglobus fulgidus* phosphate
Glyceric acid derivatives	
Cyclic-2,3-bisphosphoglycerate	*Methanothermus fervidus, Methanobacterium thermoautotrophicum , Methanopyrus kandleri*
Mannosylglycerate	*Thermus thermophilus, Rhodothermus marinus, Rubrobacter xylanophilus, Pyrococcus, Palaeococcus, Thermococcus, Archaeoglobus, Aeropyrum, Stetteria*
Mannosylglyceramide	*Rhodothermus marinus*
Glucosylglycerate	*Agmenellum quadruplicatium, Erwinia chrysanthemi, Persephonella marina*
Glucosyl-(1,6)-glucosylglycerate	*Persephonella marina*
Mannosyl-(1,2)-glucosylglycerate	*Petrotoga miotherma*
Polyols	
Sorbitol	*Zymomonas mobilis*
Mannitol	*Pseudomonas putida*
Glucosylglycerol	*Pseudomonas mendocina, Stenotrophomonas rhizophila, Synechocystis*

Glycine betaine spans the three domains of life, even though in many prokaryotes it is not synthesized *de novo*.

1.3.1 Glycine Betaine

Glycine betaine (N, N, N-trimethylglycine) is a widely distributed biologically active compound (Roberts, 2005, Boltyanskaya et al., 2005). It was recognized in the early 1980s as the most important organic osmotic solute in photosynthetic purple bacteria and halophilic cyanobacteria (Ventosa et al., 1998a). In addition, it is considered to be a common product of a highly salt tolerant phototrophic eubacteria, and as a primary product in halophilic archaebacterial methanogens (Galinski and Trüper, 1994). The ability to synthesize glycine betaine *de novo* was detected in several strains of moderately and extremely halophilic methanogens (Lai et al., 1991)

The extremely halophilic methanogens were also found to synthesize high concentrations of β-glutamine, and Nε-acetyl-β-lysine, and also accumulate potassium ion to balance the external osmotic pressure (Lai and Gunsalus, 1992). Betaine was detected by means of ^{13}C-NMR, when *Halomonas campisalis* was grown in medium containing yeast extract (Boltyanskaya et al., 2005). *Halomonas* species accumulates high concentrations of glycine betaine at low- salinity (5% NaCl), whereas intracellular ectoine and hydroxyectoine (see section1.3.2) are accumulated by synthesis at higher salinities (Severin et al., 1992, Wohlfarth et al., 1990).

A study was carried out to evaluate the effect of five different osmoprotectors (proline, glycine, glutamic acid, proline, glycerol), on the growth of *Aeromonas trota* and *A. hydrophila* under high salt concentrations. The results showed that only betaine (1mM) was able to stimulate the growth of *A. trota* and *A. hydrophila* in the presence of 0.51 M NaCl, and allowing its growth on 0.85 M NaCl (Delamare et al., 2003).

Among all aerobic heterotrophic bacteria examined, only *Actinopolyspora halophila* was able to synthesize betaine from carbon sources such as glucose (Severin et al., 1992). Glycine betaine and proline increase the cytoplasmic volume and free water content of the cells at high osmolality (Kempf and Bremer, 1998). Glycine betaine was found to be a major organic compatible solute in extremely halophilic phototrophic bacteria of the genus *Ectothiorhodospira* isolated from alkaline soda lakes of the Wadi Natrun in Egypt (Galinski et al., 1985), when the organisms were grown on yeast extract medium, while ectoine was present at a lower concentration in complex medium than in mineral medium (Wohlfarth et al., 1990). *E. coli* accumulate glycine betaine during osmotic stress but this glycine betaine originates from the choline component of the yeast extract (Landfald and Strøm, 1986). The control mechanism of the choline-glycine betaine pathway of *E. coli* K10 is shown in Figure 1.8.

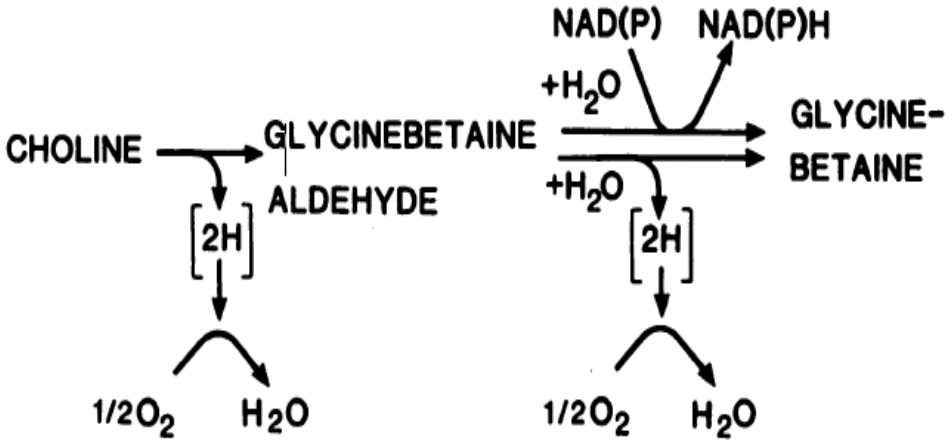


Figure 1-8 The choline-glycine betaine pathway of *E. coli* K10 (Landfald and Strøm, 1986).

1.3.2 Ectoine and Hydroxyectoine

Ectoine is a heterocyclic amino acid or a partially hydrogenated pyrimidine derivative (1, 4, 5, 6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) (Galinski et al., 1985). It is considered as an excellent osmolyte solute of aerobic heterotrophic bacteria (Galinski and Trüper, 1994). Ectoine was first detected in the extremely halophilic phototrophic bacterium (*Ectothiorhodospira halochloris*) and characterized using ^{13}C nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry and infrared spectroscopy (IR) (Galinski et al., 1985). Moreover, ectoine has been found throughout different halophilic and halotolerant microorganisms such as, photosynthetic bacteria of the genus *Halorhodospira*, and aerobic chemoheterotrophic bacteria of the genus *Halomonas* (Roberts, 2005). Bacterial species of the family Halomonadaceae are able to survive at high NaCl concentration (up to 32%, w/v) by synthesizing mainly ectoine and hydroxyectoine as osmolytes (Van-Thuoc et al., 2010). Ectoine synthesis is via the reactions of diaminobutyric acid transaminase (EctB), diaminobutyric acid acetyltransferase (EctA), and ectoine synthase (EctC) that together convert the precursor L-aspartate-β-semialdehyde into ectoine (Figure 1.9) ((Van-Thuoc et al., 2010).

The other osmoprotectant compound being almost chemically identical to ectoine is hydroxyectoine (Pastor et al., 2010). This compatible solute was discovered in the actinomycin D producer *Streptomyces parvulus* (Inbar and Lapidot, 1988). It is often synthesized together with ectoine by many other ectoine-producing species (Pastor et al., 2010). For instance, *Halomonas elongata*, *Chromohalobacter salexigens* and *Streptomyces griseus* accumulate hydroxyectoine in response to temperature upshift, providing evidence that hydroxyectoine functions as a thermoprotectant *in vivo* (García-

Estepa et al., 2006, Malin and Lapidot, 1996, Wohlfarth et al., 1990). Ectoines and hydroxyectoines can only be obtained by biotechnological procedures (Margesin and Schinner, 2001b).

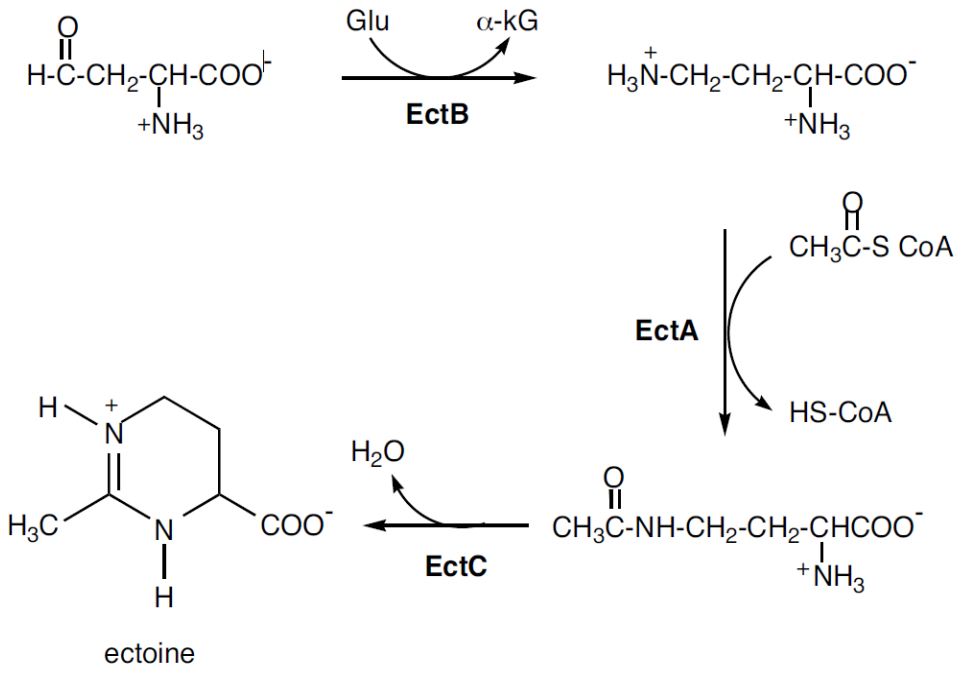


Figure 1-9 Pathway for the synthesis of ectoines (adapted from Roberts, 2005).

1.3.3 Osmoregulation of compatible solutes in prokaryotic cells

Microorganisms in natural saline environments must cope with changing of different conditions such as availability of nutrients, varying temperatures and pH, and finally osmolarity (Pflüger and Müller, 2004). The cytoplasmic membrane of bacterial cells is permeable to water, ions and organic molecules but forms barriers for most solutes in and out of cells (Poolman and Glaasker, 1998)

Water always tend to move from a high to a low activity until the potential gradient is abolished (Grammann et al., 2002). Thus, an increase in the levels of external salinity can cause deep effect on the physiology of the bacterial cell and changes the composition of the cell membrane (Cummings and Gilmour, 1995, Adams and Russell, 1992, Kates, 1986). Similarly, a lowering of external water activity (hyper-osmatic condition) causes water flows into the cells and increase the cytoplamic volume and/or development of turgor pressure (Poolman and Glaasker, 1998, Wood et al., 2001).

Prokaryotic microorganisms dwell in both natural and artificial environments with diverse and fluctuating of physical properties, such as osmolalities, salinities and temperatures (Wood et al., 2001). i.e, Halophilic organisms living in natural saline environments such as salt lakes, are challenged by two stress factors, the high inorganic ion concentration and the low water potential (Grammann et al., 2002). Thus, nonadapted microorganisms have evolved mechanisms to adapt to osmotic stress ranging from high water activity (low solute concentrations in spring water) to low water activity (high solutes concentrations) (Vreeland, 1987). The principal of these mechanisms is to lower the potential of cytoplasmic water, avoiding the loss of water from the cell and achieving osmotic strength similar to that of the surrounding medium (Grammann et al.,

2002). Microorganisms exposed to environments where the extracellular solute concentration exceeds that of the cell cytoplasm, accumulate compatible solutes together with K^+, that increase osmatic strength and minimise osmatic dehydration and death (Lai et al., 1991). It has been reported by Kempf and Bremer (1998) that microorganisms accumulate organic compatible solutes up to molar concentrations. In many bacterial cells, Intracellular K^+ ion increases rapidly with the salinity of the growth medium (Empadinhas and da Costa, 2008). This contributes to both the osmotic balance across the membrane and the stabilization of the cellular turgor pressure (Empadinhas and da Costa, 2008).

Halophilic and halotolerant bacteria use two different strategies for osmoregulation when exposed to high osmolarity: One is the so-called "salt-in cytoplasm" strategy, which involves accumulation of inorganic ions, mainly concentrations of K^+ and Cl^- in their cytoplasm to maintain osmotic balance (Galinski, 1993, Pflüger and Müller, 2004, Oren, 2008). However, this strategy is used by relatively few groups of halophiles. The organisms that employ this strategy are (i) the extremely aerobic halophilic *Archaea* of the family *Halobacteriaceae*, which includes the extreme halophiles of genera such as *Halobacterium, Haloarcula, Haloquadratum, Halorhabdus, Natronobacterium,* and *Natronococcus;* (ii) the anaerobic halophilic Bacteria of the order *Haloanaerobiales*, and (iii) the bacteruim *Salinibacter ruber* (Galinski, 1993, Oren, 2002a, Oren, 2008, Empadinhas and da Costa, 2008). Such salt-in-cytoplasm adaptation requires that the intracellular enzymatic machinery is altered to cope with the high intracellular saline conditions (Oren, 2002a, Oren, 2008).

The second type (salt–out cytoplasm) is more flexible and widespread among many microorganisms in natural environments (Oren, 2008, Galinski and Trüper, 1994, Oren, 2002a). It is based on excluding salt from the cytoplasm as much as possible and accumulating compatible solutes to provide osmotic balance (Oren, 2008, Oren, 2002a). In this type, less far – reaching adaptations of intracellular enzymatic machinery are needed (Oren, 2008). Many halophilic bacteria are able to synthesize their own compatible solutes such as glycine betaine, and ectoine (Grammann et al., 2002). However, halophilic microorganisms are also able to take up compatible solutes from the surrounding medium which is a far more economical way to accumulate osmoprotectants (Grammann et al., 2002).

1.4 Molecular Method Used to Identfiy Microorganisms

Ribosomal RNAs (Figure 1.10) are key constituents of ribosomes in all cellular organisms, and ribosomes are the sites of protein synthesis in all living cells. This process occurs in all organisms and is not affected by environmental changes (Rosselló-Mora and Amann, 2001). Bacteria have three different genes that code for 5S, 16S, and 23S rRNAs (Pontes, 2007). The chain lengths of all these components are about 120, 1650 and 3300 nucleotides, respectively (Rosselló-Mora and Amann, 2001). 5S and 16S rRNAs have proven to be of most interest in microbial ecology (Øvreås, 2000). Analysis of ribosomal 16 rRNA sequences using reference sequence data bases provides a valuable tool for determination of the extensive microbial populations in natural ecosystems (Øvreås, 2000, Bull and Hardman, 1991). Small amounts of DNA such as 16S rDNA can be

amplified by polymerase chain reaction (PCR) and microorganisms occurring in low numbers in the environment can be detected (Øvreås, 2000).

However, the study of the 16S rRNA gene only, is insufficient to describe fully the classification of prokaryotic microorganisms at species level (Rosselló and Amann, 2001). In 1991, Bull and Hardman reported that assessment of phylogenetic affinities can be measured using sequence analysis of 5S rRNA and defined sequences of the l6S rRNA molecules with reference sequence data bases, and they add that DNA-DNA hybridization, 16S rRNA sequencing and PCR technologies have also been used to determine the phylogenetic comparisons of organisms in the natural environments.

DNA-DNA hybridization and 16S rRNA sequence technique has been used by Arahal , et al. (2001), to compare the phylogenetic relationship of two moderate halophilic bacteria isolated from high saline and/or alkaline pH aquatic environmnets, with species of the genus *Chromohalobacter* or to *Halomonas elongat*a (Arahal et al., 2002).

In recent years, the study of microbial diversity in natural habitats has been facilitated by the analysis of 16S ribosomal RNA genes; these genes have been amplified by polymerase chain reaction (PCR) with DNA extracted directly from naturally occurring samples (Kamekura, 1998). In addition, this technique has improved detection levels and has enabled the sequence analysis of genes from organisms that are difficult to cultivate, and to permit the detection of even dominant and/or minority of components within the population (Bull and Hardman, 1991). The increase of known biodiversity is based on both laboratory cultures of isolated microorganisms and on the characterization of 16S rDNA sequences recovered directly from the environments (Seckbach, 2000). Thus, the study of prokaryotic diversity based on direct PCR amplification of the 16S rRNA gene

from environmental DNA sample provided a practical assessment to microbial communities in extreme environments (Øvreås, 2000).

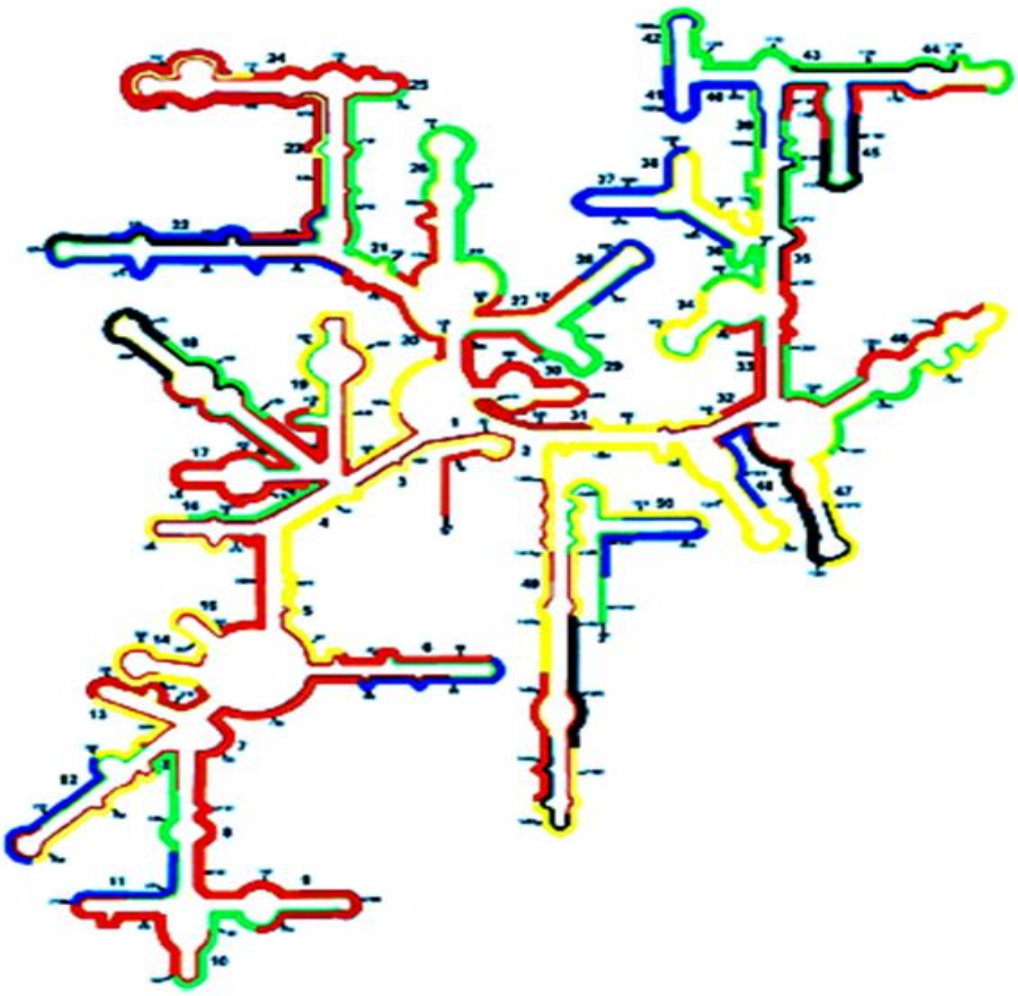


Figure 1-10 Predicted secondary structures of *E. coli* 16S rRNA. Numbers attached to nucleotides indicate sequence numbering (Fuchs et al., 1998).

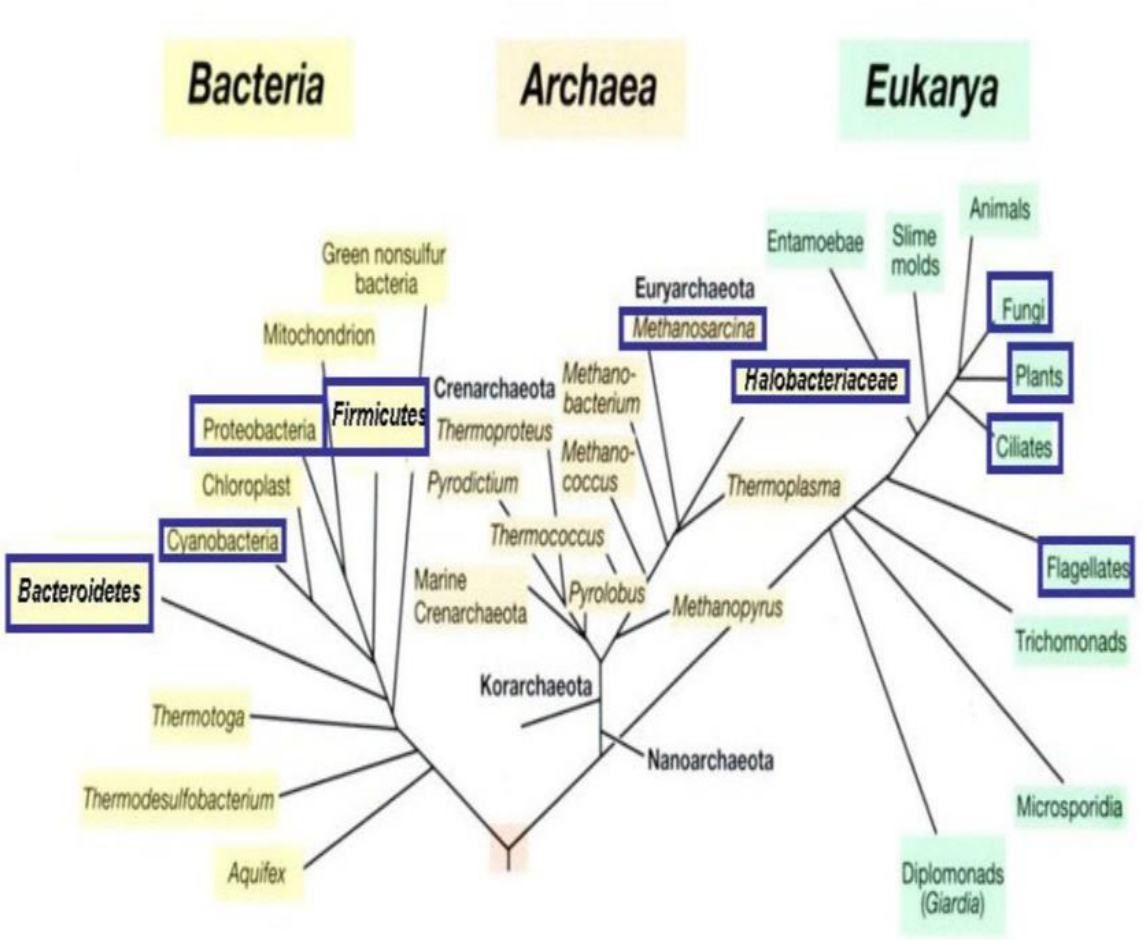


Figure 1-11 The universal phylogenetic tree of life as based on small subunit rRNA gene sequences, and the distribution of halophilic microorganisms within the tree. Groups marked with blue boxes contain at least one halophilic representative. The tree is based on Fig 11.16 in Madigan and Martinko, 2006 (brook biology of microorganisms 11th ed).

1.5 Biotechnological Applications of Halotolerant and Halophilic Microorganims

Halophilic microorganisms have been considered as a group of extremophiles with a biotechnological potential similar to that of other extremophilic microorganisms (Ventosa and Nieto, 1995). The halobacteria (extremely halophilic aerobic Archaea), the moderate halophiles (Bacteria and some methanogens) and several eukaryotic algae, are already used for some biotechnological processes (Ventosa and Nieto, 1995). For instant, the green microalga *Dunaliella,* which is produced in large amounts, can accumulate high levels of glycerol and β-carotene (Ventosa and Nieto, 1995, Ben-Amotz and Avron, 1990). This is an important commercial sources which has been widely used in the pharmaceutical, chemical and food industries (Ventosa and Nieto, 1995).

In addition, microorganisms that inhabit extreme environments can produce a variety of products which can be used across different industries such as, production of antibiotics, production of anti-tumour agents in pharmaceutical industries, and as biopesticides, anti-parasite agents and food-processing agents in the agricultural sector (Singh, 2010).

1.5.1 Compatible solutes

In 1995, Ventosa and Nieto reported that compatible solutes are easily produced by biotechnological processes. For example, a novel biotechnological process called "bacterial milking" has been established for the production of ectoine and hydroxyectoine by the extremely halotolerant bacterium *Halomonas elongata* (Margesin and Schinner, 2001b, Ma et al., 2010). Ectoine is an ingredient of many cosmetics and skin care products and is becoming increasingly important in medicinal preparations (Graf et al., 2008). In addition, these compounds are able to protect lactate dehydrogenase and other enzymes against high and low temperatures, salt and desiccation (Lippert and Galinski, 1992, Kuhlmann et al.,

2008). Thus, industrial use of compatible solutes is a very promising field (Ventosa and Nieto, 1995, Oren et al., 1992).

1.5.2 Enzymes

The discovery of extremophilic microorganisms and their enzymes has had a great potential for industrial biotechnology processes and scientific research (Demirjian et al., 2001). There are many useful extremophile enzymes that have been applied in the detergent industry (washing powders), and other biotechnological processes were derived from bacteria growing in saline alkaline lakes (Ventosa et al., 1998b, Ma et al., 2010). The most common interesting extremozymes that are active and stable under unusual environments are esterases/lipases, glycosidases, aldolases, nitrilases/amidases, phosphatases and racemases (Demirjian et al., 2001). In addition, salt-resistant enzymes such as the amylase, are produced by a number of halotolerant bacteria such as *Bacillus* sp; this is stable at 60°C and 5 M NaCl and could be used in the treatment of effluents containing starchy or cellulosic residues (Khire and Pant, 1992). Furthermore, alkalophilic organisms which are able to survive in alkaline environments produce important enzymes for biological detergents, especially alkaline proteases and cellulases (Demirjian et al., 2001).

1.5.3 Bioremediation

Hypersaline environments (including hypersaline lakes and soils) are often contaminated with harmful toxic compounds as a result of industrial activities (Ventosa and Nieto, 1995). There are a large number of moderate halophiles and halobacteria which have been isolated from contaminated hypersaline environments (Ventosa and Nieto, 1995). Thus, the ability of halophiles/halotolerant bacteria to oxidize hydrocarbons is useful for biological treatment of petroleum products in saline ecosystems (Margesin and Schinner, 2001b).

The biological treatment of saline ecosystems containing toxic compounds is clearly desirable (Ventosa and Nieto, 1995, Margesin and Schinner, 2001b). The halobacterial strain

(EH4) , isolated from a salt-marsh in the south of France, was found to be able to biodegrade a variety of *n*-alkanes and aromatic hydrocarbons in the present of salt (Bertrand et al., 1990, Oren et al., 1992). In addition, bioremediation of oil spills has been achieved successfully in marine, arctic, and antarctic environments (Margesin and Schinner, 1999). Thus, the biodegrading potential of halophiles will become increasingly important in the future.

In addition, nitrate is considered as a common contaminant compound in shallow groundwater (Smith et al., 1994). Nitrate (NO_3^- N) is commonly released into the soils and groundwater by fertilizer additions and crop applications within the agricultural process (Jacinthe et al., 2000). It is used most widely by living organisms, including higher plants, algae, fungi, and bacteria as a N source (Guerrero, 1981).

Many industries contribute to produce wastes that contain high levels of nitrate (Glass and Silverstein, 1999). Concentrations of nitrate in surface waters are usually below 5 mg NO_3^- - N/L, whereas higher concentrations have been found in groundwater (Fraser et al., 1980). The contamination of surface and ground water with fertilizer N and P is now recognized as a major water quality problem, and presents a potential risk to public health (Sharpley et al., 1987, Kapoor and Viraraghavan, 1997). High concentrations of nitrate can cause methemoglobinemia ``blue baby disease" in infants. Thus, in the U.S and Europe, nitrate is limited in drinking water to 10 mg/L N and to 12 mg/L N, respectively (Glass and Silverstein, 1999). Biological denitrification is a treatment method that can be used to remove nitrates from drinking water (Kapoor and Viraraghavan, 1997). Most of the *Halomonas* species isolated were capable of degrading aromatic compounds or/and possessing denitrifying properties (Bouchotroch et al., 2001, García et al., 2004, Mormile et al., 1999). Furthermore, nitrate reduction and denitrification occurred widely, and were carried out by both *Thioalkalivibrio denitrificans* and chemo-organotrophic halomonads (Grant et al., 2004). Therefore, these

organisms hold promise for the treatment of saline, alkaline waste due to their denitrification ability, wide range of carbon utilization, high salinity and pH tolerance (Mormile et al., 1999). Removal of nitrate, involves the reduction of NO_3^- to NO_2^- (nitrate to nitrite) by nitrate reductase enzyme, and further reduced to NH_4^+ (ammonia) by nitrite reductase enzyme (Guerrero, 1981).

1.6 Aims of the project

The importance of Qabar - oun and Um - Alma lakes in the south Libyan Sahara desert has been neglected as good hunting grounds for interesting types of halophilic bacteria. Studies of such halophilic microorganisms offer potential applications in various fields of biotechnology because they may produce compounds of industrial interest. There was very little published information on microbial community of these lakes, prior to the present project. The location of these lakes is difficult to access and this may well be one of the reasons why only a few studies have been conducted in this area. The aims of this study were to isolate and characterize bacteria from Qabar - oun and Um - Alma lakes, with a view to screening for halophilic and halotolerant bacteria types. In addition, the project involved collection and analysis of chemical/physical properties of water samples from both lakes. Furthermore, microbial isolation and identification will be determined by using classical and molecular techniques, the latter based on the analysis of 16S rRNA gene sequences. Further physiological characterizations of isolated bacteria will be carried out.

CHAPTER 2

Materials and Methods

2 Materials and Methods

2.1 Study Sites

Qabar - oun and Um - Alma Lakes (Figures 2.1 and 2.2), are located in the south Libyan Sahara close to the city of Ubari, and surrounded by high sand dunes within Ubari's sand sea (Fezzan region) (Figure 2.3). Qabar - oun Lake is the largest of a series of small lakes, 250 m by 300 m, and up to 7 m deep. The salt concentrations are about 6 times higher than seawater, and can be compared with the Dead Sea in terms of overall solute concentration. The climate of the region is characterized by low rainfall and extreme temperature with a high light intensity. The lakes are subject to high levels of evaporation due to the high temperatures and high light intensities (average temperatures of 15 and 40°C for the coldest and hottest seasons, respectively). Qabar - oun and Um-Alma lakes form closed drainage basins that represent typical examples of extreme environments, which may support a wide set of ecological niches for halophilic microorganisms.

2.2 Sample Collection

Water samples were collected in January 2009 from different points of the surface water of Qabar - oun and Um - Alam lakes using 500 ml sterile bottles. Each sample was labelled at the time and a photograph taken at each sampling site (Figures 2.1 and 2.2). The samples were then transferred to the lab in the UK within three days. Samples were stored at 4°C until required. Chemical/physical analysis of water samples was carried out by the chemical laboratory of Yara LTD Company in the UK.

Figure 2-1 Qabar - oun Lake.

Figure 2-2 Um - Alma Lake.

Figure 2-3 Map of Libya showing the location (red circle) of Qabar - oun and Um - Alma lakes.

2.3 Isolation of Microorganisms

2.3.1 Growth media and culture conditions

All media used for cultivation and isolation of microorganisms were prepared with distilled water and sterility was achieved by autoclaving for at 121°C for 20 minutes. Cultivation and isolation of halophilic strains from water samples of Qabar - oun and Um - Alma water lakes was carried out using slightly modified Luria - Bertani (LB) medium (liquid and plates) which contained the ingredients shown in section 2.3.1.1. Approximately 5 ml of raw environmental water samples of Qabar - oun and Um - Alma lakes was added to 2 x 250 ml sterile conical flasks, containing 50 ml of LB medium, 1.5 M NaCl and, pH 7.8. Flasks were then shaken at 250 rpm at 30°C overnight.

2.3.1.1 Luria - Bertani (LB) medium

Luria - Bertani (LB) liquid medium contained in 1 litre the following ingredients: 5 g yeast extract (Oxoid L21), 10 g tryptone (Oxoid, LP0042), 87.66 g NaCl, and the pH was adjusted to 7.8 with 1 M NaOH before autoclaving at 121°C for 20 minutes. For solid medium, 15 g of bacteriological agar No1 (Oxoid, LP0011) was also added per litre.

2.3.1.2 M9 minimal salt medium

M9 minimal salt liquid medium was prepared by dissolving 11.3 g M9 minimal salts (Sigma M-6030) and 1.5 g NaCl in 900 ml of distilled water and the pH was adjusted to 7.8 with 1 M NaOH, and the volume was made to 980 ml using distilled water and autoclaved at 121°C for 20 minutes. Other ingredients were separately prepared by dissolving in distilled water as shown in Table 2.1, and were then autoclaved at 121°C for 20 minutes. 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 M9 minimal salt medium as follows: 1 ml (1 M $MgSO_4$), 9 ml (5g NH_4Cl in 45 ml), 9 ml (15 g glucose in 45 ml), 1 ml (100 mM $CaCl_2$). The pH value of liquid media was checked after autoclaving and shown not alter by more than 0.05 pH units.

M9 minimal salt medium plates were prepared by dissolving 11.3 g M9 minimal salts (Sigma M-6030) and 1.5 g NaCl in 500 ml of distilled water, the pH was adjusted to 7.8 with 1 M NaOH. A separate gelling solution was prepared by dissolving 15 g agar No1 (Oxoid, LP0011) in 400 ml of distilled water to avoid the medium turning brown after autoclaving, and the overall volume was adjusted with distilled water to 980 ml. Both bottles were autoclaved at 121°C for 20 minutes. After autoclaving, both solutions were mixed, and the following solutions were added ($MgSO_4$ 1 ml of 1 M, NH_4Cl 9 ml (5 g in 45 ml), glucose 9 ml (15 g in 45 ml), $CaCl_2$ 1 ml of 100 mM, before plates were poured.

Table 2-1 Composition M9 minimal salt medium

Substrates	Amount/distilled water or concentration
M9 minimal salts (Sigma M-6030)	(11.3 g in 980 ml)
$MgSO_4$	1M
NH_4Cl	(5 g in 45 ml)
Glucose	(15 g in 45 ml)
$CaCl_2$	100 mM

Table 2-2 Composition of M9 minimal salts (Sigma M-6030)

Composition of M9 minimal salts medium (Sigma M-6030)	Weight (w/v)
Na_2HPO_4	6.78 g/l
KH_2PO_4	5 g/l
NaCl	0.5 g/l
NH_4Cl	1 g/l

2.3.2 Initial isolation of microorganisms

Initial isolation of halophilic microorganisms was carried out as follows: Portions of 0.2 ml from different dilutions (initial culture) were spread on plates. Plates were closed with Parafilm to reduce evaporation and incubated in the dark room at 30°C. Growth was monitored daily for 3 days and any single colonies arising were streaked onto a second set of fresh plates using sterile loops in order to produce a pure culture of cells. Once these plates were grown, single colonies from each isolate were aseptically removed and inoculated into 5 ml of the appropriate LB medium and incubated aerobically at 30°C for 24 hours. These cultures were then transferred into 250 ml conical flasks containing 50 ml of medium, and shaken at 250 rpm at 30°C.

2.3.3 Maintenance of the halophilic microorganisms

All strains isolated were subcultured repeatedly for purification and maintenance using both LB and M9 minimal salt medium at 0.5 - 1.5 M NaCl.

2.4 Initial Identification of Microorganisms

2.4.1 Cell morphology

Cells morphology was determined by Gram stain using light microscopy; a diluted sample of the cells was examined using a Nikon light microscope at x 40 and x 100 magnification. This technique was used as the first step for identification of ABQ1, ABQ2, ABU1, ABU2 and ABU3 bacteria isolated from water samples of Qabar-oun and Um-Alma lakes. Typically, this involved the following steps: a droplet of water was placed onto a slide microscope and a single colony from each isolate was obtained using

inoculating loop and smeared on the slide. The slide was allowed to air dry or by passing gently through a Bunsen flame. The cells were then treated by adding the following dyes: crystal violet for 1 minute, iodine solution for approximately 1 minute. The cells were then rinsed with ethanol to decolourise the stain. Safranin solution was added to counterstain the cells for 1 minute, and then rinsed with water. The slides were examined under a light microscope at 1000 times magnification (oil immersion). Gram - negative bacterial cells were the most predominant with pink colour.

2.4.2 Motility

Motility was examined using hanging drop preparations under the light microscope and by looking for flagella.

2.5 Molecular Identification of Environmental Isolates

2.5.1 Genomic DNA extraction

Extraction of genomic DNA from isolated strains was carried out using the commercially available KeyPrep DNA extraction kit (Anachem) following the manufacturer's protocols (see detailed procedure in Appendix A). Five 1 ml aliquots of bacterial cells from well-grown overnight cultures in LB medium (e.g. OD = 1.2 - 1.4) at a salt concentration of 0.5 M NaCl and pH 8 were transferred into 1.5 ml Eppendorf tubes and then harvested by centrifugation in a bench top centrifuge at 6,000 × g for 2 minutes at room temperature.

The supernatant was poured off immediately and then each pellet was re-suspended (washed) two times in 1 ml sterile distilled water (this step was carried out to remove all residual salt as it may affect the quality of DNA). A portion (100 µl) of Buffer R1 was

added to the pellet to re-suspend the cells completely by gently pipetting up and down. The cell suspension was then treated by adding 10 µl of lysozyme (50 mg/ml) mixed thoroughly and incubated in a bench hot block at 37°C for 20 minutes. Digested cells were pelleted by centrifugation at 10,000 × g for 3 minutes, and the supernatant discarded immediately and completely. Protein denaturation was carried out by resuspending the pellet in 180 µl of Buffer R2 followed by adding 20 µl of Proteinase K. Then, mixed thoroughly and incubated at 65°C for 20 minutes in a shaking water bath with occasional mixing every 5 minutes.

Removal of RNA achieved by adding 4 µl of RNase A (100 mg/ml), mixed gently and incubated at 37°C for 5 minutes. Two volumes (~ 410 µl with RNase A treatment) of Buffer BG were added and mixed thoroughly by inverting tubes several times until a homogeneous solution is obtained, and then incubated for 10 minutes at 65°C in water bath. The samples were then treated by adding 200 µl of absolute ethanol and mixed immediately and thoroughly. The samples were then transferred into a column assembled in a clean collection tube (provided as part of the kit). Then, the samples were centrifuged at 10,000 × g for 1 minute, and flow through discarded.

The columns were washed with 750 µl of wash Buffer and centrifuged at 10,000 × g for 1 minute, and flow through discarded. The columns were centrifuged at 10,000 × g for 1 minute to dry them and to remove residual ethanol. The columns were then placed into clean microcentrifuge tubes, and 50 µl of preheated Elution Buffer and TE buffer (recipe)were added directly onto column membrane and left to stand for 2 minutes. Genomic DNA was eluted by centrifugation at 10,000 × g for 1 minute. Genomic DNA was transferred to 1.5 ml Eppendorf tubes and stored at minus 20°C until required.

2.5.2 Agarose gel electrophoresis

All DNA samples were analysed by 1% gel electrophoresis with ethidium bromide staining. In order to prepare an agarose gel, 1 g agarose was added to 2 ml of 50 X TAE buffer (recipe) in conical flasks and then distilled water was added to make the final volume 100 ml. This solution was then heated in a microwave until the agarose completely melted and 6 µl of ethidium bromide was added when the gel had cooled to 50 - 55°C. The molten gel was poured into the gel tank with a 30 well comb and left to set for about 20 minutes. The comb and casting plates were removed from tank and gel completely covered with 1X TAE running buffer. The voltage was set at 80 V and left to run for 45 minutes. Gels were visualised and photographed under ultra violet light with the Uvitec "Uvidoc" mounted camera system.

A successful genomic DNA extraction was verified by resolving 2 µl of genomic DNA with 8 µl of a blue loading dye by gel electrophoresis against 2 µl GeneRuler 1 Kb ladder (Fermentas International Inc, Canada). Genomic DNA was kept in 1.5 ml Eppendorf tubes and stored at minus 20°C for short term until required. Long term storage was done at minus 80°C.

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

Following extraction of genomic DNA, polymerase chain reaction (PCR) was carried out in order to amplify the 16S rRNA gene. The reaction was performed in a final volume of 50 µl using universal primers for Bacteria and Archaea. The sequences of the forward primers were 5'-AGRGTTTGATCCTGGCTCAG-3' (20) for the Bacteria and 5'-TCCGGTTGATCCTGCC-3' (16) for the Archaea. The sequences of the reverse primers were 5-CGGCTACCTTGTTACGACTT-3' (20) for the Bacteria and 5'-

GGCTACCTTGTTACGACTT-3' (20) for the Archaea, These primers are described in detail by (Weisburg et al., 1991). The amplification (PCR) mixture contained the following reagents in a 0.2 ml thin walled PCR tube: 39 µl of sterile Mill1Q water, 5 µl 10X PCR Buffer, 2.5 µl 50 mM MgCl2, 0.5 µl Forward Primer, 0.5µl Reserve Primer, 1 µl (25 mM dNTPs), 1 µl genomic DNA, 0.5 µl Taq DNA polymerase (Fermentase). Amplifications were carried out in a MyCycler thermocycler (BioRad Laboratories, Inc., USA). This began with PCR cycling steps which consisted of a 3 minutes initial pre-incubation step at 94°C followed by 30 cycles of a denaturation step at 94°C for 1 minute, 1 minute annealing step at 50°C, and a 1 minute elongation step at 72°C followed by a final extension step at 72°C for 5 minutes.

Portions of 5 µl from the successful amplification of the expected fragment (approximately 800 bp), stained with addition of 2 µl of loading dyes were checked by electrophoresis in 1% (w/v) agarose gel against 7 µl of 1 kb GeneRuler ladder (Fermentase) to confirm the correct sized product had been amplified. This was achieved by running gel at 80 V for 45 minutes.

2.5.4 Purification of PCR products

The PCR product was purified and cleaned up using KeyPrep PCR purification kit following the methods stated in the protocol. All steps were carried out at room temperature. Five volumes of buffer PCR were added to the sample (PCR product) and mixed thoroughly by vortexing or inverting several times. The samples were then transferred into a column (maximum 1 ml) assembled in a clean collection tube (provided with kit). They were then centrifuged at 10,000 x g for 1 minute, and the flow through was discarded. The PCR product was washed with 750 µl wash buffer in a spin column

and centrifuged at 10,000 x g for 1 minute and then the flow through was discarded. The samples were dried by centrifugation at 10,000 x g for 1 minute in order to remove residual ethanol. DNA was eluted by moving spin columns into clean microcentrifuge tubes and 50 µl of preheated Elution buffer (at 65°C) was added onto the centre of the column membrane, left to stand for 2 minutes and then centrifuged at 10,000 x g for 1 minute. The products were run on a 1% agarose gel against 2 µl of 1 kb GeneRuler as a marker ladder to ensure that the correct sized product had been purified.

2.5.5 DNA sequencing and phylogenetic analysis

Purified PCR products were prepared and sent for sequencing according to the Eurofins's company sample submission guide as stated below. The sequence was then compared to other sequences using the NCBI Blast function http://www.ncbi.nih.gov/BLAST/.

- **Purified PCR products**

  < 300 bp: 2 ng/ µl in a minimum volume of 15 µl

  300 – 1000 bp: 5 ng/ µl in a minimum volume of 15 µl

  > 1000 bp: 10 ng/ µl in a minimum volume of 15 µl

2.6 Electron Microscopy

2.6.1 Preparation of cells for scanning electron microscopy (SEM)

1 ml aliquots of cells from overnight cultures in LB medium and M9 minimal salt medium were harvested in 1.5 ml Eppendorf tubes at 3000 x g for 10 minutes. Samples were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer for 4 hours at 4°C. The

specimens were then washed in 0.1 M phosphate buffer. Cells were then washed twice with 15 intervals at 4°C.

Secondary fixation was carried out in 2% osmium tetroxide aqueous for 1 hour at room temperature. Wash step was repeated. Dehydration was carried out through a graded series of ethanol solutions, followed by 100% ethanol dried over anhydrous copper sulphate for 15 minutes. All the above steps were carried out at room temperature. The specimens were then air dried. Next they were placed in a 50/50 mixture of 100% ethanol and hexamethyldisilazane for 30 minutes followed by 30 minutes in 100% hexamethyldisilazane. Cells were then allowed to air dry overnight before mounting. Upon completion of drying, the cells were mounted on 12.5 mm diameter stubs and attached with Sticky Tabs and then coated in an Edwards S150B sputter coater with approximately 25 nm of gold. The specimens were examined in a Philips XL - 20 Scanning Electron Microscope at an accelerating voltage of 20 Kv.

2.6.2 Transmission electron microscopy (TEM)

Specimens of cells was minced finely into pieces approxmately 1 mm^2 fixed in fresh cold 3% glutaradehyde in 0.1 M phosphate buffer overnight at 40°C. The specimens were then washed in 0.1 M phosphate buffer two times with 30 minute intervals at 40°C.

Secondary fixation was carried out in 2% aqueous osmium tetroxide for 2 hours at room temperature, washed in buffer as above. Then followed by dehydration through a graded series of ethanol solutions and then 100% ethanol dried over anhydrous copper sulphate twice for 15 minutes. All the above steps were carried out at room temperature. The specimens were then placed in an intermediate solvent, propylene oxide, for two changes of 15 minutes duration. Infiltration was accomplished by placing the specimens in a

50/50 mixture of propylene oxide/Araldite resin. The samples were left in this 50/50 mixture overnight at room temperature, and then were left in full strength Araldite resin for 6 - 8 hours at room temperature after which they were embedded in fresh Araldite resin for 48 - 72 hours at 60°C.

Araldite resin

CY212 resin 10 ml

DDSA hardener 10 ml

BDMA accelerator 1 drop per 1 ml of resin mixture.

Semi-thin sections approximately 0.5 nm thick were cut on a Reichert Ultracut E ultramicrotome and stained with 1% Toluidine blue in 1% Borax for around on a hotplate until the stain begins to evaporate. Then, it was differentiate the stain in 50% alcohol for around 15 - 20 seconds, wash in water and dry on a hot plate. Mount in DPX with a coverglass. Ultrathin sections, approximately 85 nm thick, were cut on a Reichert Ultracut E ultramicrotome and stained for 30 minutes with 3% aqueous uranyl acetate followed by staining with Reynold's lead citrate for 10 minutes. The sections were examined using a FEI Tecnai Transmission Electron Microscope at an accelerating voltage of 80 Kv. Electron micrographs were taken using a Gatan digital camera. NB Glutaraldehyde should be made freshly every week and stored at 40°C. Specimens were placed in approximately 10 times volume of fixative to tissue volume.

2.7 Growth experiments

2.7.1 Anaerobic growth

The ability of ABQ1 and ABU3 bacterial cells to grow anaerobically was examined using LB medium plates with 0.5 M NaCl, and pH 8 or 9, respectively. Portions of 0.2 ml of overnight fresh cultures from each strain were spread on LB plates, left to dry for a few seconds and then placed in the GasPak anaerobic jar system (BBL) and incubated at 30°C for five days.

2.7.2 Determination of optimum salinity

Determination of salinities in which optimum growth of the bacteria was observed was revealed using M9 minimal salt medium containing glucose at a range of salt concentrations (0, 0.05, 0.1, 0.25, 0.5, 1, 1.5, 2, and 2.5 M NaCl). 5 ml of overnight cultures grown in M9 medium at 0.25 M NaCl and pH 7.8 to mid-exponential phase were harvested and washed twice time in sterile distilled water. The washed cells were resuspended in 3 ml of sterile distilled water and 1 ml was then inoculated into 3 x 50 ml M9 minimal medium at appropriate concentrations of NaCl. All flasks were then incubated at 30°C with shaking (250 rpm), and the optical density of the cells was monitored at 600nm using a spectrophotometer.

2.7.3 Determination of pH range for growth

Determination of the pH range for growth was carried out in M9 minimal salt medium in which the pH had been adjusted to 5.5, 6, 7, 8, 9 and 10 with either NaOH or HCl (1 M) before autoclaving and was checked afterwards to ensure the pH levels had not changed. 1 ml from an overnight culture grown in M9 minimal medium at 0.5 M NaCl, pH 7.8,

was inoculated into 4 × 50 ml M9 minimal medium at the same salinity, with pH values as described above. All flasks were incubated on a shaker at 250 rpm, at 30°C. Optical density (OD600nm) of grown cells was measured over the course of the experiment.

2.7.4 Growth at optimum temperature

The influence of temperature on growth was studied at 25, 30 and 37°C. The optical density (OD600nm) of bacteria at each temperature was determined over the course of the experiment.

2.7.5 Determination of growth curve

Determination of growth curves of bacterial cells was carried out in M9 salt minimal medium at salt concentration range 0.5 - 2 M NaCl. Bacterial cells were pre-adapted by being grown overnight at appropriate pH value and concentration of NaCl to high density at 37°C. In order to measure growth curve, exactly 50 ml of each NaCl concentration in M9 minimal salt medium were added to (4 x 250 ml) conical flasks. All the flasks were labelled in addition to normal information (A, B, C and D), and inoculated with 3 - 4 ml of same NaCl concentration adapted cells from an overnight culture in order to give an initial optical density (OD600nm) of 0.1 for each flask. All flasks were put on the shaker (250rpm) at 37°C and the optical density of each flask was measured every hour throughout the day.

2.7.6 Carbon source utilization

The carbon sources utilization test was performed using Biolog GN2 Microplates. The experiment was carried out to investigate utilization of different carbon sources by ABQ1 and ABU3. The strains were grown overnight in M9 minimal salt medium at 0.5 M NaCl, pH 9, and at 37°C. 5 ml of bacterial cells from each strain were centrifuged at 3000 g for ten minutes. The cells suspensions were then washed three times using M9 minimal salt medium free of glucose. Then, samples were diluted using fresh M9 minimal medium free of glucose in sterile 15 ml Falcon tubes until the optical density (OD 600nm) reached the desired dilution (e.g. OD = 0.2), using a spectrophotometer. The contents of Falcon tubes were then dispensed into a sterile multichannel pipette reservoir. 150 µl of the inoculum were then transferred from the reservoir into each well of the plate using a multichannel pipette. The initial reading of each plate was recorded by microplate reader software (Wallac Viktor2). All plates were then incubated overnight at 37°C, and read again in the microplate reader.

2.8 Measurement of Oxygen Uptake in the Bacterial Cells

2.8.1 Preparation and calibration of oxygen electrode

Respiration rate was measured using a modified Clark-type oxygen electrode described by Delieu and Walker (1972). Schematic drawings of the oxygen electrode unit are shown in Figure 2.4. The oxygen electrode was prepared for use by adding a few drops of 2.3 M KCl to the disk and placing approximately 1 inch square piece of cigarette paper on top of the centre of the disk. The electrode Teflon membrane was places on the top of this and forced carefully onto the electrode disk using a membrane applicator and held in place by an O-ring. The disk was then placed into the chamber and connected to the control box.

The temperature of the reaction chamber was maintained constant at 30°C by circulating water from a temperature controlled water bath. A tungsten halogen lamp (12 V, 100 W) weas used to illuminate the chamber when required, with an approximate output light intensity of 1800 µmol m^{-2} s^{-1}. The calibration of the oxygen electrode was achieved by adding 2 ml of distilled water in the reaction chamber for approximately 15 minutes and setting the chart recorder to 100% saturation point and then using sodium dithionite to remove all oxygen from air saturated water. The sample in the reaction chamber, working volume 2 ml, was stirred continually by a small magnetic stirring rod (Figure 2.4).

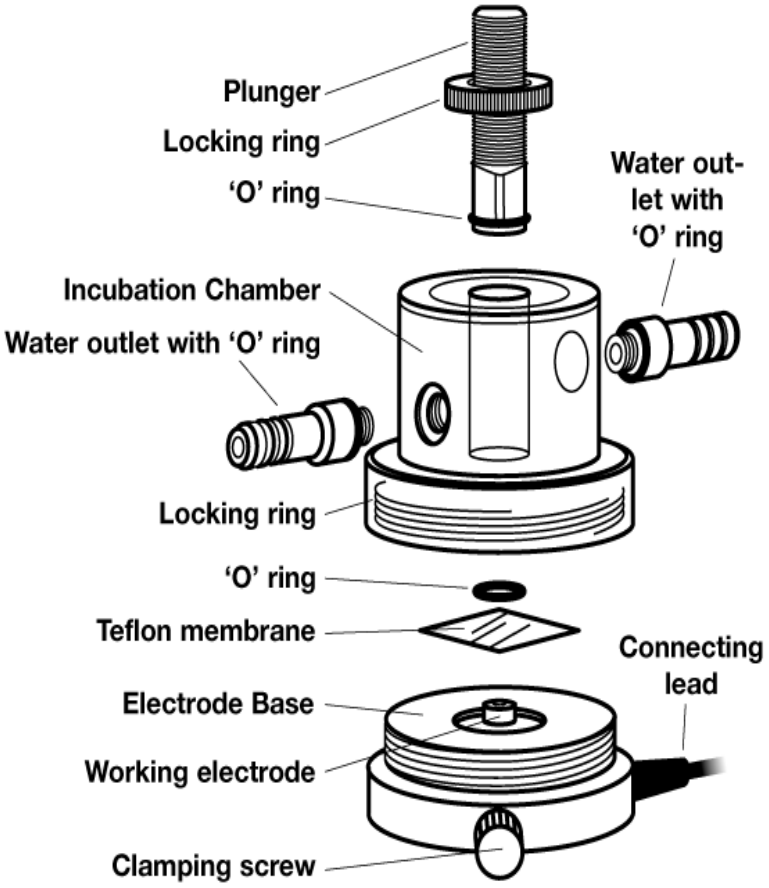


Figure 2-4 Schematic diagram illustrating the key components of an oxygen electrode. Figure adapted from: http:/www.rankbrothers.co.uk/prod1exp.

2.8.2 Oxygen (O2) uptake calculation

The rate of oxygen uptake was calculated using the following equation:

Respiration rate (µmoles mg^{-1} protein h^{-1})

$$\text{Respiration rate} = \frac{\text{Standard}}{\text{range}} \times \frac{\text{number of units}}{\text{time}} \times \frac{60}{\mu\text{g protein in sample}}$$

- Standard: oxygen solubility in 2 ml (sample) = (0.660 µmoles O2 2 ml^{-1} at 30°C).
- Range: Units taken from calibration (the different between 0% and 100%).
- Number of units: Number of units read directly in a period of 3 minutes.
- Time: The length of time in minutes for which the sample was measured.
- 60: this converts the time from minutes to hours
- µg Protein present in sample: this relates to amount of protein in a sample of 2 ml from Bradford assay (see section 2.9).

2.9 Determination of Protein Content

2.9.1 Determination of standard curve

The standard curve of protein was produced by dissolving 250 mg of Bovine Serum Albumin (BSA) in 50 ml of distilled water. The final concentration of protein in the stock solution was 5 mg ml^{-1} (5 µg $µl^{-1}$). A range of protein concentrations was prepared by series of dilutions using the stock solution as shown in Table 2.3.

Table 2-3 Components in the test tubes which were needed to make a standard protein curve.

Tube number	BSA (5 mg ml^{-1}) Stock solution	Distilled Water (µl)	Total volume in each tube	OD595nm	Mass of protein (µg)
1	0	100	100	0.000	0 (Blank)
2	2.5	97.5	100	0.102	12.5
3	5	95	100	0.196	25
4	7.5	92.5	100	0.231	37.5
5	10	90	100	0.294	50
6	15	85	100	0.498	75
7	20	80	100	0.724	100

Each test tube above has three replicates. 3 ml of Bradford Reagent (Sigma B6916) was added to each test tube, thoroughly mixed and left on the bench for 5 minutes. 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 E) was plotted from which the protein concentration for each sample can be determined. Standard error for each sample were calculated and shown on the curve.

2.9.2 Determination of protein in the bacterial cells

Determination of the amount of protein in a sample was measured using the Bradford assay (Bradford, 1976). 0.1 ml of samples was places into a test tube and 0.9 ml of 1M NaOH 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 prior to centrifugation at 3000 x g for 10 minutes. 0.3 ml of the resulting supernatant was added to 3 ml Bradford's reagent, agitated and the optical density measured in the Unicam Heliosα spectrophotometer against 0.3 ml water plus 3 ml

Bradford's reagent blank at 595 nm after a minimum of 5 minutes incubation. The protein content of the sample was determined by reading (µg protein) from the standard curve (Appendix E) divided by 0.3 to get µg protein ml^{-1} and then multiplied by 10 to take into account the dilution by NaOH.

2.10 Effect of NaCl Concentrations on Oxygen uptake

The effect of NaCl concentrations on oxygen uptake was examined in ABQ1 and ABU3 strains both shocked and pre-adapted cultures. For shock experiment, ABQ1 and ABU3 strains were grown overnight to exponential phase in M9 minimal salt medium at 0.5 M NaCl. Then, 5 ml of each overnight culture from both strains 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 cells were then subjected to 9 ml fresh M9 minimal salt medium at different salt concentrations over the range 0.5 - 2.5 M NaCl.

For pre-adapted experiment, cells from ABQ1 and ABU3 strains were grown to exponential phase in M9 minimal salt medium over the range 0.5 - 2.5 M NaCl. Cells of each strain were then subjected to the same medium after centrifugation as mentioned above. The oxygen uptake of the cells was measured immediately by adding 2 ml of each concentrated cell sample into the electrode chamber, at optimum temperature 37°C. The electrode chamber was rinsed thoroughly with distilled water before the next sample was applied. Each sample was repeated in triplicate. A Bradford assay (section 2.9) was performed on the cell suspensions to determine the protein concentration for use in calculating the respiration rate of the cells.

2.11 Effect of Temperature on Oxygen Uptake

The effect of temperature on respiration rate of ABQ1 and ABU3 strains was determined at optimum salinity (0.5 M NaCl), and optimum pH (pH 9). Measurement of oxygen uptake was carried out at temperature 15, 25, 30, 37 and 45°C. Methods of measurement of O_2 uptake was described previously in section 2.8.

2.12 Pre-adapted Effect of pH on Oxygen Uptake

Measurement of respiration rate was carried out in pre-adapted cultures of ABQ1 and ABU3 cells grown in M9 minimal salt medium at various pH values (5.5, 6, 7, 8, 9 and 10). In pre - adapted experiments, 5 ml samples from overnight cultures (50 ml) were concentrated from each pH level by centrifugation (15 minutes, 3000 x g), and cells were then resuspended in 9 ml of fresh M9 medium at pH values described above. The rates of oxygen uptake were measured immediately after the cells were subjected to the same pH media by the oxygen electrode.

2.13 Shock Effect of pH on Respiration Rate

Rates of oxygen uptake was measured in ABQ1 and ABU3 cells grown in M9 minimal salt medium at pH 9 using the oxygen electrode. In shock experiments, a portion of 5 ml of 50 ml from an overnight culture in M9 minimal salt medium at pH value 9 was harvested by centrifugation (15 minutes, 3000 x g), then the cells were resuspended in 9 ml of fresh M9 media ranging from pH 5.5 to 10 and rates of oxygen uptake of bacterial strains were measured immediately.

2.14 Preparation of Cell-Free Extracts (CFE)

To allow the measurements of enzyme activity (see section 2.15), cell-free extracts of bacterial cells were prepared from 50 ml volumes of overnight cultures grown in M9 minimal salt medium at 0.5 M NaCl, and pH 8 or 9. Cells were transferred into 50 ml Falcon tubes and harvested by centrifugation at 3000 x g in a bench top centrifuge for 15 minutes. Supernatant was removed and the bacterial pellet was re-suspended in 4 ml of fresh growth medium of the same composition. The bacterial cells were disrupted by sonication for 2 × 20 seconds with 10 seconds breaks between each 20 seconds of sonication to allow cooling of the sample. The cell debris (cell extracts) were then centrifuged immediately in a bench top microfuge at full speed (10000 x g) for 1 minute. Finally, the supernatant (CFE) was collected into 1.5 ml microcentrifuge tubes and kept on ice until required. The protein content of CFE was determined for use in calculating the specific activity of the enzymes using the Bradford Assay (Bradford 1976) as described previously.

2.15 Determination of Intracellular Enzyme Activities of Bacteria

2.15.1 Malate dehydrogenase

Malate dehydrogenase (EC 1.1.1.37) (MDH) was assayed in the direction of oxaloacetate reduction by measuring OD at 340 nm at room temperature.

L-Malate + NAD^+ $\longleftrightarrow$ oxaloacetate + NADH + H^+

The assay mixture contained:

Assay mixture	Volume in ml
M9 minimal salt medium, pH 8.5	2.0
1.5 mM NADH	0.2
7.5 mM oxaloacetate (pH 7.5)	0.2
Cell – free extract	0.1
Distilled water (to make final volume 3 ml	0.5

The reverse reaction is normally measured by providing a crude cell-free extract (described in section 2.14) with oxaloacetate and NADH. Assay of enzyme activity was carried out using a Unicam Helisα spectrophotometer at room temperature (20 - 25°C) using both glass and quartz 4.0 ml cuvettes with 1 cm light path in all cases.

The reaction rate was initiated by the addition of all assay mixture reagents to the 4 ml cuvettes with the exception of oxaloacetate and the absorbance at 340 nm was measured for 2 minutes against water blank. Then rate of decrease of OD 340nm due to the oxidation of NADH was measured upon the addition of oxaloacetate. The unit of activity of MDH is expressed as 1 µmole NADH oxidised min^{-1} mg^{-1} of protein at 25°C. The extinction coefficient of NADH at 340 nm is 6.22 x 103 litre $mole^{-1}$ cm^{-1}.

2.15.2 NADH oxidase

NADH oxidase activity can be measured by following the decrease in OD340nm, which corresponds to the decrease in NADH in the following reaction:

NADH Oxidase

$$NADH + H^+ + O_2 \longrightarrow NAD^+ + H_2O_2$$

The assay mixture contained:

Assays	Volume in ml
M9 minimal salt medium, pH 8.5	2.0
1 mM FAD	0.3
2 mM NADH	0.2
Cell – free extract	0.2
Distilled water (to make final volume 3 ml)	0.3

The reaction mixture in 3 ml was initiated by the addition of the cell-free extract and measuring the decrease in the absorbance at 340 nm by a spectrophotometer after 20 minutes incubation at room temperature (25°C). A control mixture was set up for each assay lacking cell free extract to determine the background rate of NADH disappearance.

Activity of NADH oxidase can be calculated by using the following equation.

1- Enzyme Activity = [(ΔA/min test – ΔA/min control) × Assay vol] / ε × CFE]

2- Specific Activity (μmoles min^{-1} mg^{-1} protein) = Enzyme Activity / mg Protein from Bradford Assay.

2.16 Sensitivity to Antibibtics

Sensitivity of both strains (ABQ1 and ABU3) to antibiotics was tested by using LB solid medium containing 1M NaCl, at pH 8 or 9, respectively. The following antibiotic discs (µg) were obtained and tested: tetracycline (50), ampicillin (25), carbenicillin (100), kanamycin (30), erythromycin (15), rifampicin (30), cefoxitin (30) and nalidixic acid (30). Antibiotic sensitivity was tested by spreading 0.2 ml of the fresh cells on LB plates, left to dry for a few seconds and then antimicrobial disks were put on the bacterial film (one in each quarter of the plate). Three plates (for each antibiotic) were incubated at 37°C for two days. The results were recorded by measuring the size of inhibition zones on plates by ruler in mm and then the mean and standard error of inhibition zones for each antibiotic were calculated.

2.17 Compatible Solutes

2.17.1 The effect of compatible solutes on bacterial growth at high sodium chloride concentrations

To determine the effect of compatible solutes on ABQ1 and ABU3 strains in highly saline media, M9 minimal medium was prepared containing 1.5 M and 2 M NaCl, at pH 8 and 9. The compatible solutes to be examined were added to 3 x 49 ml aliquots of this medium to give final concentration of 1 mM. An inoculum of 2 ml of cells grown in M9 minimal medium at 1.5 and 2 M NaCl, respectively, was added into (3 × 3 × 50 ml) to give initial reading (OD600nm) of approximately 0.1. Three flasks were prepared without addition of compatible solutes as control. The experiment was conducted at 37°C in orbital shaker (250 rpm), and growth was monitored by optical density measurements at 600 nm over the next 10 hours.

2.17.2 The effect of compatible solutes (1 mM) on oxygen uptake

The effect of compatible solutes on oxygen uptake was examined using M9 minimal salt media containing 1 mM betaine, ectoine and proline. ABQ1 and ABU3 strains were grown on M9 medium to exponential phase (i.e. OD = 1.5) at 1.5 and 2 M NaCl. 5 ml samples of 50 ml overnight cultures were harvested by centrifugation (15 minutes, 3000 x g) and resuspended in 9 ml of M9 medium containing different compatible solutes as described above. Control Falcon tubes were set up without addition of compatible solutes. Measurement of O_2 uptake was performed as described in section 2.8.

2.18 Nuclear Magnetic Resonance (NMR)

2.18.1 Compatible solute analysis

Five ml of mid exponential phase bacterial cultures (grown in LB and M9 minimal salt medium at salinities ranging from 0.25 - 2.5 M NaCl) were harvested and centrifuged at 3000 x g for 10 minutes. After supernatant was discarded, the resulting pellets were re-suspended (washed) twice in 1 ml sterile distilled water and vortexed for 1 minute at room temperature. The washed cells were then transferred into 1.5 ml Eppendorf tubes and sonicated 2 × 20 seconds with 10 second breaks between each 20 seconds of sonication to allow cooling of the sample. Samples were then centrifuged at 6000 x g for 10 minutes, and resulting supernatant was transferred into 1.5 ml Eppendorf tubes, and stored in freezer at – 80°C. After a minimum of 2 hours at minus 80°C, the samples were freeze - dried and stored at room temperature until required for NMR analysis. The dried samples were prepared for NMR analysis by dissolving them in 500 µl of D2O, and 5 µl of trimethyl styl propionate (TSP) using 1.5 ml Eppendorf tubes. The dissolved samples were transferred into NMR tubes for the analysis.

2.18.2 Growth with glycine betaine as sole source of carbon

The ability of ABQ1 and ABU3 strains to grow with betaine as carbon source was examined using M9 minimal medium. Glycine betaine was added to 49 ml M9 medium to give a final concentration of 17 mM, at salt concentrations of 0.5 and 2 M NaCl, and pH values 8 and 9, respectively. 5 ml of glucose grown cells in M9 minimal salt medium were obtained and centrifuged at 3000 x g for 15 minutes. The pellets were then re-suspended (washed) two times in 5 ml sterile distilled water. Portions of 1 ml from washed cells were then inoculated into 2 x 3 x 50 ml sterile conical flasks containing betaine (17 mM) at salt concentrations 0.5 M and 2 M NaCl, and pH values 8 or 9, respectively. Growth was measured by spectrophotometer at 600 nm over three days.

The ability of ABQ1 and ABU3 strains to grow with betaine as carbon source was examined using M9 minimal medium. Glycine betaine was added to 49 ml M9 medium to give final concentration of 17 mM, at salt concentrations of 0.5 and 2 M NaCl, and pH values 8 and 9 respectively. 5 ml of glucose grown cells in M9 minimal salt medium were obtained and centrifuged at 3000 x g for 15 minutes. The pellets were then re-suspended (washed) two times in 5 ml sterile distilled water. Portions of 1 ml from washed cells were then inoculated into 2 x 3 x 50 ml sterile conical flasks containing betaine (17 mM) at salt concentrations 0.5 M and 2 M NaCl, and pH values 8 or 9, respectively. Growth was measured by spectrophotometer at 600 nm over three days.

2.18.3 Determination of compatible solutes in betaine grown cells

This experiment was carried out to investigate accumulation of compatible solutes by ABQ1 and ABU3 strains grown in M9 minimal salt medium containing glycine betaine (17 mM) as carbon source, at salt concentrations of 0.5 and 2 M NaCl at pH 9. The bacterial cells from both strains were pre-adapted in M9 minimal salt medium with glycine betaine (17 mM), and

sub-cultured twice (for two days) on the same medium. Analysis of compatible solutes in glycine betaine grown cells at 0.5 and 2 M NaCl was carried out using NMR as described in section 2.18.1.

2.19 Bioremediation

2.19.1 Effect of urea and nitrate concentrations on bacterial growth

The effect of urea [CO $(NH_2)_2$] and sodium nitrate ($NaNO_3$) concentrations on the growth of ABU3 strain was measured using slightly modified M9 minimal salt medium (free of NH_4Cl) as shown in Table 2.5. The concentrations of CO $(NH_2)_2$ and $NaNO_2$ used were 2, 4, 8, 12 and 16 mM, at pH 9. 5 ml of cells grown on normal M9 minimal medium (containing NH_4Cl), 0.5 M NaCl, at pH 9, were harvested and washed twice in 5 ml sterile distilled water using 15 ml Falcon tubes. The washed cells were then resuspended in 5 ml sterile distilled water and 1 ml aliquots was then inoculated into 2 x 50 ml volumes of M9 medium containing 2 mM urea and sodium nitrate. All flasks were then incubated at 37°C with shaking (at 250 rpm) for two days to allow growth. After two days incubation, 1 ml of pre - adapted culture was inoculated into 3 x 250 conical flasks containing 50 ml of M9 minimal salt medium at appropriate concentrations of urea and nitrate described above. All flasks were then incubated at 37°C with shaking (at 250 rpm), and the optical density of the bacterial growth was monitored at 600 nm.

2.19.2 Nitrate concentration curve

A standard curve was created using $NaNO_3$ in the range of 0 - 2 mM by dissolved 0.69 g of $NaNO_3$ in 1 litre of M9 minimal salt medium free of nitrogen (i.e. no NH_4Cl). The final concentration of $NaNO_3$ in the stock solution was 8 mM. The stock solution was used to

make a range of NO_3-N concentrations by a series of dilutions as shown in Table 2.4. Nitrate standard curve is shown in Appendix F.

Table 2-4 Concentrations of $NaNO_3$ determined by spectrophotometry method.

$NaNO_3$ (mM/100 ml)	**$NaNO_3$ Stock Solution (ml)**	**M9 minimal medium (ml)**	**OD600nm**
0.0	0.0	100	0.008
0.04	0.5	99.5	0.087
0.08	1	99	0.137
0.16	2	98	0.278
0.24	3	97	0.399
0.32	4	96	0.516
0.4	5	95	0.652
0.48	6	94	0.741
0.56	7	93	0.885
0.64	8	92	1.005
0.72	9	91	1.167
0.8	10	90	1.22

Table 2-5 Slightly modified M9 minimal salt medium.

Ingredient	g litre-1
$Na_2 HPO_4$	6.8 g
$KH_2 PO_4$	3 g
NaCl	87.66 g
Na NO_3	0.69 g
pH	9
$MgSO_4$	1ml (1M in 50 ml)
$CaCl_2$	1ml (100 mM in 50 ml)

2.19.3 Nitrate reduction by strain ABU3

Nitrate reduction experiments were conducted using slightly modified M9 minimal salt media (Table 2.5). In addition to the medium components, glucose, sodium lactate and sodium acetate were used as carbon sources. The final concentrations of $NaNO_3$ and carbon sources in the media were 8 mM and 15 mM, respectively.

3 - 4 ml of adapted cells from an overnight culture were inoculated into 3 x 3 x 50 ml of slightly modified M9 minimal salt medium (Table 2.5) containing different carbon sources in order to give an initial optical density (OD600nm) of 0.1 for each flask. This was followed by measuring growth curve, and nitrate content in the media as described in the next section.

2.19.4 Measurement of nitrate reduction in the medium

Rate of nitrate reduction in M9 minimal medium tested was determined in parallel to measuring growth curve of ABU3. The initial OD of nitrate was first measured in M9 minimal medium (Table 2.5) by taking 1 ml from 50 ml of the medium and centrifuging at 6000 x g in a bench top microcentrifuge for 10 minutes, and then 0.5 ml volumes of supernatant were transferred into 15 ml Falcon tubes and diluted 1:10. Then, the initial OD of nitrate content in the sample was measured in 1 ml of diluted supernatant at 232 nm against a blank medium using 4 ml glass cuvette. All flasks were put on the shaker (250 rpm) at 37°C, and the optical density (OD) of bacteria growth against nitrate reduction was measured every two hours through the day at 600 nm and 232 nm, respectively (See Appendix G) (Measurement of growth curve was described in section 2.7.5).

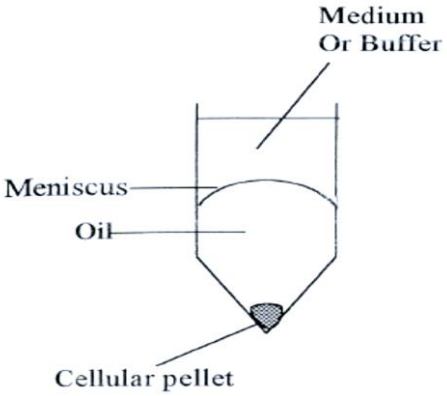
2.20 Silicone Oil Technique

Centrifugation through silicone oil was used to completely separate cells from the medium. 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 2.5).

2.20.1 Determination of intracellular volume (ICV) of bacteria

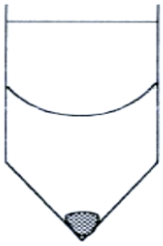
Determination of cell volume was carried out using the silicone oil technique based on the method described by (Rottenberg, 1979).

Two 1 ml samples of concentrated cells (2 x 20 ml of cells from an overnight culture grown in M9 minimal salt medium at pH 9 were transferred into 50 ml Falcon tubes and harvested by centrifugation at 3000 x g for 10 minutes and then the bacterial pellet was resuspended in 9 ml of fresh M9 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 20 µl of ^{14}C - dextran (MW = 70000, 740 KBq ml^{-1}) were added to second sample of concentrated cells to give 14.8 KBq ml^{-1}. Both samples were thoroughly vortexed. 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 silicon oil mixture (right density) in fresh 1.5 ml Eppendorf tubes. The samples were then centrifuged for 1 minute at 6000 x g in a bench top microcentrifuge. From the aqueous fraction, triplicate samples (50 µl) were taken and placed into separate scintillation vials containing 5 ml of FluoranSafe scintillation fluid (VWR) and labelled appropriately.



(a). Oil is not dense enough.

(b). Oil is too dense.



(c). Oil is in the right density.

Figure 2-5 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 (c), silicone oil of the incorrect density (a and b).

The lower half of the 1.5 ml Eppendorf tubes, which contain the pellet of bacteria, were carefully cut off using a razor blade and placed upside down into 1.5 ml Eppendorf tubes containing 300 µl distilled water. They were then centrifuged for approximately 15 seconds at 6000 x g) in order to remove the pellet from the tips into the distilled water, which was then discarded. Each pellet was resuspeneded 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 $3H_2O$ 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 2.6). The pellet volume (PV) and extracellular volume (ECV) were calculated from the ratio of $3H_2O$ and ^{14}C - dextran in the pellet and supernatant fractions respectively using the following equations given in (Hard and Gilmour, 1996).

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

$$\text{Extracellular volume (ECV) (µl)} = \frac{^{14}C\text{-dextran in pellet}}{^{14}C\text{-dextran in supernatant x } 6} \times 300$$

dpm = disintegrations per minute

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

ICV = PV – ECV (µl)

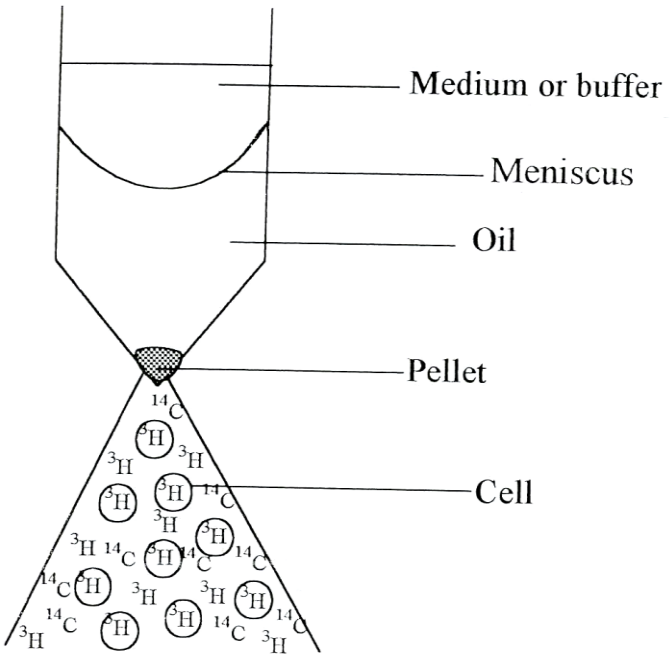


Figure 2-6 Intracellular volume (ICV) determination using $3H_2O$ and ^{14}C - dextran. The $3H_2O$ distributes throughout the pellet and ^{14}C - dextran is only found in the spaces between cells in the pellet.

2.20.2 Measurement of isotope uptake over time

In order to ensure an accurate measurement of the ICV of ABU3 cells, a time course experiment of the uptake of both $3H_2O$ and ^{14}C - dextran was carried out. Essentially the silicone oil method as described above was carried out using only one isotope. The normal volume of $3H_2O$ and ^{14}C - dextran was added to four 1 ml samples of cells and the silicone oil procedure carried out at 1, 5, 10 and 20 minutes intervals. Twenty four 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 disintegrations per minute (dpm) for pellet and supernatant triplicates per time point were calculated and plotted against time.

2.20.3 Measurement of membrane potential (ΔΨ) in bacterial cells

Determination of membrane potential was carried out using the silicone oil technique as described by (Rottenberg, 1979, Rottenberg, 1989). The experimental procedure was similar to the method used to determine intracellular volume (ICV), 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 = A (ao)

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) = 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) = C (ai)

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

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

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 In to log 10 (x2.303) $\longrightarrow \Delta\Psi\ (mV) = -\ 58.8 \times \log\ \frac{ai}{a_。}$

2.20.4 Determination of internal pH (pHi) 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 microorganism (Rottenberg, 1979, Rottenberg, 1989, Kashket, 1985). The internal pH of the cells was measured using a weak base. ^{14}C - methylamine was used when external pH is higher than pH 7.0 in order to obtain a measurable accumulation of the isotope. The silicone oil method used was identical to that described in Section 2.20.1, except that 5 µl of ^{14}C - methylamine were added to 1 ml of cells suspension (sample) to give a final concentration of 18.5 kBq ml^{-1}.

The calculations were exactly the same as those used in the previous section to calculate the ratio ai/ao.

1) Dpm ^{14}C - methylamine in 1 µl = Dpm ^{14}C - methylamine of supernatant divided by 50 = A (ao)

2) ^{14}C - methylamine 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) = B

3) Dpm μl^{-1} cell volume = Dpm ^{14}C - methylamine in pellet minus B and divided by intracellular volume in µl (calculated from parallet samples treated with $3H_2O$ and ^{14}C-dextran) = C (ai)

4) Ratio of $\frac{C}{A}$ = concentration of ^{14}C - methylamine inside the cells (ai) / concentration of ^{14}C-methylamine outside cells (ao)

To determine the internal pH from the ai/ao ratio calculated ^{14}C - methylamine, the following 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:

$$\text{pHi} = -\log\left[\frac{a_i}{a_o}\left(10^{-pk} + 10^{-pH0}\right) - 10^{-pk}\right]$$

2.21 Statistics

Most of the experimental data in this study were conducted in triplicate and error bars represent standard errors of the means using sigmaplot software. If no error bars are shown, they were smaller than symbol used to repreasent the mean. Figures and graphs were also created using sigmaplot graphing software.

CHAPTER 3

Water Analysis and Isolation of Microorganisms

3 Water Analysis and Isolation of Microorganisms

3.1 Introduction

The alkaline saline or soda lakes (pH 8 to > 11) are defined as ecosystems that form in closed drainage basins exposed to high evaporation rate (Jones et al., 1998). These habitats are characterized by large amounts of sodium carbonates (Na_2CO_3 + $NaHCO_3$) with high buffering capacity (Grant WD, 1986). The neutral saline lakes with a pH 6 - 8.5 are defined as those which have Na^+ and Mg^{2+} as major cations and (Cl^- and SO_4^{2-}) as major anions with low buffering capacity (Grant WD, 1998). Halophilic and halotolerant microorganisms have been isolated from a variety of hypersaline lakes, while haloalkaliphilic species were obtained from alkaline saline lakes (Galinski and Trüper, 1994, Grant, 2004, Banciu et al., 2004, Chen et al., 2007).

Extreme environments such as soda lakes and saline lakes are naturally occurring as a result of complex interactions of the geological, climatic, and biogeochemical conditions (Banciu et al., 2004, Jones et al., 1998). They are often located in dry climate zones with high temperature and evaporation rate. Accordingly, they create extreme pH environments because of high daily light intensities and unlimited supply of CO_3 (Grant, 1992, Jones, 1999). In arid areas, high evaporation rate exceeding inflow leads to the accumulation of salts to high concentrations (Jones et al., 1994).

Saline and hypersaline lakes that form closed drainage basins are widely distributed. They are considered extreme environments for life (Jiang et al., 2006) because of the effects of high salinity on water activity (Jiang et al., 2006). Some examples of well-known soda lakes in Africa are those located in the East African Rift Valley of Kenya-Tanzania, in Egypt (Wadi Natrun), in the Libyan Desert (Qabar-oun, Um-Alma) (Sorokin and Kuenen, 2005). The Wadi Natrun in Egypt contains a number of shallow lakes, most of them a half

metre deep (Oren, 2002b). Microbial community studies on soda lakes of the East African Rift Valley and Wadi Natrun in Egypt have been carried out intensively over many years (Jones et al., 1998, Rees et al., 2004, Mwirichia et al., 2011, Taher, 1999).

The previous studies of microbial diversity in hypersaline environments such as the Great Salt Lake in Utah, the Dead Sea, established that halophilic members of *Archaea* and *Bacteria* are dominant (Jiang et al., 2006, Cui et al., 2011). i.e. The Wadi Natrun saline lakes in Egypt were found to be populated by phototrophic purple bacteria, halophilic and alkalophilic *Archaea,* cyanobacteria and green alga *Dunalialla salina* (Oren, 2002b).

Qabar-oun and Um-Alma saline lakes (pH 9.2 - 9.8, respectively) are closed drainage basins located in the south Libyan Desert. The lakes represent an ideal site for the isolation of halophilic and halotolerant microorganisms. The location of these lakes is difficult to access and this may be one of the reasons why only few studies have been carried out on these lakes. This chapter provides data on the chemical/physical characterization of water from Qabar-oun and Um-Alma lakes.

3.2 Results and Discussion

3.2.1 Chemical/ physical properties of water samples

Water samples of Qabar-oun and Um-Alam lakes were obtained using clean, sterile 50-ml Falcon plastic tubes from the water surface. The samples were then transported to the lab within three days of collection. Analysis of the water samples was conducted at Landcrop laboratories, Yara UK Limited, in 2010 (Table 3.1).

Table 3-1 Chemical/ physical properties of Qabar-oun and Um-Alam lakes waters and comparison with the Wadi Natrun lake in Egypt. (nd = not detected, nm = not measured)

Chemical/physical parameters	Qabar-Oun lake	Um-Alma lake	Wadi Natrun lakes
pH	9.2	9.8	9.26
Mg^{2+} (mg/l)	144	20	nd
Mn (mg/l)	0.03	0.04	2000
B (mg/l)	29.21	53.22	nd
Cu (mg/l)	< 0.01	< 0.01	nd
Mo (mg/l)	< 0.01	0.04	nd
Fe (mg/l)	0.08	0.11	60000
Zn (mg/l)	< 0.01	< 0.01	100000
S (mg/l)	2883	2180	nd
P (mg/l)	< 1	2.000	nd
K (mg/l)	2726	2653	nd
Ca (mg/l)	13	1	nd
NO_3^- N (mg/l)	0.2	0.2	371000
E.C. (mmhos/cm)	280	314	nm
NH_3 N (mg/l)	0.08	0.03	nm
Na^+ (mg/l)	20463	21874	295210
Cl^- (mg/l)	79521	87906	93000
HCO_3^- (mg/l)	3022	14909	640

The chemical composition of The Wadi Natrun lake obtained from Table 1 (sample 2) in paper (Taher, 1999).

3.2.2 Isolation of microorganisms

In order to cultivate and isolate halophileic microorganisms, media with high salinity must be used. Bacterial halophiles were cultivated and isolated under high salinities using the modified Luria-Bertani (LB) medium as described in section 2.1.2.1. The medium had an adequate salt concentration for isolating moderate halophiles from these environments. Environmental samples of water were collected from different points on Qabar-oun and Um-alma lakes (See Figures 2.1and 2.2).

Cultivation of halophiles from Qabar-oun and Um-Alma lakes in liquid medium was carried out using 250 ml flasks. 5 ml volumes of each water sample were inoculated into 50 ml of LB medium at salinity of 1.5 M NaCl, at pH 7.8. Flasks were incubated overnight at 30°C with shaking (250 rpm).

In order to isolate pure cultures of bacteria from Qabar-oun and Um-Alma waters, serial dilutions ($10^0 – 10^{-6}$) of overnight culture were made, and a 100 µL aliquot of each diluted sample was spread onto LB agar plates containing 1.5 M NaCl, at pH 7.8. Samples were prepared in triplicate and plates were incubated at 30°C for three days. All colonies were selected according to approximate colony size and colour, designated as strains: ABQ1, ABQ2, ABU1, ABU2 and ABU3 (see Table 3.2). Isolated colonies were sub-cultured successively and repeatedly on the same medium to ensure purity. It should be noted that the isolation of microorganisms in pure culture requires time and skill, and remains one of the prime challenges for microbiologists (Rosselló-Mora and Amann, 2001). M9 minimal salt medium was used for further subcultures to maintain the microorganisms.

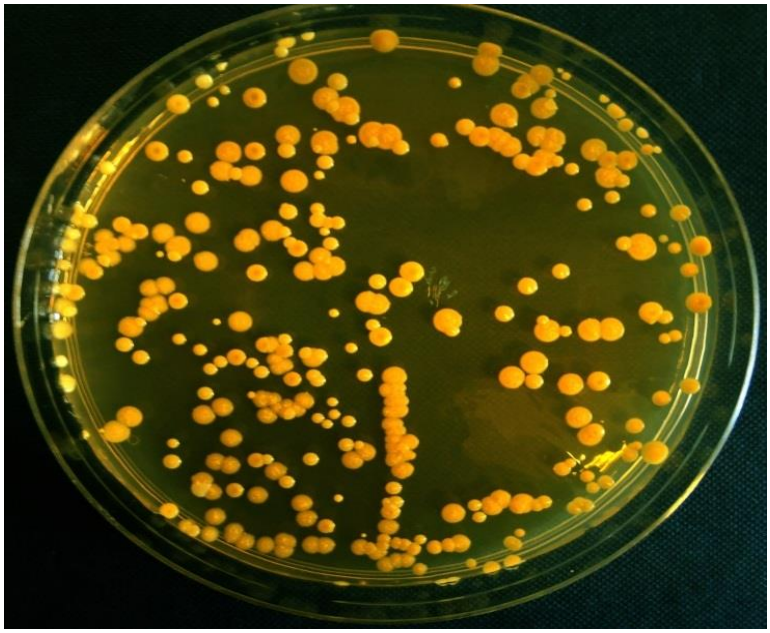


Figure 3-1 Creamy and white colonies cultivated from Qabar-oun Lake water sample.

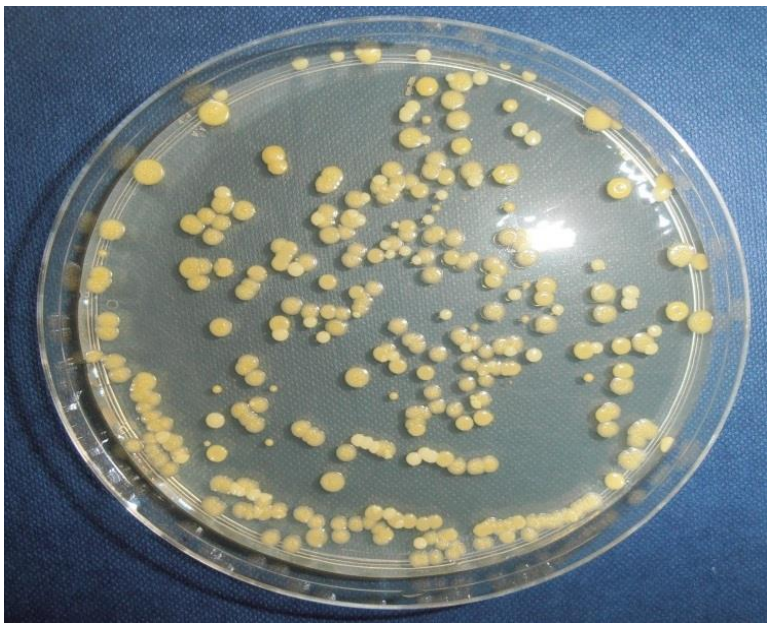
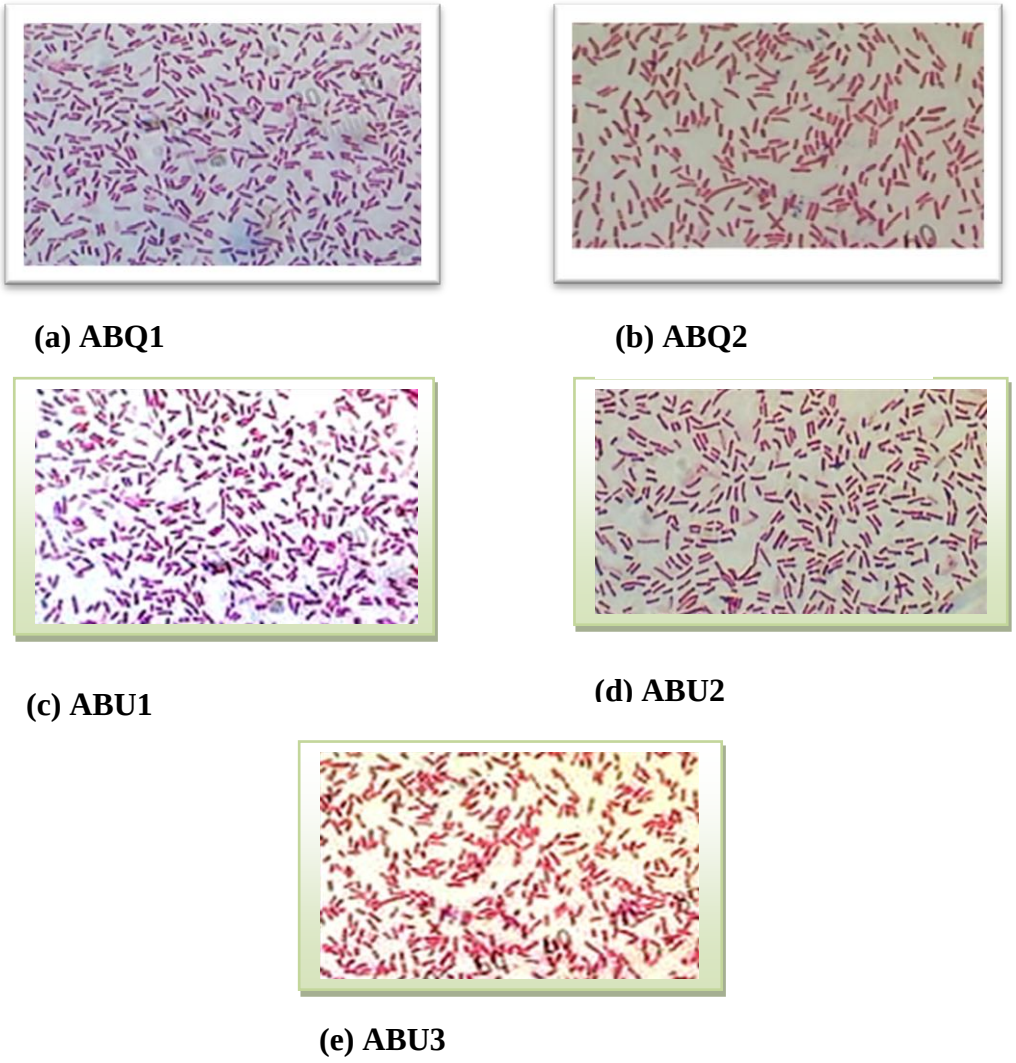


Figure 3-2 Creamy and white colonies cultivated from Um-Alma Lake water sample

3.2.3 Gram stain (Microscopy)

Cells from colonies on agar plates were picked up by loops and mixed with drop of distilled water over microscope slide followed by gentle drying through a Bunsen flame. The Gram staining technique was carried out as described in section 2.4.1. Cultures were examined using a Nikon light microscope equipped with lens at x 40 and x 100 magnification (Figure 3.3).



(a) ABQ1 **(b) ABQ2**

(c) ABU1 **(d) ABU2**

(e) ABU3

Figure 3-3 Short rod, Gram-negative, non-spore forming bacteria with polar flagella were seen under the microscope. Cells were grown overnight in LB medium containing 0.5 M NaCl, at 30°C**.** All ABQ strains are isolated from Qabar-oun Lake and ABU strains are isolated from Um-Alma Lake.

Table 3-2 The initial characterization of isolated strains

Name of Bacterium	Gram stain	Motile	Morphology	Pigmentation
ABQ1	-	+	rod	creamy
ABQ2	-	+	rod	creamy
ABU1	-	+	rod	white
ABU2	-	+	rod	white
ABU3	-	+	rod	white

Examination by light microscopy showed that all cells are motile. The bacteria was found to be Gram negative, non-spore forming and able to grow in LB and M9 minimal salt media at salt concentrations ranging from 0.5 - 2.5 M NaCl, at pH 7.5 - 9. Cells were shaken at 250 rpm in a 30°C constant temperature room overnight.

3.3 Conclusions

Five strains of Gram-negative bacteria were isolated from the water samples taken from the Qabar-oun and Um-Alma Lakes. The strains were selected for their ability to grow at a range of salinities on M9 minimal medium as well as on rich LB medium. All strains looked similar under the microscope and were also mainly similar for colony morphology with the exception of ABQ2.

In the next chapter, molecular methods will be used to identify the five strains of bacteria successfully isolated.

CHAPTER 4

Molecular Identification

4 Molecular Identification of Strains ABQ1, ABQ2, ABU1, ABU2 and ABU3 Using 16S rDNA Sequencing

4.1 Introduction

The 16S rRNA gene has a size of about 1500 base pairs (bp) and encodes for the 16S part of the small subunit of the prokaryotic ribosome (Cai et al., 2003). It is considered to be a major source of information for phylogenetic studies of microorganisms (Arahal et al., 2002), because it is distributed among all prokaryotic microorganisms (Drancourt et al., 2000, Chakravorty et al., 2007). Before the advent of molecular identification techniques, phenotype methods were used as "classical methods" in most microbiology laboratories for prokaryotic classification (Rosselló-Mora and Amann, 2001). The phenotype method was based on morphology, physiology and biochemical features of microorganisms and this information was used to construct a framework for an accurate identification of each organism (Rosselló-Mora and Amann, 2001, Vandamme et al., 1996). The morphological characteristics of a bacterium include cell shape, presence/absence of endospores, motility (presence/absence of flagella), type of inclusion bodies, Gram staining and colony form (colour, dimensions, shape). The physiological and biochemical description includes information on growth at different temperatures, pH values, salinities, or atmospheric conditions, sensitivity/resistance to antimicrobial agents, and data for the activity of different enzymes and metabolic pathways. It has been reported that many of these characteristics are irrelevant as parameters for genetic relatedness, however, they provide descriptive information which enables the recognition of taxa (Vandamme et al., 1996).

The earliest attempts to detect relationships between distantly related bacteria were carried out by cataloguing ribosomal ribonucleic acids (rRNA) in the late 1970s

(Stackebrandt, 1985). In the 1980s, the analysis of full sequences of 16S rRNA genes was used as a powerful tool to determine phylogenetic relationships between different bacteria and to provide a satisfactory framework for prokaryotic classification from various sources, such as environmental or clinical specimens (Cai et al., 2003, Mignard and Flandrois, 2006).

In recent years, the polymerase chain reaction (PCR) has been used universally to amplify 16S rRNA genes and as such it is a powerful tool for the identification and classification of bacteria (Yeung et al., 2009, Kamekura, 1998). The application of PCR mostly depends on the availability of specific DNA sequences (primers) that match parts of the genomic DNA that flank the whole 16S rRNA gene or that flank areas of interest within the 16S rRNA gene (Yeung et al., 2009, Vandamme et al., 1996). As well as being distributed among all organisms, 16S rDNA contains species-specific variable regions that allow species identification (Cai et al., 2003, Drancourt et al., 2000, Mignard and Flandrois, 2006, Petti et al., 2005). One of the limitations of using phenotype analysis is that the full information of a prokaryotic genome is never expressed (Rosselló-Mora and Amann, 2001). In addition, the 16S rRNA gene approach can be used as a method for detecting species in environmental samples that cannot be cultured (Mignard and Flandrois, 2006).

In this chapter, 16S rRNA gene analysis will be used to identify the five isolates from Qabar-oun and Um-Alma lakes which were described in Chapter 3.

4.2 Results and Discussion

4.2.1 Genomic DNA extraction

The genomic DNA extraction was carried out as described in section 2.5.1, in order to obtain high molecular weight and high quality genomic DNA from the five unidentified bacterial isolatess (ABQ1, ABQ2, ABU1, ABU2 and ABU3) derived from Qabar-oun and Um-Alma lakes. Genomic DNA was obtained successfully (Figure 4.1) from all five unidentified bacterial cultures grown on LB medium at 0.5 M NaCl. The genomic DNA extraction method involves breakdown of the cell wall, centrifugation to remove the cell fragments and debris, precipitation of nucleic acid from the pelleted cells and purification. Figure 4.1 demonstrates the presence of high molecular weight of DNA free of RNA contamination and in large enough quantities for PCR amplification.

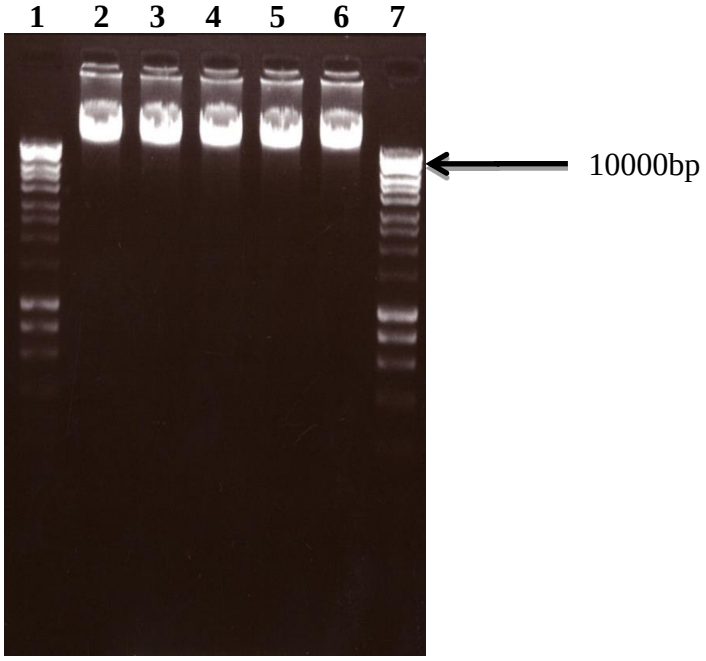
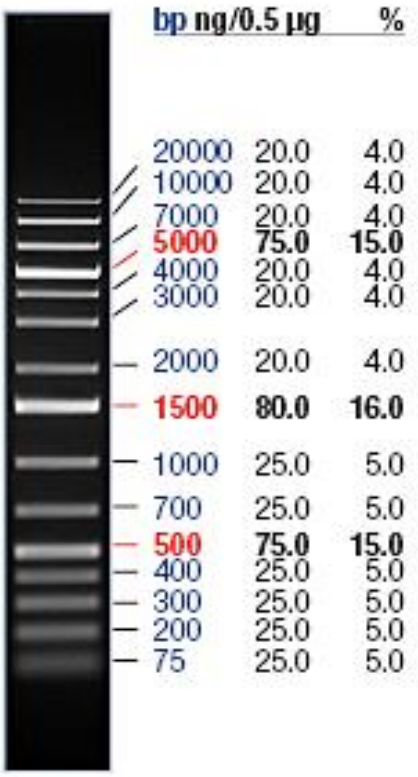


Figure 4-1 Agarose gel (1%) electrophoresis with 6 µl ethidium bromide (EB) and bands of 10 kb DNA ladders (lanes 1 and 7), and total genomic DNA extracts with sizes over 10000 base pairs from five strains ABQ1, ABQ2, ABU1, ABU2 and ABU3, respectively (lanes 2 – 6).

4.2.2 PCR amplification of 16S rRNA gene

The 16S rRNA was amplified from DNA samples of isolated strains using PCR (see section 2.5.3). Only the bacterial primers which were described in section 2.5.3 gave a clear and sufficient PCR product for all five DNA samples. The 16S rRNA was amplified by using the universal bacterial forward primer 5'AGRGTTTGATCCTGGCTCAG-3' and universal bacterial reverse primer 5-CGGCTACCTTGTTACGACTT-3' as described by (Mwirichia et al., 2011). The presence of 16S rRNA gene products was determined by analysing 5 µl of PCR product on 1% agarose gels stained with ethidium bromide. The products were compared with a 1 kb GeneRuler ladder (Figure 4.2) that has bands of molecular weights ranging from 200 to 10,000 bp and is used for easy quantification as well as size determination of DNA (Mwirichia et al., 2011).

According to the results, the genomic DNA of all five isolated strains was amplified successfully with the correct fragment band size of 16S rRNA, is roughly 1.5 kb. The results of amplification of 16S rRNA from genomic DNA samples are shown in Figure 4.3. PCR products were then purified using KeyPrep PCR purification kit (section 2.5.4). Figure 4.4 shows that the same sized bands were present after purification and they were sent for sequencing.



- GeneRuler 1kb Plus DNA Ladder
- Supplied in a ready-to-use format
- Each lane (0.5µg) provides 720ng of DNA

Figure 4-2 Standard GenRuler DNA Ladder produces a pattern of 15 regularly spaced bands (20000, 10000, 7000, 5000, 4000, 3000, 2000, 1500, 1000, 700, 500, 400, 300, 200 and 75 bp).

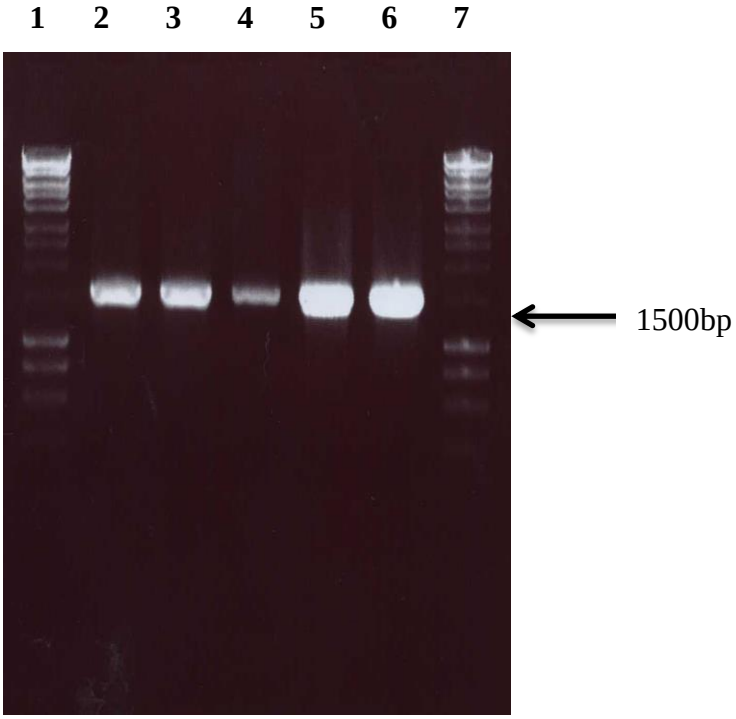


Figure 4-3 Ethidium bromide stained 1% agarose gel displaying amplification of 16S rRNA (approximately 1.5 kbp) using genomic DNA from strains ABQ1, ABQ2, ABU1, ABU2 and ABU3, respectively (lanes 2 - 6). Lanes 1 and 7 represent the 10 kb GeneRuler DNA ladder.

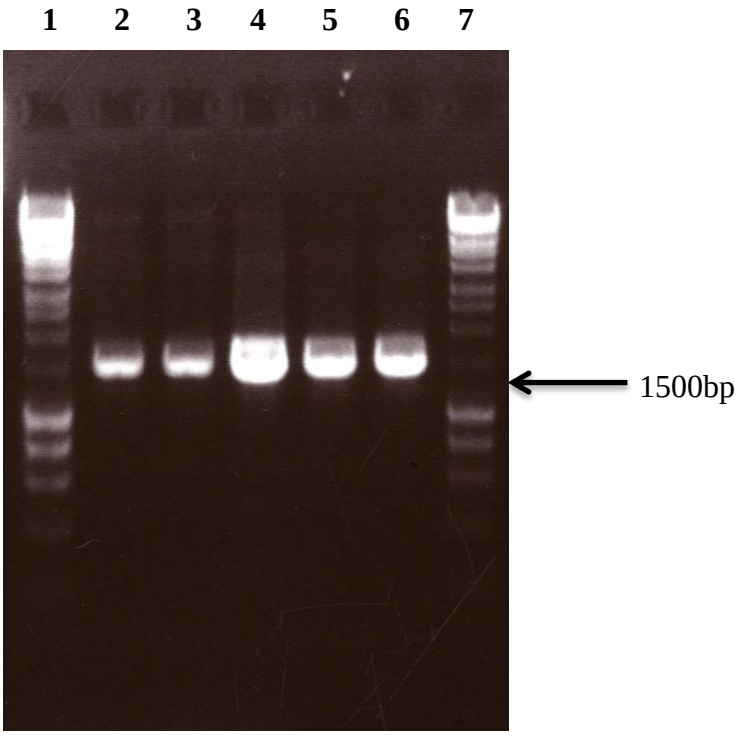


Figure 4-4 Agarose gel (1%) with ethidium bromide showing the resolution of approximately 1.5 kb of PCR products after purification of the 16S rRNA genes using KeyPrep PCR clean-up Kit. Lanes 2 - 6 are ABQ1, ABQ2, ABU1, ABU2 and ABU3, lanes 1 and 7 are DNA ladder (10 kb).

4.2.3 Sequencing of 16S rRNA Gene of Isolated Bacteria

As shown in section 4.2.2, the 16S rDNA of each strain was successfully amplified and purified. Two strains (ABQ1 and ABU2 were sequenced by NCIMB (Aberdeen, Scotland) and these sequences are 600 and 486 bp long, respectively (Figures 4.5 and 4.8). The relatively short reads reflect the primers used by the NCIMB, which are designed to amplify one of the variable regions of the 16S rDNA.

The other three sequences for ABQ2, ABU2 and ABU3 were obtained from Eurofins MWG, a commercial sequencing company. These reads are longer, which reflects the fact that our primers (see section 2.5.3) were used in the sequencing reactions (Figures 4.6, 4.7 and 4.9).

ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGAAACGAAAGCTT
GCTTCCAGGCGTCGAGCGGCGGACGGGTGAGTAATGCATAGGAATCTGCCCGAT
AGTGGGGGATAACGTGGGGAAACTCACGCTAATACCGCATACGTCCTACGGGAG
AAAGCAGGGGATCTTCGGACCTTGCGCTATCGGATGAGCCTATGTCGGATTAGCT
AGTTGGTGAGGTAACGGCTCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGA
TGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG
TGGGGAATATTGGACAATGGGGGAAACCCTGATCCAGCCATGCCGCGTGTGTGA
AGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAAGAAGGCCTGAGGGCTA
ATACCCTTCAGGAAGGACATCACTCGCAGAAGAAGCACCGGCTAACTCCGTGCC
AGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAA
AGCGCGCGTAGGTGGCTTGATAAGCCGGTTGTGAAAGCCCCGGGCTCAACCTGG
GAACGG

Figure 4-5 16S rRNA gene sequence of facultative anaerobic strain ABQ1 obtained from NCIMB, Aberdeen. The sequence is 600 base pairs long.

CATGCAGTCGAGCGGTAACAGGGGTAGCTTGCTACCCGCTGACGAGCGGCGGA
CGGGTGAGTAATGCATAGGAATCTGCCCGGTAGTGGGGGATAACCTGGGGAAA
CCCAGGCTAATACCGCATACGTCCTACGGGAGAAAGGGGGCTTCGGCTCCCGCT
ATTGGATGAGCCTATGTCGGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGG
CAACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACATCGGGACTGAGACA
CGGCCCGAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAA
AGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCAC
TTTCAGCGAGGAAGAACGCCTAGTGGTTAATACCCACTAGGAAAGACATCACTC
GCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGT
GCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGCTTGATAAG
CCGGTTGTGAAAGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACTGTCAAGC
TAGAGTGCAGGAGAGGAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAGA
GATCGGGAGGAATACCAGTGGCGAAGGCGGCCTTCTGGACTGACACTGACACT
GAGGTGCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGATGTCGACCAGCCGTTGGGTGCCTAGCGCACTTTGTGGCGAAGTTA
ACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATG
AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACG
CGAAGAACCTTACCTACTCTTGACATCCTGCGAACTTGTGAGAGATCACTTGGT
GCCTTCGGGAACGCAGAGACA

Figure 4-6 16S rRNA gene sequence of facultative anaerobic strain ABQ2 obtained from EurofinsMWG. The sequence is 984 base pairs long.

GATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGAAACGATCCT
AGCTTGCTAGGAGGCGTCGAGCGGCGGACGGGTGAGTAATGCATAGGAATCTG
CCCGGTAGTGGGGGATAACTTGAGGAAACTCAAGCTAATACCGCATACGCCCTA
CGGGGGAAAGCAGGGGCTCTTCGGACCTTGCGCTATTGGATGAGCTTATGTCGG
ATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAGCTGGTCT
GAGAGGATGATCAGCCACATCGGGACTGAGACACGGCCCGAACTCCTACGGGA
GGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTGATCCAGCCATGCCG
CGTGTGTGAAGAAGGCCCTCGGGTTGTAAAGCACTTTCAGTGAGGAAGAACGCC
TGTCGGTTAATACCCGGYAGGGGAGACATCACTCACAGAAGAAGCACCGGCTA
ACTCC

Figure 4-7 16S rRNA gene sequence of ABU2 strain obtained from NCIMB, Aberdeen. The sequence is 486 base pairs long.

CTACCATGCAAGTCGAGCGGAACGATGGAAGCTTGCTTCCAGGCGTCGAG
CGGCGGACGGGTGAGTAATGCATAGGAATCTGCCCGATAGTGGGGGATA
ACGTGGGGAAACTCACGCTAATACCGCATACGTCCTACGGGAGAAAGCA
GGGGATCTTCGGACCTTGCGCTATCGGATGAGCCTATGTCGGATTAGCTA
GTTGGTGAGGTAACGGCTCACCAAGGCGACGATCCGTAGCTGGTCTGAGA
GGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
GGCAGCAGTGGGGAATATTGGACAATGGGGGAAACCCTGATCCAGCCAT
GCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGA
AGAAGGCCTGAGGGCTAATACCCTTCAGGAAGGACATCACTCGCAGAAG
AAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCG
AGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGCTTGATAA
GCCGGTTGTGAAAGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACTGT
CAGGCTAGAGTGCAGGAGAGGAAGGTAGAATTCCCGGTGTAGCGGTGAA
ATGCGTAGAGATCGGGAGGAATACCAGTGGCGAAGGCGGCCTTCTGGAC
TGACACTGACACTGAGGTGCGAAAGCGTGGGTAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGTTCCTT
GAGAACTTTGTGGCGCAGTTAACGCGATAAGTCGACCGCCTGGGGAGTAC
GGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGG
TGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCCT

Figure 4-8 16S rRNA gene sequence of anaerobic facultative strain ABU3 obtained from EurofinsMWG. The sequence is 940 base pairs in length.

4.2.4 Phylogenetic Analysis

Phylogenetic analysis of the 16S rRNA gene sequences of the five isolated strains was carried out by comparing them with highly similar sequences available from NCBI GenBank library using BLAST program. The amount of identical 16S rRNA gene sequence obtained for the five strains ranged from 98.3% - 100%. 16S rRNA gene sequences comparison showed that all five isolated strains belong to the family of *Halomonadacea*e. The 16S rRNA sequence of ABQ1 strain produced by NCIMB showed the highest sequence similarity to the type strain of *Halomonas pacifica* (99.7 %) (Table 4.1 and Figure 4.10). This identification was carried out by NCIMB using the MicroSeq database. Strain ABQ2 has 99% sequence similarity to *Halomonas species* WB-1 with BLAST accession number GU377269 (Figures 4.11 and 4.12).

The 16S rRNA gene sequences of the second group of strains from Um-Alma lake (designated as ABU1, ABU2 and ABU3) displayed the following similarities. The 16S rDNA sequence of ABU1 has 100% similarity with *Halomonas salifodinae* (BLAST Accession number EF527873.1) (Figures 4.13 and 4.14). The closest match to the ABU2 strain (sequenced by NCIMB) on the MicroSeq database was *Halomonas elongata* at 98.3% similarity (Table 4.2 and Figure 4.15). The sequence of strain ABU3 is most closely related to *Halomonas campisalis* (BLAST accession number DQ289061.1) with 99% similarity (Figure 4.14).

Table 4-1 ABQ1 Top 10 Hits in the MicroSeqTM 500 Database.

Sequence name	% Match	Sequence name	% Match
Halomonas pacifica	99.65	*Halomonas elongate*	93.41
Cobetia marina	94.65	*Chromohalobacter canadensis*	92.76
Halomonas halophila	94.05	*Halomonas venusta*	92.23
Halomonas salina	94.05	*Chromohalobacter marismortui*	92.12
Halomonas cupida	93.68	*Halomonas aquamarina*	91.92

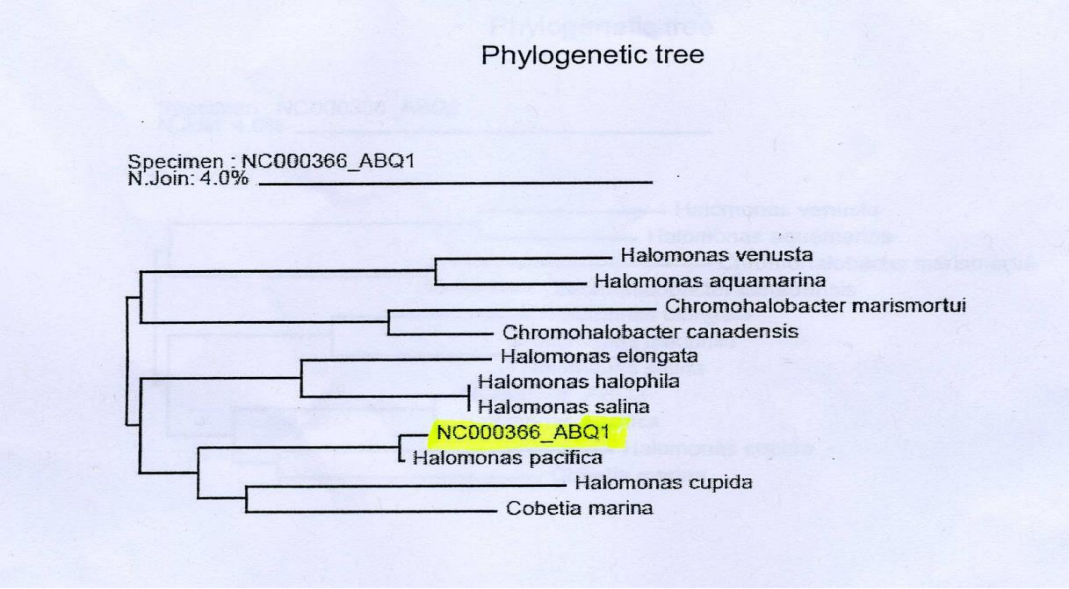


Figure 4-9 Phylogenetic tree based on the 16S rRNA gene sequence of ABQ1 compared with the most closely related organisms. ABQ1 is highlighted in yellow and the closest match is with *Halomonas pacifica*. NC000366 is the NCIMB job number for this sequence.

Halomonas sp. WB-1 16S ribosomal RNA gene, partial sequence, Length = 1439, Score = 1790 bits (969), Expect = 0.0, Identities = 979/984 (99%), Gaps = 0/984 (0%), Strand = Plus/Plus.

```
Query  1    CATGCAGTCGAGCGGTAACAGGGGTAGCTTGCTACCCGCTGACGAGCGGCGGACGGGTGA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  17   CATGCAGTCGAGCGGTAACAGGGGTAGCTTGCTACCCGCTGACGAGCGGCGGACGGGTGA  76
Query  61   GTAATGCATAGGAATCTGCCCGGTAGTGGGGGATAACCTGGGGAAACCCAGGCTAATACC  120
            |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  77   GTAATGCATAGGAATCTGCCCGATAGTGGGGGATAACCTGGGGAAACCCAGGCTAATACC  136
Query  121  GCATACGTCCTACGGGAGAAAGGGGGCTTCGGCTCCCGCTATTGGATGAGCCTATGTCGG  180
            |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  137  GCATACGTCCTACGGGAGAAAGGGGGCTCCGGCTCCCGCTATTGGATGAGCCTATGTCGG  196
Query  181  ATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCAACGATCCGTAGCTGGTCTGAGAGG  240
            |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  197  ATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCAACGATCCGTAGCTGGTCTGAGAGG  256
Query  241  ATGATCAGCCACATCGGGACTGAGACACGGCCCGAACTCCTACGGGAGGCAGCAGTGGGG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  257  ATGATCAGCCACATCGGGACTGAGACACGGCCCGAACTCCTACGGGAGGCAGCAGTGGGG  316
Query  301  AATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTC  360
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  317  AATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCCTC  376
Query  361  GGGTTGTAAAGCACTTTCAGCGAGGAAGAACGCCTAGTGGTTAATACCCACTAGGAAAGA  420
              ||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  377  GGGTTGTAAAGCACTTTCAGCGAGGAAGAACGCCTAGTGGTTAATACCCATTAGGAAAGA  436
Query  421  CATCACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  437  CATCACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGG  496
Query  481  TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGCTTGATAAGCCGGT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  497  TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGCTTGATAAGCCGGT  556
Query  541  TGTGAAAGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACTGTCAAGCTAGAGTGCAGG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  557  TGTGAAAGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACTGTCAAGCTAGAGTGCAGG  616
Query  601  AGAGGAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAATACCAG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  617  AGAGGAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGGAGGAATACCAG  676
Query  661  TGGCGAAGGCGGCCTTCTGGACTGACACTGACACTGAGGTGCGAAAGCGTGGGTAGCAAA  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  677  TGGCGAAGGCGGCCTTCTGGACTGACACTGACACTGAGGTGCGAAAGCGTGGGTAGCAAA  736
Query  721  CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACCAGCCGTTGGGTGCCT  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  737  CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACCAGCCGTTGGGTGCCT  796
Query  781  AGCGCACTTTGTGGCGAAGTTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAG  840
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  797  AGCGCACTTTGTGGCGAAGTTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAG  856
Query  841  GTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC  900
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  857  GTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC  916
Query  901  GATGCAACGCGAAGAACCTTACCTACTCTTGACATCCTGCGAACTTGTGAGAGATCACTT  960
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  917  GATGCAACGCGAAGAACCTTACCTACTCTTGACATCCTGCGAACTTGTGAGAGATCACTT  976
Query  961  GGTGCCTTCGGGAACGCAGAGACA  984
            |||||||||||||||||||||||||
Sbjct  977  GGTGCCTTCGGGAACGCAGAGACA  1000
```

Figure 4-10 Alignment of the 16S rRNA gene sequence of ABQ2 with the most closely related matching species - *Halomonas* WB-1 (BLAST accession number GU377269).

Halomonas salifodinae strain, isolate BC7 16S ribosomal RNA gene, partial Sequence, Length =1428, Score = 1441 bits (780), Expect = 0.0, Identities = 780/780 (100%), Gaps = 0/780 (0%) Strand=Plus/Plus

```
Query  1    ACTCACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  110  ACTCACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGC  169

Query  61   TATCGGATGAGCCTATGTCGGATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  170  TATCGGATGAGCCTATGTCGGATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACG  229

Query  121  ATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  230  ATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTC  289

Query  181  CTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGAAACCCTGATCCAGCCATGCC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  290  CTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGAAACCCTGATCCAGCCATGCC  349

Query  241  GCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAAGAAGGCCTGAGG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  350  GCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAAGAAGGCCTGAGG  409

Query  301  GCTAATACCCTTCAGGAAGGACATCACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  410  GCTAATACCCTTCAGGAAGGACATCACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAG  469

Query  361  CAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  470  CAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCG  529

Query  421  TAGGTGGCTTGATAAGCCGGTTGTGAAAGCCCCGGGCTCAACCTGGGAACGGCATCCGGA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  530  TAGGTGGCTTGATAAGCCGGTTGTGAAAGCCCCGGGCTCAACCTGGGAACGGCATCCGGA  589

Query  481  ACTGTCAGGCTAGAGTGCAGGAGAGGAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  590  ACTGTCAGGCTAGAGTGCAGGAGAGGAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGT  649

Query  541  AGAGATCGGGAGGAATACCAGTGGCGAAGGCGGCCTTCTGGACTGACACTGACACTGAGG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  650  AGAGATCGGGAGGAATACCAGTGGCGAAGGCGGCCTTCTGGACTGACACTGACACTGAGG  709

Query  601  TGCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  710  TGCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG  769

Query  661  TCGACTAGCCGTTGGGTTCCTTGAGAACTTTGTGGCGCAGTTAACGCGATAAGTCGACCG  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  770  TCGACTAGCCGTTGGGTTCCTTGAGAACTTTGTGGCGCAGTTAACGCGATAAGTCGACCG  829

Query  721  CCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCG  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  830  CCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCG  889
```

Figure 4-11 Alignment of 16S rRNA gene sequence of ABU1 with the most closely related matching species – *Halomonas salifodinae.*

Table 4-2 ABU2 Top 10 hits in the MicroSeqTM 500 Database

Sequence name	% Match	Sequence name	% Match
Halomonas elongata	98.32	*Halomonas aquamarina*	93.09
Halomonas halophila	95.83	*Halomonas cupida*	92.65
Halomonas salina	95.83	*Cobetia marina*	92.29
Chromohalobacter canadensis	93.3	*Chromohalobacter salexigens*	91.41
Halomonas pacifica	93.22	*Halomonas venusta*	91.38

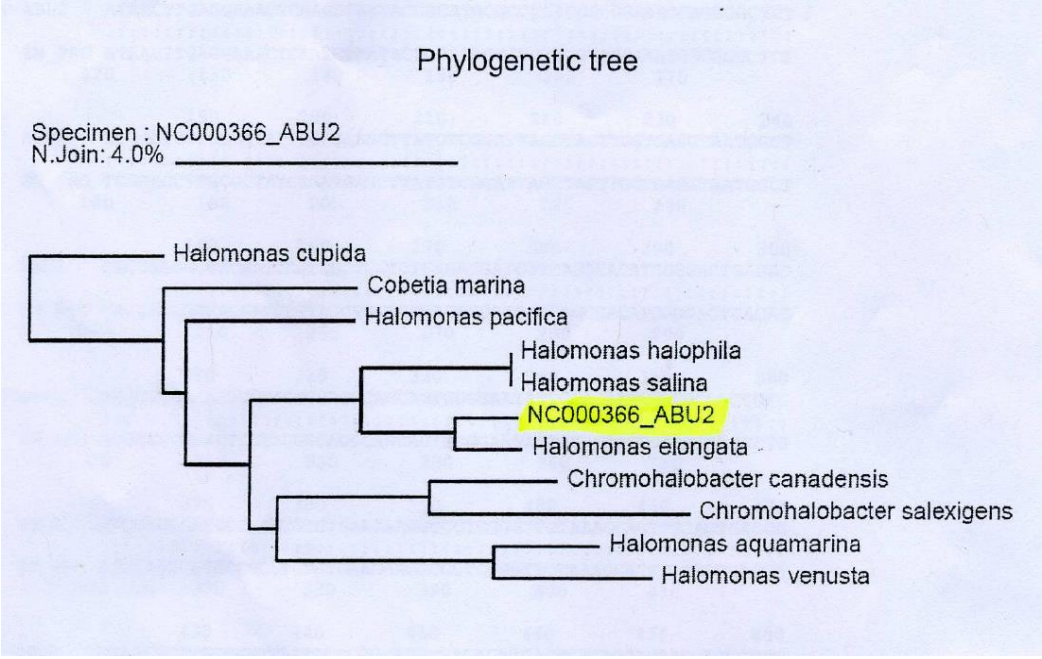


Figure 4-12 Phylogenetic tree based on 16S rRNA gene sequence from strain ABU2 with the closest matching organisms. ABU2 is highlighted in yellow and the closest match (98.32 % sequence similarity) is with *Halomonas elongata*. NC000366 is the NCIMB job number for this sequence.

Halomonas cf. campisalis 'campaniae' isolate HKS 4-1 16S ribosomal RNA gene, partial sequence, Length =1433, Score = 1720 bits (931), Expect = 0.0, Identities = 938/941 (99%), Gaps = 2/941 (0%). Strand=Plus/Plus

```
Query  1    CTAC-CATGCAAGTCGAGCGGAACGATGGAAGCTTGCTTCCAGGCGTCGAGCGGCGGACG  59
            ||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  9    CTACACATGCAAGTCGAGCGGAACGATGGAAGCTTGCTTCCAGGCGTCGAGCGGCGGACG  68

Query  60   GGTGAGTAATGCATAGGAATCTGCCCGATAGTGGGGGATAACGTGGGGAAACTCACGCTA  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  69   GGTGAGTAATGCATAGGAATCTGCCCGATAGTGGGGGATAACGTGGGGAAACTCACGCTA  128

Query  120  ATACCGCATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTATCGGATGA  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  129  ATACCGCATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTATCGGATGA  188

Query  180  GCCTATGTCGGATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATCCGTAGCT  239
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  189  GCCTATGTCGGATTAGCTGGTTGGTGAGGTAACGGCTCACCAAGGCGACGATCCGTAGCT  248

Query  240  GGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  249  GGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG  308

Query  300  CAGCAGTGGGGAATATTGGACAATGGGGGAAACCCTGATCCAGCCATGCCGCGTGTGTGA  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  309  CAGCAGTGGGGAATATTGGACAATGGGGGAAACCCTGATCCAGCCATGCCGCGTGTGTGA  368

Query  360  AGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAAGAAGGCCTGAGGGCTAATACCC  419
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  369  AGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAAGAAGGCCTGAGGGCTAATACCC  428

Query  420  TTCAGGAAGGACATCACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGT  479
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  429  TTCAGGAAGGACATCACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGT  488

Query  480  AATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGCTT  539
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  489  AATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGCTT  548

Query  540  GATAAGCCGGTTGTGAAAGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACTGTCAGGC  599
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  549  GATAAGCCGGTTGTGAAAGCCCCGGGCTCAACCTGGGAACGGCATCCGGAACTGTCAGGC  608

Query  600  TAGAGTGCAGGAGAGGAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGG  659
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  609  TAGAGTGCAGGAGAGGAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGGG  668

Query  660  AGGAATACCAGTGGCGAAGGCGGCCTTCTGGACTGACACTGACACTGAGGTGCGAAAGCG  719
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  669  AGGAATACCAGTGGCGAAGGCGGCCTTCTGGACTGACACTGACACTGAGGTGCGAAAGCG  728

Query  720  TGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCC  779
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  729  TGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCC  788

Query  780  GTTGGGTTCCTTGAGAACTTTGTGGCGCAGTTAACGCGATAAGTCGACCGCCTGGGGAGT  839
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  789  GTTGGGTTCCTTGAGAACTTTGTGGCGCAGTTAACGCGATAAGTCGACCGCCTGGGGAGT  848

Query  840  ACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG  899
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  849  ACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG  908

Query  900  TGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCCT  940
            |||||||||||||||||||||||||||||||||||| |||
Sbjct  909  TGGTTTAATTCGATGCAACGCGAAGAACCTTACCTAC-CCT  948
```

Figure 4-13 Alignment of 16S rRNA gene sequence of ABU3 strain with the closest matching species - *Halomonas campisalis*.

Table 4-3 Identification of the bacteria isolated and their similarity to the closest match based on 16S rRNA gene sequences.

Isolate	Closest Matching Species	Similarity
ABQ1	*Halomonas pacifica*	99.7%
ABQ2	*Halomonas species WB-1*	99%
ABU1	*Halomonas salifodinae*	100%
ABU2	*Halomonas elongata*	98.3 %
ABU3	*Halomonas campisalis*	99%

4.3 Conclusions

Experimental methods based on PCR amplification and analysis the 16S rRNA sequences of microbial DNA allows determination of the microbial diversity in natural environments (Benlloch et al., 1996, Bull and Hardman, 1991). In the current work, five strains of bacterial species were isolated from saline alkaline waters of Qabar-oun and Um-Alma lakes by classical techniques based on culture and isolation of microorganisms. This was followed by identification of the strains using molecular techniques based on genomic DNA extraction, PCR amplification and purification of 16S rRNA gene.

Phylogenetic analysis based on 16S rRNA gene sequence show that all isolates belong to the family of *Halomonadaceae* and the genus *Halomonas* (Table 4.3). The overall results of 16S rRNA gene sequence among these species ranged from 98.3% to 100% similarities. All five isolates were identified as *Halomonas pacifica* (ABQ1) with 99.7% similarity, *Halomonas* species WB-1 (ABQ2) with 99% sequence similarity, *H. salifodinae* (ABU1) with 100% similarity, *H. elongata* (ABU2) with 98.3% similarity and *H. campisalis* (ABU3) with 99% similarity. It has been previously described that organisms which have 70% homology of their total genomic DNA will share at least 96% 16S rDNA sequence similarity (Stackebrandt and Goebel, 1994, Rosselló-Mora and Amann, 2001). On this basis, any 16S rDNA match at 97% similarity or above is taken as being a true identification. Therefore all the strains have been properly identified (Table 4.3).

The family *Halomonadaceae* belongs to the γ-subclass of the *Proteobacteria* and was proposed to accommodate the moderately halophilic and marine bacteria of the genera *Halomonas* and *Deleya* (Franzmann et al., 1988). However, further studies were carried out and it was proposed that the genera *Halomonas* and *Deleya* were joined into the single genus *Halomonas* (Dobson and Franzmann, 1996). At the present time, the family

Halomonadaceae includes two genera of halophilic bacteria *Halomonas* and *Chromohalobacte*r, and also a non-halophilic bacterial genus *Zymobacter* (Arahal et al., 2002, Franzmann et al., 1988, Dobson and Franzmann, 1996). The genus *Halomonas* comprises two separate phylogenetic groups that currently include larger numbers of species (Mata et al., 2002, de la Haba et al., 2010). During past few years, many novel species are being described within the family *Halomonadaceae* (Arahal et al., 2002, Detkova and Boltyanskaya, 2007, Arahal et al., 2007).

Species belonging to the genus *Halomonas*, are heterotrophic, Gram-negative, rod-shaped bacteria which have been isolated from various saline environments and/or alkaline pH such as seawater, hypersaline soils, soda lakes, salt lakes and the Dead Sea (Dobson and Franzmann, 1996, Ghozlan et al., 2006, Jones, 1999). *Halomonas species* are often characterised as being obligately aerobic, but some strains have the capacity for facultative anaerobic growth in the presence of nitrate (Dobson and Franzmann, 1996). All the isolates from the Qabar-oun and Um Alma lakes were halophilic, Gram-negative, rod-shaped bacteria able to grow on LB and M9 minimal salt medium at pH values of 8 - 10 and at wide range of salt concentration of 0 - 2.5 NaCl and at temperatures between 25 and 37 °C.

4.3.1 Identification of strain ABQ1 as *Halomonas pacifica*

The closest match to the ABQ1 strain from Qabar–oun lake is *Halomonas pacifica*. This match was made by the NCIMB using the MicroSeq database. Therefore, we do not have the full sequence to compare with the closest match as we have for ABQ2, ABU1 and ABU3. *H. pacifica* was originally isolated from a marine environment and named *Alcaligenes pacificus* (Baumann et al., 1972). It was then transferred to a new genus *Deleya* as *D. pacifica* (Baumann, 1983). There is only one published paper with *D. pacifica* in the title, and that is by Suzuki et al. (1991) who found a restriction enzyme (Dpal – isoschizomer of Scal) in large

amounts in *D. pacifica.* Dobson and Franzmann (1996) transferred all species of the genus *Deleya* to *Halomonas,* thus it became *H. pacifica*. There are no published papers with *H. pacifica* in the title. *H. pacifica* was described as slightly halophilic by Arahal and Ventosa (2006), but it is halotolerant up to 20 % NaCl (approx. 3.4 M) with optimum growth at 3% NaCl (0.5 M). *H. pacifica* has been associated with community DNA taken from a well head (1700 m below the sea floor) in California (Orphan et al., 2000). *Halomonas pacifica* has also been isolated from surface seawater on the Hawaiian coast (Kaye and Baross, 2004).

4.3.2 Identification of strain ABQ2 as *Halomonas* WB-1

The closest match to the ABQ2 strain from Qabar-oun lake is *Halomonas* WB-1. The information on strain WB-1 is very sparse. There is no publication associated with this strain in the BLAST database, it is a direct submission from Liu and Yang of the China Agricultural University, Beijing and the isolation source is given as a saline lake in China.

4.3.3 Identification of strain ABU1 as *Halomonas salifodinae*

The closest match to the ABU1 strain from Um-Alma lake is *Halomonas salifodinae* BC7, which was originally isolated from a soil sample collected from a salt mine in north-western China (Wang et al., 2008). *H. salifodinae* is a moderately halophilic aerobic Gram-negative, motile, rod-shaped bacterium requiring 3% (w/v) salt (0.5 M NaCl) for optimum growth with a growth range from 0.5 - 20 % (w/v) salt i.e. 0.1 to 3.4 M NaCl. Growth of *H. salifodinae* takes place between pH 6.0 - 9.0, and the temperature range for growth is 4 - 48°C. Optimum growth for *H. salifodinae* BC7is found at 0.5 M NaCl, pH 7.0 at 30°C (Wang et al., 2008). A more recent report (Sahay et al., 2011), also found *H. salofodinae* in Pulicat Lake (a brackish-water lake in South-East India) along with a number of other *Halomonas species,* including *H. pacifica* (see section 4.3.1). *H. salofodinae* strain from Pulicat lake also grew in up to 3.4 M NaCl and no growth in the absence of salt was observed. However, unlike *H.*

salofodinae BC7, the Indian strain was moderately halophilic growing optimally at 1.5 to 2.6 M NaCl (Sahay et al., 2011)

During the isolation and initial characterization procedure, it was found that *H. salifodinae* ABU1 isolated from Um – Alma lake showed good growth at salt concentrations ranging from 0.5 - 1.5 M NaCl (data not shown).

4.3.4 Identification of strain ABU2 as *Halomonas elongata*

The closest match to the ABU2 strain from Um-Alma lake is *Halomonas elongata* which is one of the most studied strains of the genus *Halomonas* (Vreeland et al., 1980, Ventosa et al., 1998b, Arahal et al., 2001). H. elongata is a highly adaptable organism, growing in the presence of a wide range of salt concentrations, and able to grow anaerobically with nitrate as the electron acceptor, forming nitrite (Ventosa et al., 1998b, Vreeland et al., 1980). In addition, *H. elongata* has been used as a model organism for the study of osmoregulatory mechanisms in halophilic bacteria (Ventosa et al., 1998b). Furthermore, it has become one of the most popular organisms found in biotechnology applications (e.g. it is able to produce nearly pure β-carotene), because of its ability to grow over a wide range of salinities (Rodríguez-Sáiz et al., 2007).

4.3.5 Identification of strain ABU3 as *Halomonas campisalis*

The closest match to the ABU3 strain from Um-Alma lake is *Halomonas campisalis* which was first isolated from a soda lake in Grant County, Washington, USA (Mormile et al., 1999, Aston and Peyton, 2007). The strain was described as a haloalkaliphile organism able to grow aerobically and under denitrifying conditions over a wide range of salinity from 0.2 - 4.5 M NaCl, with optimal growth occurring at 1.5 M NaCl. No growth was found when NaCl was absent from the medium. The optimum pH was 9.5, but growth was possible from pH 6 to 12 (Mormile et al., 1999, Aston and Peyton, 2007, Arahal and Ventosa, 2006).

The optimum temperature for growth was 30°C and growth was possible from 4 to 50°C (Mormile et al., 1999).

Based on the above information, it was decided to concentrate on two strains for the further studies described in Chapters 5 and 6: *Halomonas pacifica* ABQ1 and *Halomonams campisalis* ABU3.

CHAPTER 5

Physiological Characterization

5. Physiological Characterization of *Halomonas pacifica* (ABQ1) and *Halomonas campisalis* (ABU3)

5.1 Introduction

Halophilic and halotolerant microorganisms belonging to the family *Halomonadaceae,* typically exist in naturally occurring saline lakes, solar salt facilities, saline soils and marine ecosystems, and haloalkaliphilic members of the *Halomonadaceae* are found in soda lakes and alkaline soils (Arahal and Ventosa, 2006). The genus *Halomonas* is a ubiquitous genus, heterogeneous, that comprises the largest number of species within the *Halomonadaceae* (Arahal and Ventosa, 2006). Within the family *Halomonadaceae*, many novel species have been described during the past few years (Arahal et al., 2007). Very recently, Wang et al. (2009) described *Halomonas salifodinae* isolated from a salt mine in China, Arenas et al. (2009) described *Halomonas illicicola* isolated from a saltern in southern Spain, and Kim et al. (2010) described *Halomonas jeotgali* isolated from traditional Korean fermented seafood.

Of the five isolates from Qabar-oun and Um-Alma lakes, which were identified in the work described in the previous chapter, it was decided to conduct further studies on *H. pacific* (ABQ1) and *H. campisalis* (ABU3). As noted in section 4.3.1. *H. pacifica* has been little studied, and there are no published papers with *H. pacifica* in the title. The reasons for choosing *H. campisalis* for further study are that it is a haloalkaliphilic strain that can utilize nitrate under anaerobic condions (section 4.3.5). It is also interesting to note that according to Arahal and Ventosa (2006), *H. pacifica* and *H. campisalis*, do not fall into either of the two major groups of *Halomonas* that have been described based on 16S and 23S rDNA sequencing. It is possible that further information may suggest that the species of *Halomonas* that are not part of the major

groupings are transferred to a new genus. Therefore, it is important to further characterize these less well studied strains of *Halomonas*.

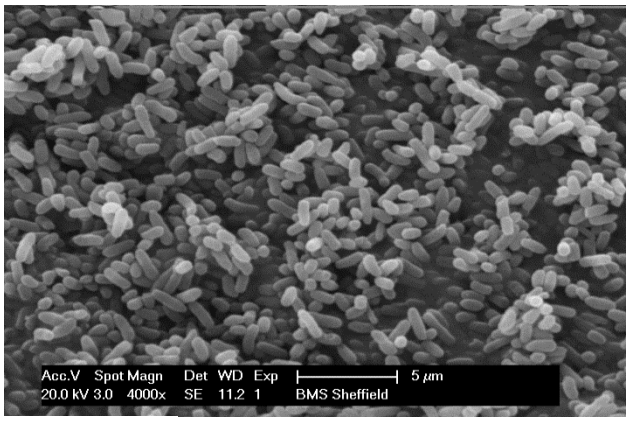
This chapter aims to study the physiological characterization of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) isolated from Qabar-oun and Um-Alma lakes in the south Libyan Sahara. The responses of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) to changes in salinity, temperature and pH will be measured, in order to determine their ability to grow under different conditions and to assess the optimal set of growth conditions to be used in further experiments (Arahal et al., 2007).

5.2 Results and Discussion

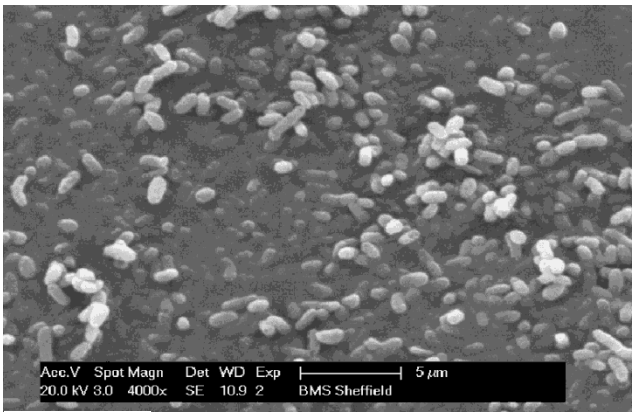
5.2.1 Cell morphology

Cell morphology was examined by transmission and scanning electron microscopy (TEM and SEM). Cells were prepared for electron microscopy by carrying out a conventional glutaraldehyde and tetroxide aqueous osmium fixation, as described in Chapter 2 (sections 2.6.1 and 2.6.2). Electron micrographs were prepared using a Philips XL-20 Scanning Electron Microscope at an accelerating voltage of 20 kv. Examination by SEM (Figure 5.1) showed that for *H.pacifica* (ABQ1) growth on M9 minimal salt medium leads to a reduction in cell size (shorter rods) when compared to cells grown on rich LB agar medium. SEM images of *H. campisalis* (ABU3) are shown in Figure 5.3, once again there appears to be a reduction in cell size when grown on minimal medium.

Greater cell detail can be seen using the higher magnification of TEM and Figure 5.2 shows the presence of gas vacuoles in cells of *H. pacifica* (ABQ1). More and larger gas vacuoles were present in cells grown on minimal medium. Figure 5.4 shows very similar images for *H. campisalis* (ABU3) again showing large numbers of gas vacuoles, particularly in cells grown in minimal medium.



(a)



(b)

Figure 5-1 Scanning electron micrgraphs (SEM) show surface structure of *Halomonas pacifica* (ABQ1) grown in LB medium (a) and M9 minimal salt medium (b) at 0.5 M NaCl.

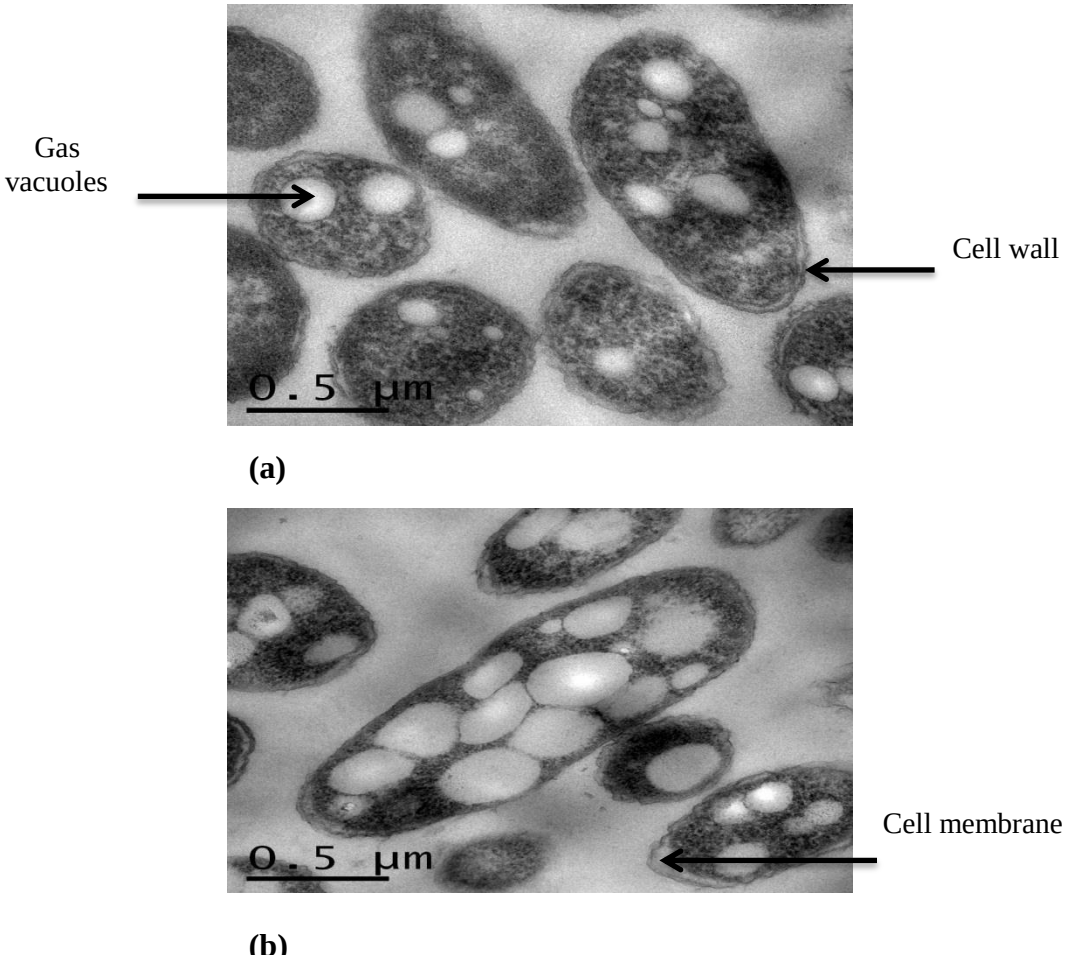
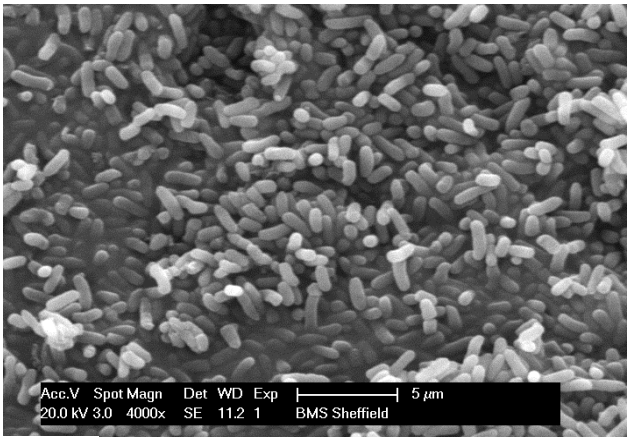
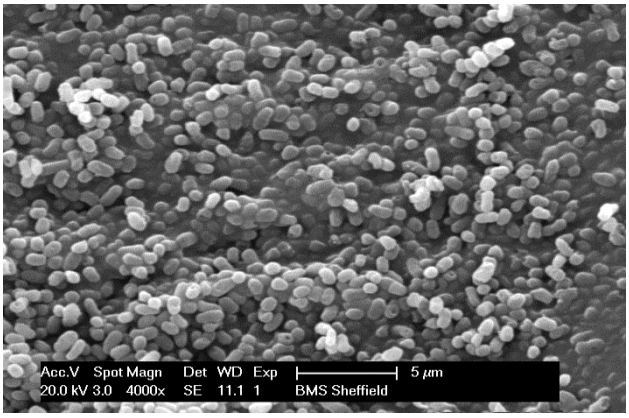


Figure 5-2 Transmission electron micrographs (TEM) of *Halomonas pacifica* (ABQ1) grown in LB medium (a) and M9 minimal salt medium (b) at 0.5 M NaCl. TEM of a thin section of *H. pacifica* (ABQ1) shows large gas vacuoles at stationary phase in M9 medium.



(a)



(b)

Figure 5-3 Scanning electron micrographs (SEM) show surface structure of *Halomonas compisalis* (ABU3) grown in LB medium (a) and M9 minimal salt medium (b) at 0.5M NaCl.

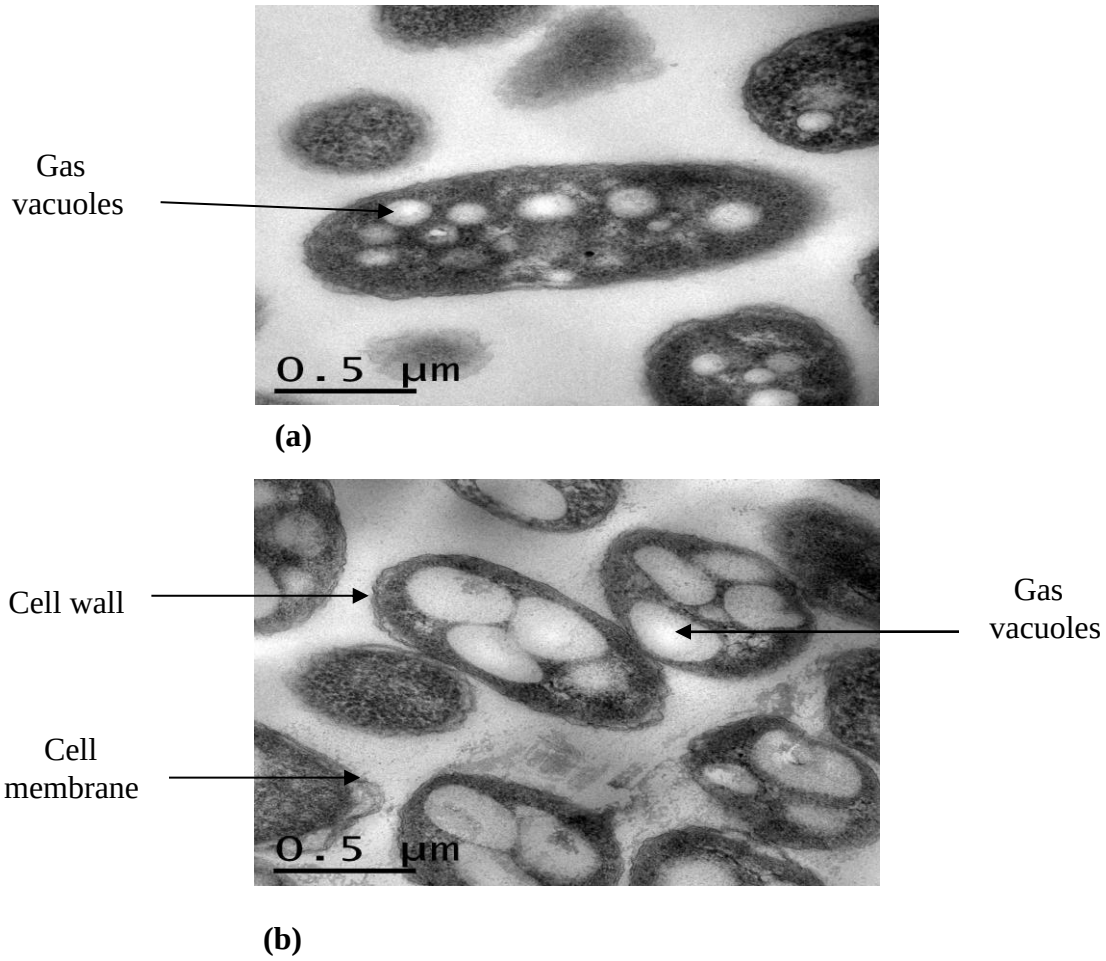


Figure 5-4 Transmission electronic micrographs (TEM) of *Halomonas campisalis* (ABU3) grown in LB medium (a) and M9 minimal salt medium (b) at 0.5M NaCl. Transmission electronic micrograph of a thin section of *H. campisalis* shows large gas vacuoles at stationary phase in M9 medium.

5.2.2 Growth characteristics of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3)

5.2.2.1 Anaerobic growth

The ability of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) to grow under anaerobic conditions was determined using LB solid medium (plates) in a Gas-Pak anaerobic jar system (BBL) that produces an anaerobic environment. The anaerobic condition was prepared by adding water to a gas generator envelope (containing sodium borohydride and sodium bicarbonate) in order to produce hydrogen gas and carbon dioxide. Anaerobic conditions were confirmed by placing a colour indicator inside the chamber. Streaked plates were placed inside the chamber and the jar was incubated at the 30°C for five days. The results showed that both *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) were able to grow under anaerobic conditions (data not shown). Thus, both strains were found to be facultative anaerobes.

5.2.2.2 Salinity range

Determination of the optimum salt concentration for growth of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) was carried out at an early stage soon after isolation. Growth after overnight incubation was measured at various concentrations of NaCl, ranging from 0 to 2.5 M, in M9 minimal salt medium containing glucose, at pH 7.8. Figure 5.5 shows that *H. pacifica* (ABQ1) required at least 0.05 M NaCl for growth, and can grow up to 2 M NaCl, with optimum growth at 0.5 M. This agrees with previous work on *H. pacifica* (Arahal and Ventosa, 2006), except the previous study found that *H. pacifica* could grow in up to 3.4 M NaCl. In contrast, *H. campisalis* (ABU3) was able to grow in the absence of NaCl and the maximum salinity for growth is 1.5 M NaCl with optimum growth at 0.25 - 0.5 M NaCl. This is in contrast to the original study on *H. campisalis* by Mormile et al. (1999), which suggested that 0.2 M NaCl was required for growth.

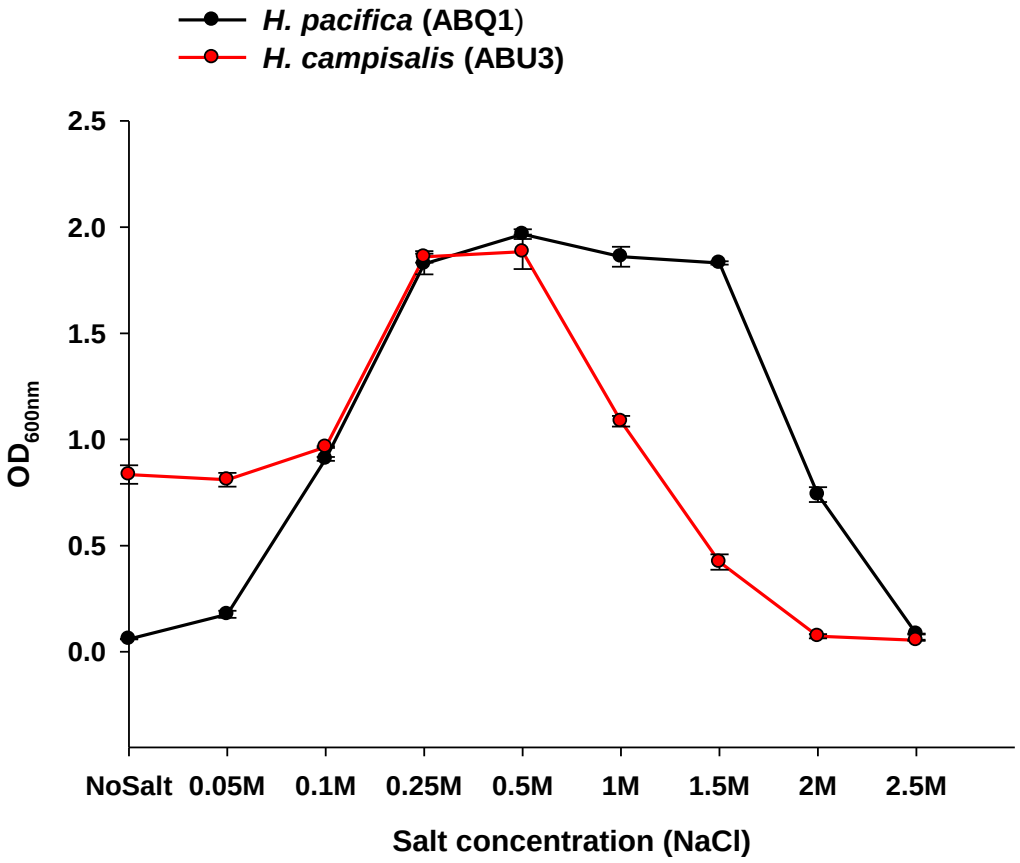


Figure 5-5 Growth curves for *H. pacifica* (ABQ1) and *H. campisalis* (ABU3). Cells were grown in M9 minimal salt medium at pH 7.8, incubated in a 37°C constant temperature room on an orbital shaker at 250 rpm overnight. The OD for each strain was measured at 600 nm. Data points are the means of triplicates and standard error. Note that the x axis is not a linear scale.

5.2.2.3 pH range

Growth of overnight cultures (final OD readings) was measured for *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) to determine the optimum external pH value for growth. For pH studies, the range of pH values used was 5.5 to 10, and media pH was adjusted with either NaOH or HCl (1 M) to give the desired pH. Figure 5.6 shows that *H. pacifica* (ABQ1) able to grow best at pH values 7 and 8, with good growth also seen at pH 9. Little or no growth was seen outside the range of pH 7 to 9. On the other hand, *H. campisalis* (ABU3) grew well at pH values from 7 to 10, with best growth at pH 9 and 10. This agrees with the results of Mormile et al. (1999) who first isolated *H. campisalis*.

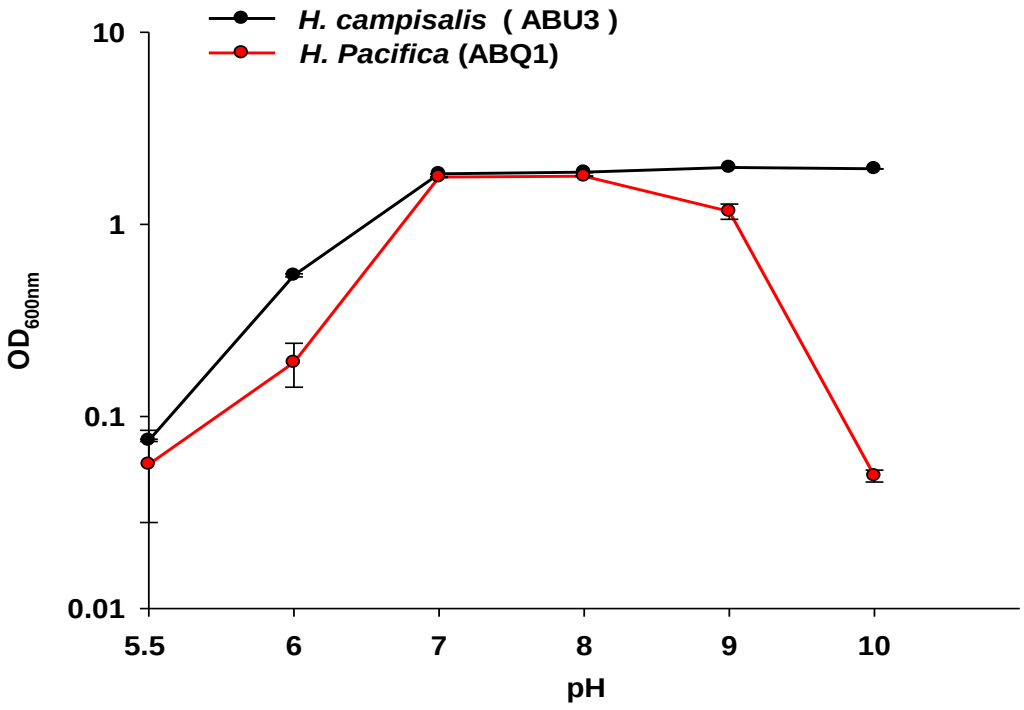


Figure 5-6 Final OD values for *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) cells grown in M9 minimal salt medium at 0.5 M NaCl, incubated in a 37°C constant temperature room on an orbital shaker at 250 rpm overnight. The OD for each strain was measured at 600 nm. Data points are the means of triplicates and standard error.

5.2.2.4 Optimum growth temperature

The effect of temperature on the growth rate of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) was measured as described in section 2.7.4. Growth was examined after overnight incubation and over the range 25, 30 and 37°C. M9 minimal salt medium was used for this test. Figures 5.7 and 5.8 show the overnight optical density (OD_{600nm}) for *H. pacifica* ABQ1 and *H. campisalis* ABU3.. According to the results, the strains were capable of growth across temperature range 25 - 37°C, and optimum temperature for growth occurred at 37°C, but in terms of final OD the values at 37°C were only very slightly higher. The results will allow suitable upper and lower limits of temperature to be chosen for further experiments. A previous study has been reported that *H. pacifica* was able to grow over a range of temperature from 2, to 30°C (Kaye and Baross, 2004). For *H. campisalis*, Mormile et al. (1999) found that 30°C was the optimum growth temperature.

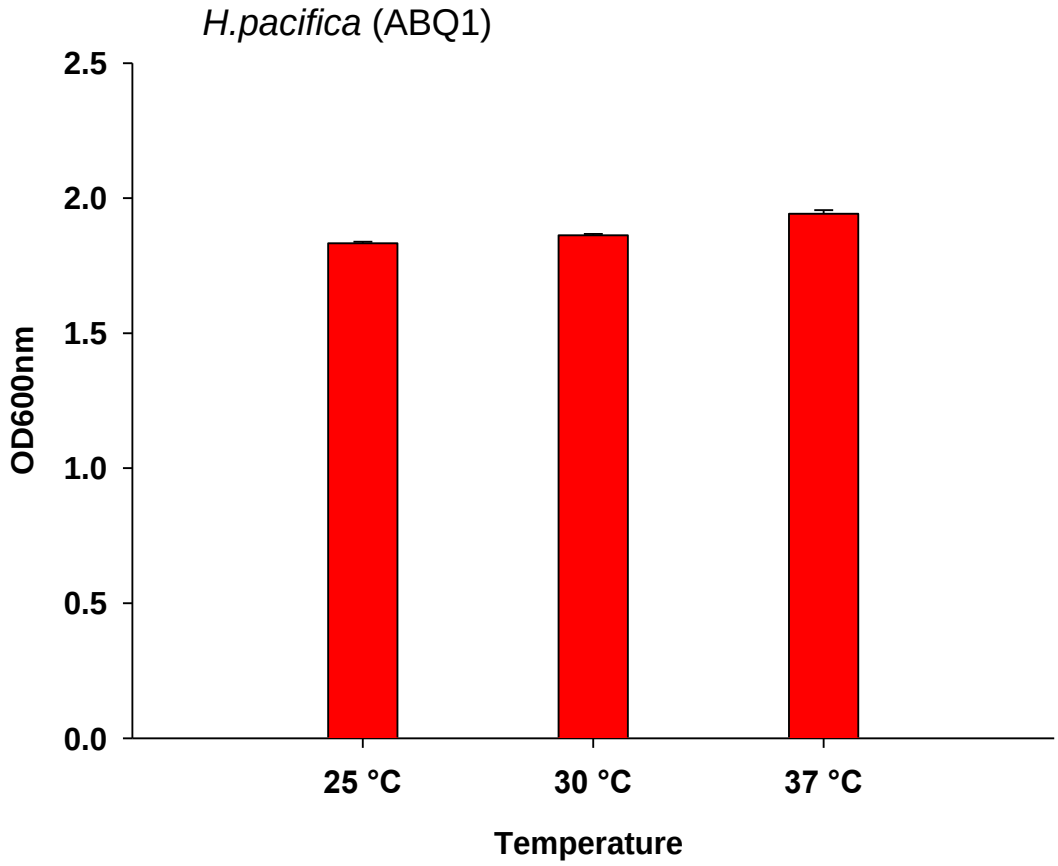


Figure 5-7 Effect of temperature on overnight growth of *H. pacifica* (ABQ1). Cells were grown in M9 minimal salt medium at 0.5 M NaCl and pH 9, incubated in a 37°C constant temperature room on an orbital shaker at 250 rpm overnight. Data are an average of three triplicates with error bars representing one standard error.

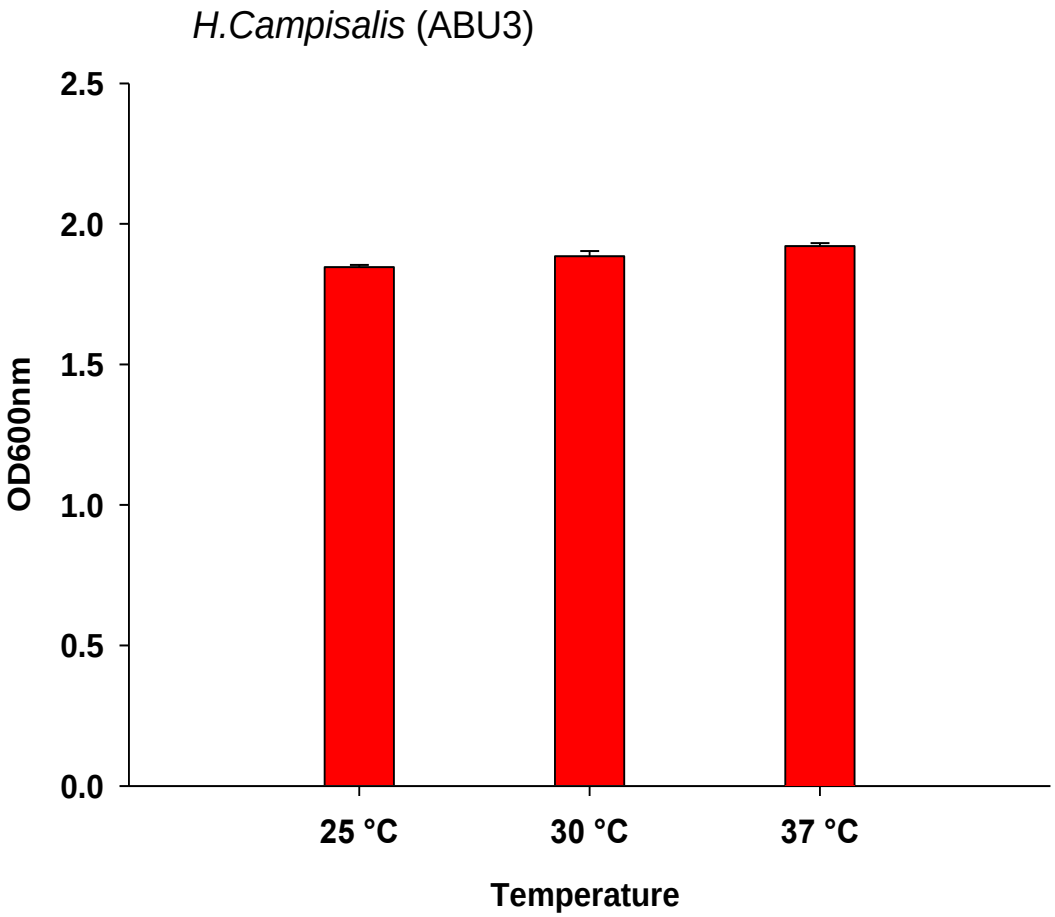


Figure 5-8 Effect of temperature on overnight growth of *H. campisalis* (ABU3). Cells were grown in M9 minimal salt medium at 0.5 M NaCl and pH 9, incubated in a 37°C constant temperature room on an orbital shaker at 250 rpm overnight. Data are an average of three triplicates with error bars representing one standard error.

5.2.2.5 Growth curves at different salt concentrations

Determination of growth curves of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) at different salinities was performed after the strains were adapted overnight to the different salt concentrations. The method was described in section 2.7.5. The generation time (g) or doubling time (t_d) for each strain was calculated in the usual way from plots of log OD_{600nm} against time (Table 5.1)

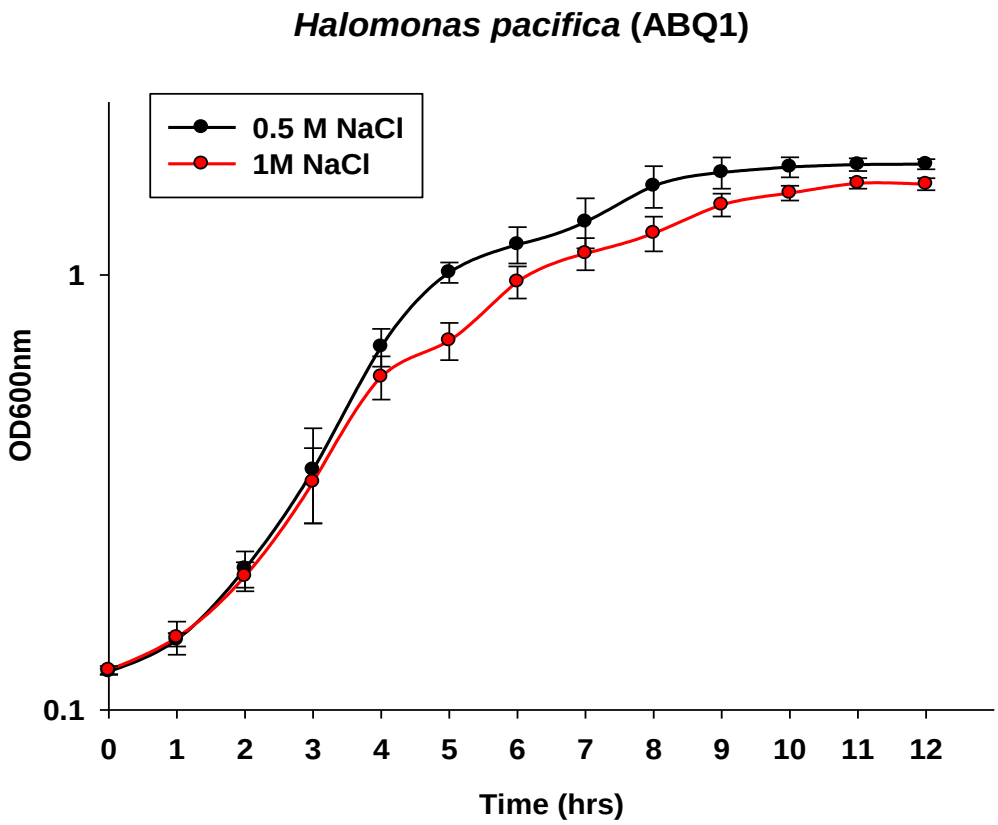


Figure 5-9 Growth curves for *H. pacifica* (ABQ1) at 0.5 and 1 M NaCl after adaptation period (overnight). Cells were grown in M9 minimal salt medium at pH 9, incubated in a 37°C constant temperature room on an orbital shaker at 250 rpm. The OD for growth was measured every hour during the day. Data represented are the means of triplicates with standard error.

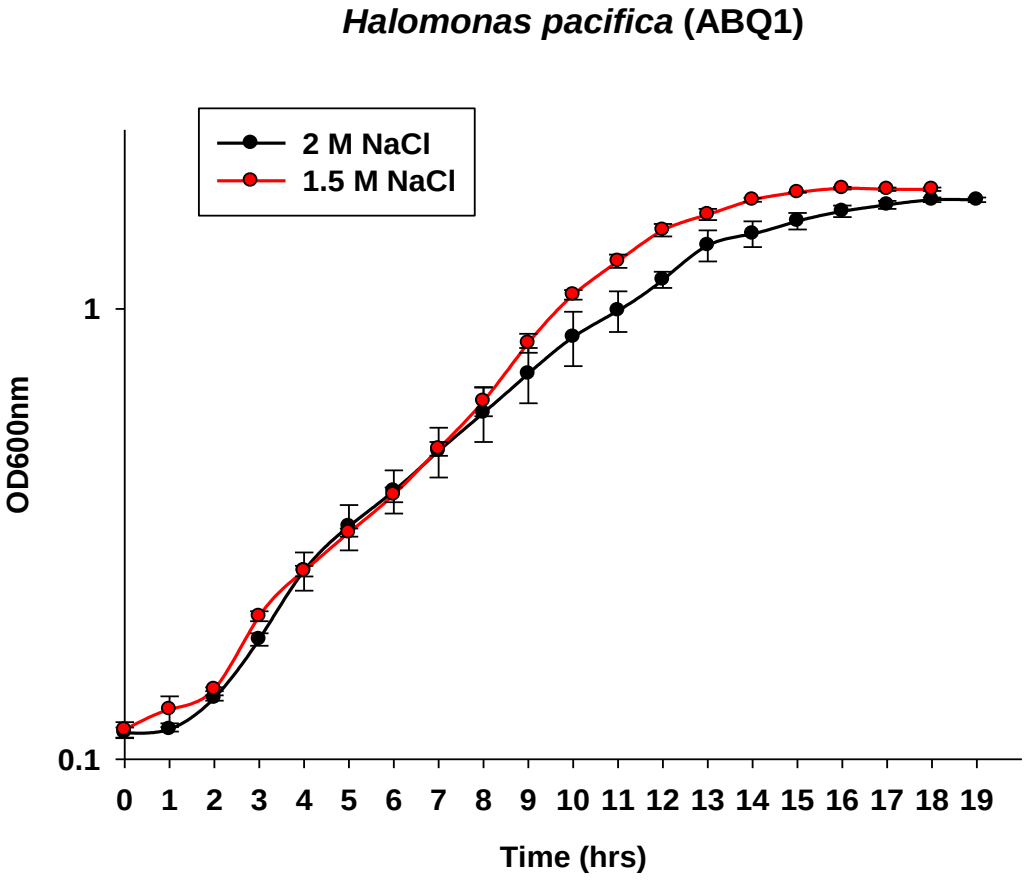


Figure 5-10 Growth curves for *H. pacifica* (ABQ1) at 1.5 and 2 M NaCl after adaptation period (overnight). Cells were grown in M9 minimal salt medium at pH 9, incubated in a 37°C constant temperature room on an orbital shaker at 250 rpm. The OD for growth was measured every hour during the day. Data represent the means of triplicates with standard error.

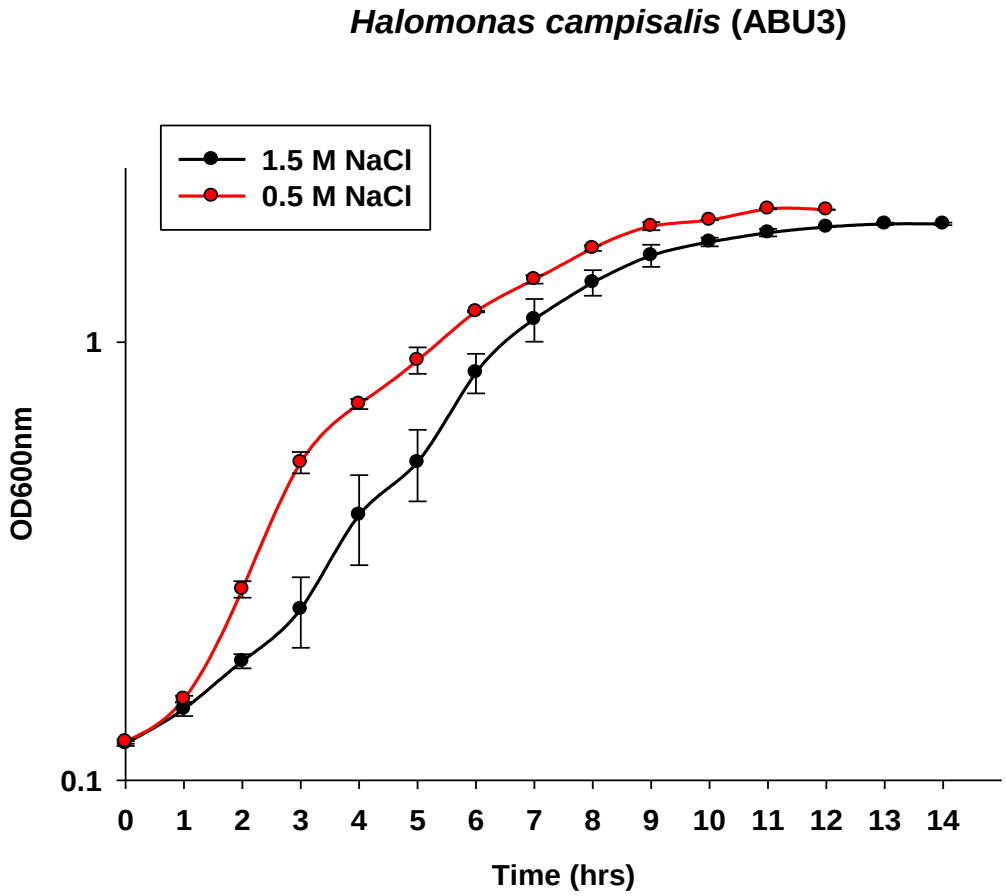


Figure 5-11 Growth curves for *H. campisalis* (ABU3) at 0.5 and 1.5 M NaCl after adaptation period (overnight). Cells were grown in M9 minimal salt medium at pH 9, incubated in a 37°C constant temperature room on an orbital shaker at 250 rpm. The OD for growth was measured every hour during the day. Data points are the means of triplicates with standard error.

Table 5-1 Doubling time of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) (minutes).

NaCl (M)	*H. pacifica* (ABQ1)	*H. campisalis* (ABU3)
0.5 M	97.2 min	90 min
1 M	120 min	-
1.5 M	156 min	108 min
2 M	168 min	-

Effect of NaCl concentration (0.5, 1, 1.5 and 2 M NaCl) on the doubling time of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3). Each point represents the average numbers from three replicate samples.

5.2.3 Carbon source utilization

A carbon substrates utilization experiment was performed using Biolog GN Microplates. This technique has been widely used to identify a wide range of aerobic Gram-negative bacteria (Miller and Rhoden, 1991). The Biolog system is used by pharmaceutical, biotech, cosmetics, and medical device companies, as well as in labs for testing human, animal and plant diseases, labs for environmental monitoring, and companies or organizations involved in the production and testing of food and drink (Weber, 2010). The test was originally based on investigating the ability of microorganisms to oxidize 96 different carbon sources and gives a characteristic reaction pattern called the "metabolic fingerprint" which is used for identification purposes (Miller and Rhoden, 1991). The Biolog method for analysing the utilization of carbon substrates was described in section 2.7.6.

The ability of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) to utilize 96 different carbon sources was determined by measuring optical density (OD) overnight using a 96 well microplate reader (absorbance at 595) (Appendix D). Consequently, reactions in wells (OD) were scored as "**+" (OD > 1)** for purple color (indicating utilization of carbon substrate), **"+/-" (OD > 0.5 – 1)** (indicating slight use of carbon substrate) and **"-" (OD ≤ 0.5)** (indicating no utilization of carbon source) with reference to the negative control well (A1) free of carbon source. The metabolic profiles of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) are shown in Plate **(a)** and **(b)** (Figure 5.12).

The different carbon substrates utilized by *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) are illustrated in Table (5.2). Based on overnight substrate utilization, 35 substrates were highly utilized by *H. pacifica* (ABQ1) and *H.campisalis*. These substrates include, Dextrin, N-acetyl-D-Glucosamine, D-Fructose, α- D-Glucose,

Maltose, D-Psicose, Sucrose, D-Trehalose, Turanose, Acetic Acid, Cie-Aconitic Acid, Citric Acid, D-Gluconic Acid, β-Hydroxybutyric Acid, p-Hydroxy Phenylacetic Acid, α-Keto Glutaric Acid, D, L-Lactic Acid, Propionic Acid, Succinic Acid, Bromosuccinic acid, D-Alanine, L-Alanine, L-Asparagine, L-Glutamic Acid, L-Histidine, L-Phenylalanine, L-Proline, L-Pyroglutamic Acid, L-Serine, Urocanic acid, Inosine, Uridine, Phenyethyl-amine, Putrescine and Glycerol.

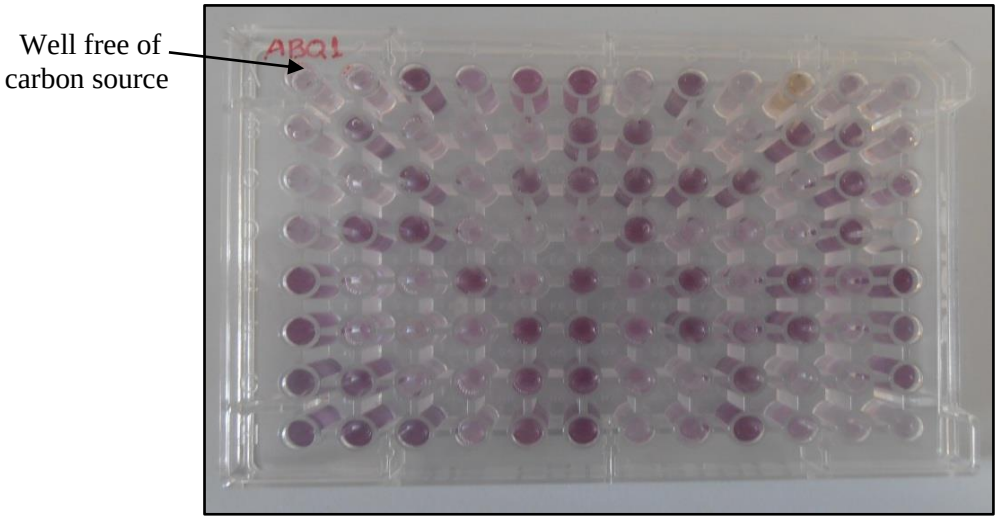
According to the results obtained, *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) used 15 carbon substrates moderately (slightly). Among these substrates were Glycogen, Tween 40, Tween 80, Succinic Acid Mono-Methyl-Ester, Itaconic Acid, Succinamic Acid, L-Alaninamide, L-Alanyl-glycine, L-Aspartic Acid, Glycyl-L-Glutamic Acid, L-Leucine, L-Omithine, γ-Amino Butyric Acid, 2,3-Butanediol and D,L-α-Glycerol Phosphate. However, there was no utilization of Itaconic Acid by *H. campisalis* (ABU3).

In addition, none of the following carbon sources were used by eitther *H. pacifica* (ABQ1) nor *H. campisalis* (ABU3) in the Biolog plates; α –Cyclodextrin, N-Acetyl-D-Galactosamine, Adonitol, L-Arabinose, D-Arabitol, D-Cellobiose, i-Erythritol, L-Fucose, D-Galactose, Gentiobiose, m-Inositol, α- D-Lactose, Lactulose, D-Mannitol, D-Mannose, D-Melibiose, β-methyl-D-Glucoside, D-Raffinose, L-Rhamnose, D-Sorbitol, Xylitol, Formic Acid, D-Galactonic Acid Lactone, D-Galacturonic Acid, D-Glucosaminic Acid, D-Glucuronic Acid, α-Hydroxybutyric Acid, y-Hydroxybutyric Acid, α-Keto Butyric Acid, α-Keto Valeric Acid, Malonic Acid, Quinic Acid, D-Saccharic Acid, Sebacic Acid, Glucuronamide, Glycyl-L-Aspartic Acid, Hydroxy-L-Proline, D-Serine, L-Threonine, D,L-Carnitine, Thymidine, 2-Aminoethanol, α-D-Glucose-1-Phosphate and D-Glucose-6-Phosphate.

All strains belonging to *Halomonas elongata* were found to oxidized sucrose, glycerol, mannose, and cellobiose (Sahay et al., 2011). The ability of *H. campisalis* to utilize carbon sources such as D-Fructose, α- D-Glucose, Maltose, Sucrose, Glycerol and acetate (Acetic acid) was also demonstrated previously in papers published by (Peyton et al., 2001, Mormile et al., 1999). In addition, a number of carbon sources oxidized by *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) in this study, were also used aerobically by a majority of the *halomonads* that have been isolated and described by (Vreeland et al., 1980).

According to the results obtained from Tables (5.2), there were a number of organic compounds and amino acids utilized that were in agreement with moderate halophilic bacteria, of the genus of *Halomanas*, isolated from different saline environments in Alexandria, Egypt (Ghozlan et al., 2006). It is noteworthy that there was only one carbon source tested (Itaconic acid) that showed a difference between *H. pacifica* (ABQ1) and *H. campisalis* (ABU3).

(a) Biolog profile of *H. pacifica* (ABQ1).



(b) Biolog profile of *H. campisalis* (ABU3).

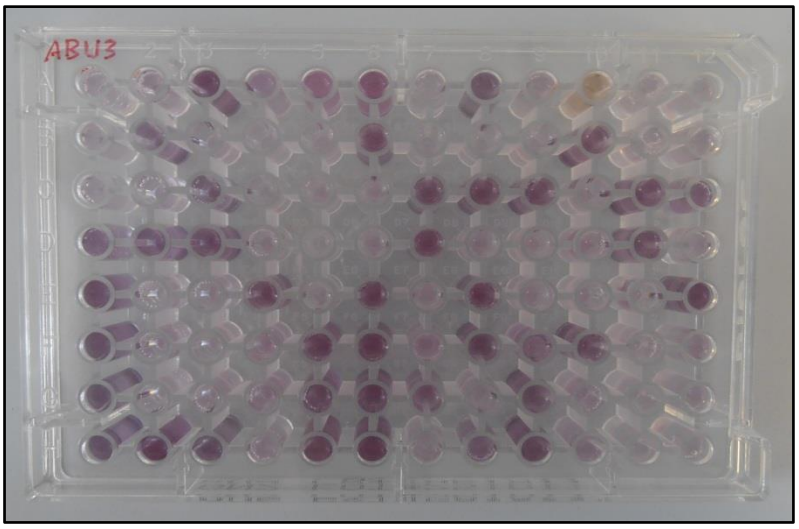


Figure 5-12 Biolog 96 well microplates inoculated with diluted cultures of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3). Each well was inoculated with 150 µl of bacterial cells free of glucose. The Biolog GN2 Microplates were then incubated overnight at a temperature of 37°C. The purple wells contain carbon sources that were used by the bacterial cells.

Table 5-2 Carbon substrates Utilization by *H.pacifica* (ABQ1) and *H.campisalis* (ABU3).

No	Carbon sources in GN2 Microplates	*H.pacifica* (ABQ1)	*H.campisalis* (ABU3)
1	Water	-	-
2	α -Cyclodextrin	-	-
3	Dextrin	+	+
4	Glycogen	-/+	-/+
5	Tween 40	-/+	-/+
6	Tween 80	-/+	-/+
7	N-Acetyl-D-Galactosamine	-	-
8	N-acetyl-D-Glucosamine	+	+
9	Adonitol	-	-
10	L-Arabinose	-	-
11	D-Arabitol	-	-
12	D-Cellobiose	-	-
13	i-Erythritol	-	-
14	D-Fructose	+	+
15	L-Fucose	-	-
16	D-Galactose	-	-
17	Gentiobiose	-	-
18	α- D-Glucose	+	+
19	m-Inositol	-	-
20	α- D-Lactose	-	-
21	Lactulose	-	-
22	Maltose	+	+
23	D-Mannitol	-	-
24	D-Mannose	-	-

Table 5.2 Continued

No	Carbon sources in GN Microplates	*H.pacifica* (ABQ1)	*H.campisalis* (ABU3)
25	D-Melibiose	-	-
26	β-methyl-D-Glucoside	-	-
27	D-Psicose	+	+
28	D-Raffinose	-	-
29	L-Rhamnose	-	-
30	D-Sorbitol	-	-
31	Sucrose	+	+
32	D-Trehalose	+	+
33	Turanose	+	+
34	Xylitol	-	-
35	Pyruvic Acid Methyl Ester	+	+
36	Succinic Acid Mono-Methyl-Ester	-/+	-/+
37	Acetic Acid	+	+
38	Cie-Aconitic Acid	+	+
39	Citric Acid	+	+
40	Formic Acid	-	-
41	D-Galactonic Acid Lactone	-	-
42	D-Galacturonic Acid	-	-
43	D-Gluconic Acid	+	+
44	D-Glucosaminic Acid	-	-
45	D-Glucuronic Acid	-	-
46	α-Hydroxybutyric Acid	-	-
47	β-Hydroxybutyric Acid	+	+
48	y-Hydroxybutyric Acid	-	-

Table 5-2 Continued

No	Carbon sources in GN2 Microplates	*H.pacifica* (ABQ1)	*H.campisalis* (ABU3)
49	p-Hydroxy Phenylacetic Acid	+	+
50	Itaconic Acid	-/+	-
51	α-Keto Butyric Acid	-	-
52	α-Keto Glutaric Acid	+	+
53	α-Keto Valeric Acid	-	-
54	D,L-Lactic Acid	+	+
55	Malonic Acid	-	-
56	Propionic Acid	+	+
57	Quinic Acid	-	-
58	D-Saccharic Acid	-	-
59	Sebacic Acid	-	-
60	Succinic Acid	+	+
61	Bromosuccinic acid	+	+
62	Succinamic Acid	-/+	-/+
63	Glucuronamide	-	-
64	L-Alaninamide	-/+	-/+
65	D-Alanine	+	+
66	L-Alanine	+	+
67	L-Alanyl-glycine	-/+	-/+
68	L-Asparagine	+	+
69	L-Aspartic Acid	-/+	-/+
70	L-Glutamic Acid	+	+
71	Glycyl-L-Aspartic Acid	-	-
72	Glycyl-L-Glutamic Acid	-/+	-/+

Table 5-2 Continued

No	Carbon sources in GN2 Microplates	*H.pacifica* (ABQ1)	*H.campisalis* (ABU3)
73	L-Histidine	+	+
74	Hydroxy-L-Proline	-	-
75	L-Leucine	-/+	-/+
76	L-Omithine	-/+	-/+
77	L-Phenylalanine	+	+
78	L-Proline	+	+
79	L-Pyroglutamic Acid	+	+
80	D-Serine	-	-
81	L-Serine	+	+
82	L-Threonine	-	-
83	D,L-Carnitine	-	-
84	y-Amino Butyric Acid	-/+	-/+
85	Urocanic acid	+	+
86	Inosine	+	+
87	Uridine	+	+
88	Thymidine	-	-
89	Phenyethyl-amine	+	+
90	Putrescine	+	+
91	2-Aminoethanol	-	-
92	2,3-Butanediol	-/+	-/+
93	Glycerol	+	+
94	D,L-α-Glycerol Phosphate	-/+	-/+
95	α-D-Glucose-1-Phosphate	-	-
96	D-Glucose-6-Phosphate	-	-

5.2.4 Effect of Sodium Chloride (NaCl) on Oxygen Uptake by *H. pacifica* (ABQ1) and *H. campisalis* (ABU3)

Effect of NaCl on respiration rate of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) was measured using a Clark type oxygen electrode. The experiment has been performed in pre-adapted-in-salt and shock-salt culture as described in section 2.10. Respiration rates were expressed as micromole of O_2 per milligram of protein per hour. Figures 5.13, 5.14 and 5.15 show O_2 uptake at different salt concentrations for *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) grown in M9 minimal salt medium at 0.1 – 2.5 M NaCl.

The results show that oxygen uptake of strains was significantly changed with increasing concentrations of NaCl. The overall result shows that respiration rates were higher in pre-adapted experiments than in shock experiments (Figure 5.13). Pre-adapted-in-salt cells of *H. pacifica* (ABQ1) respired optimally at 0.5 and 1 M NaCl, which corresponded with the growth curve data at these salt concentrations (Figure 5.5). Then, rate of respiration of the strain decreased with increasing salinity above 1 M NaCl. There was a slight decrease in the respiration rate of pre-adapted-in-salt cells of *H. pacifica* (ABQ1) from 9.18 µmoles O_2 mg^{-1} protein h^{-1} to 9.07 µmoles O_2 mg^{-1} protein h^{-1} between 0.5 and 1 M NaCl. Then, respiration rate decreased gradually from 9.18 µmoles O_2 taken up mg^{-1} cell protein h^{-1} to 2.23 µmoles O_2 taken up mg^{-1} cell protein h^{-1} between 0.5 and 2.5 M NaCl.

A significant effect of salinity on respiration rate was detected in salt-shock experiment, when cells of *H. pacifica* (ABQ1) exposed to a salt concentration range of 1, 1.5, 2 and 2.5 M NaCl. The highest rate of oxygen uptake was observed at 0.5

M NaCl and then it reduced down significantly from 7.80 µmoles O_2 mg^{-1} protein h^{-1} to 0.99 µmoles O_2 taken up mg^{-1} protein h^{-1} between 0.5 and 2.5 M NaCl.

The effect of salinity on oxygen uptake by cell suspensions of *H. campisalis* (ABU3) was performed at a salt concentration range from 0.1 to 1.5 M NaCl (Figures 5.14 and 5.15). Generally, the highest respiratory activity was noted with the strain at a salt concentration of 0.1 M NaCl in both pre-adapted-in-salt and salt-shock culture experiments. However, pre-adapted-in-salt cells demonstrated a greater amount of oxygen uptake than those subject to a salt-shock.

Rate of oxygen uptake by *H. campisalis* (ABU3) decreased gradually in pre-adapted-in-salt cells from 6.14 µmoles O_2 taken up mg^{-1} cell protein h^{-1} to 2.92 µmoles O_2 taken up mg^{-1} cell protein h^{-1} at 0.1 and 1.5 M NaCl, whereas a significant decrease in the rate of oxygen uptake was detected in the salt-shock experiment from 4.0 µmoles O_2 taken up mg^{-1} cell protein h^{-1} to 0.50 µmoles O_2 taken up mg^{-1} cell protein h^{-1} at 0.1 and 1.5 M NaCl. However, rate of respiration activity remained low and stable between 0.51 µmoles O_2 taken up mg^{-1} cell protein h^{-1} and 0.50 µmoles O_2 taken up mg^{-1} cell protein h^{-1} at 1 and 1.5 M NaCl, respectively.

Salt concentration above 1 M NaCl may cause disturbance in the metabolic activity of halotolerant bacteria due to inhibition of particular enzymes. The decrease of oxygen uptake by *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) bacteria at high levels of salt may be due to an insufficient adaptation period between the harvesting of cells and the exposure to high salt levels and the measurement of oxygen uptake (Zbigniew Mudryk, 1991). Therefore, halotolerant bacteria may need extensive adaptation periods to grow well at high concentrations of NaCl.

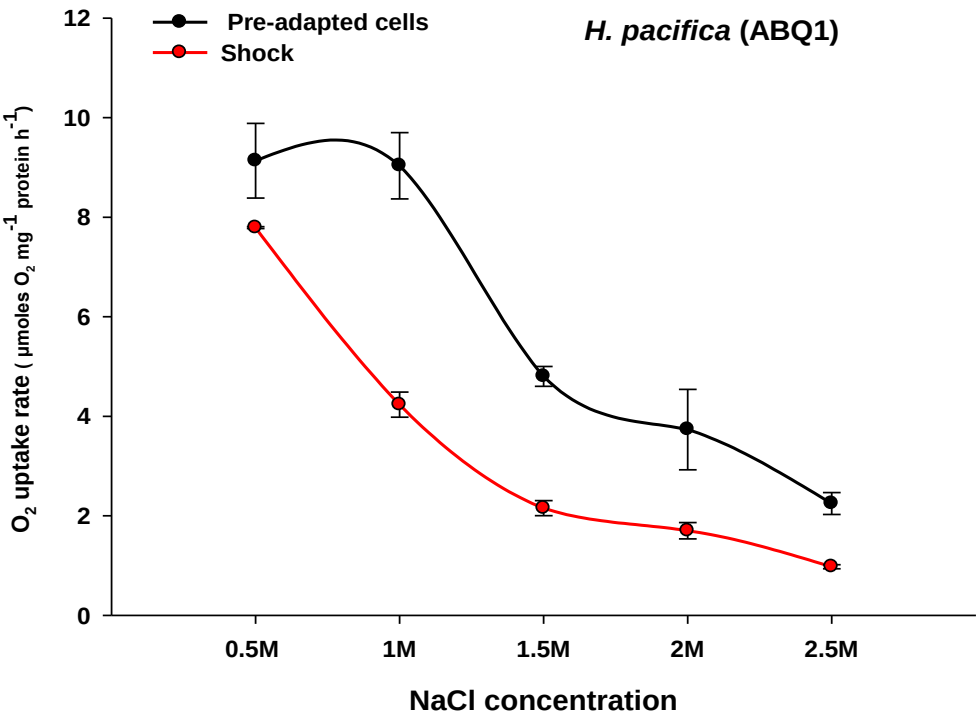


Figure 5-13 Effect of salinity on respiration rate of *H. pacifica* (ABQ1) grown in M9 minimal salt medium over a wide range from 0.5 to 2.5 M NaCl. Respiration was measured as oxygen uptake in the dark. Pre-adapted = measurement of oxygen uptake in cells grown overnight at salt concentrations range 0.5, 1, 1.5, 2 and 2.5 M NaCl. Shock = measurement immediately after cells grown at 0.5 M NaCl were subjected to different salt concentrations. Data represent the means of triplicate experiments with standard error.

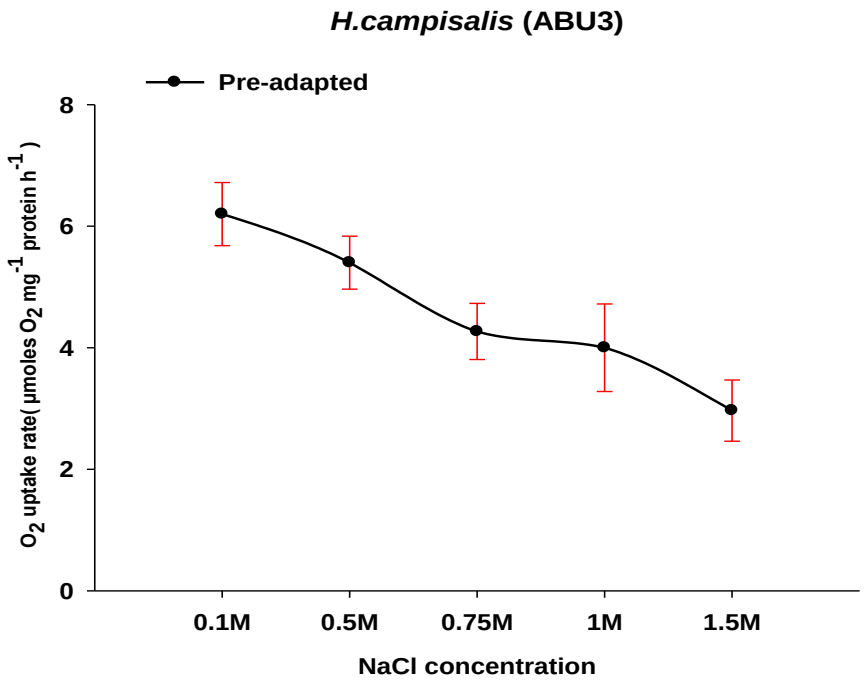


Figure 5-14 Effect of salinity on respiration rate in pre-adapted-in-salt cultures of *H. campisalis* (ABU3) grown in M9 minimal salt medium at salinity from 0.1 to 1.5 M NaCl. Data represent the means of triplicates with ± standard error.

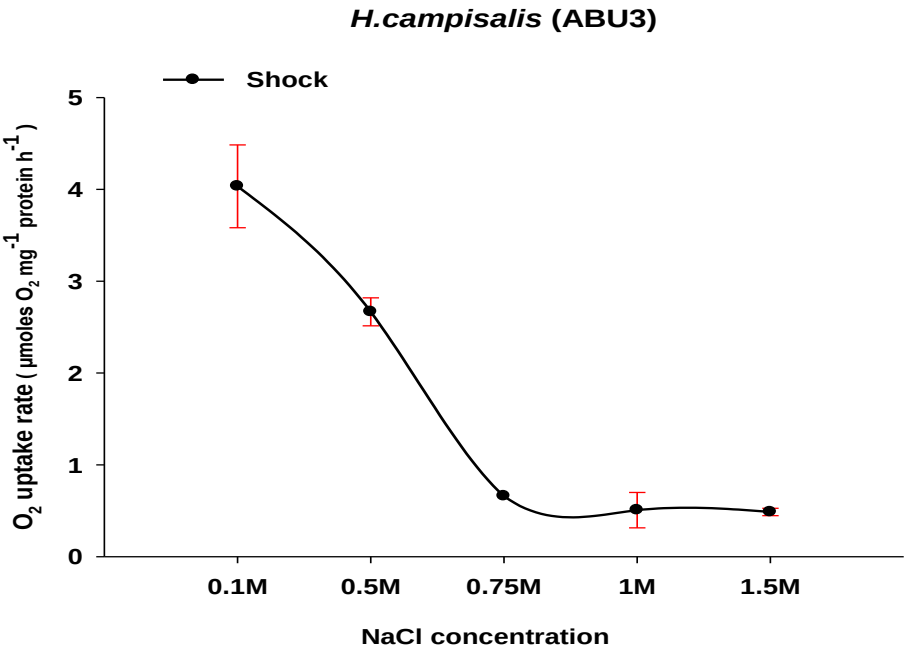


Figure 5-15 Effect of salinity (salt-shock) on respiration rate of *H. campisalis* (ABU3) grown in 0.5 M NaCl M9 minimal salt medium immediately after resuspension insalt concentrations from 0.1 to 1.5 M NaCl. Data points are the means of triplicates with ± standard error.

5.2.5 Effect of temperature on the respiration rate

As described previously in section 2.11, the effect of temperature on respiration rate of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) was tested using a Clark-type Oxygen Electrode (Figure 2.3). Cells were grown overnight at 0.5 M NaCl, under optimum temperature 37°C in M9 minimal salt medium. Respiration rate was determined at the same salinity as the growth salinity. Figure 5.16 showed that temperature had a dominant effect on the oxygen uptake in both strains with the respiration rate of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) increasing gradually with increasing temperature. Respiration rate of both strains was highest at 45°C. There was a significant increase in the rate of oxygen uptake from 0.5 µmoles O_2 taken up mg^{-1} cell protein h^{-1} at 25°C to 6 and 8 µmoles O_2 taken up mg^{-1} cell protein h^{-1} at 45°C for both *H. campisalis* (ABU3) and *H. pacifica* (ABQ1), respectively. Therefore, an increase in temperature at the optimum salt concentration (0.5 M NaCl) results in an increase in the respiration rate in both *H. campisalis* (ABU3) and *H. pacifica* (ABQ1).

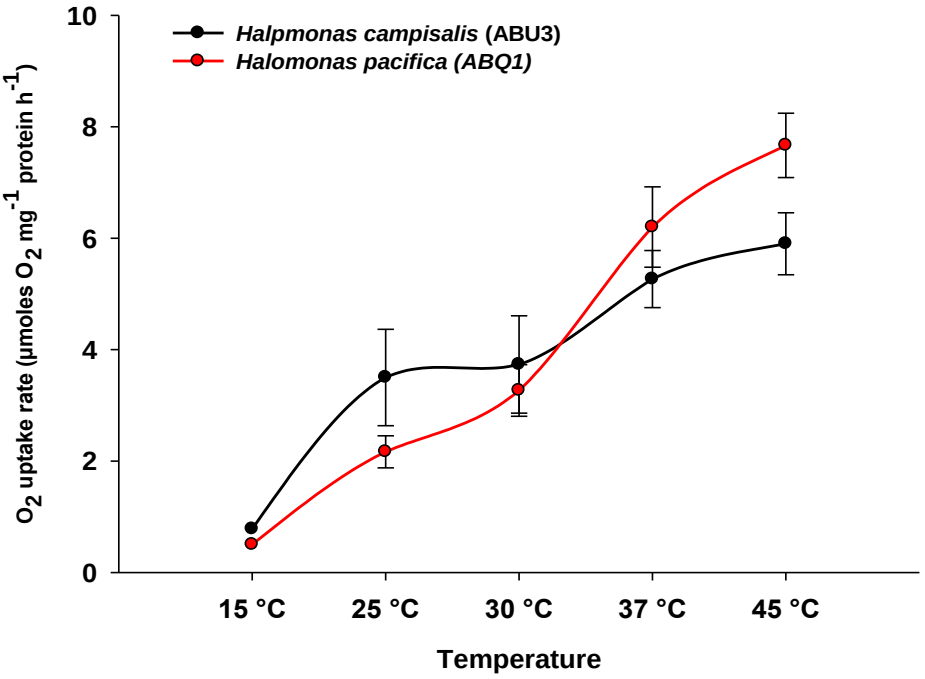


Figure 5-16 The effect of temperature on respiration rate of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3). 5 ml of cells from each strain grown overnight in M9 minimal salt medium at 37°C were harvested by centrifugation and re-suspended in 9 ml of fresh medium of the same composition. The O_2 uptake was measured as described in section 2.8. Data points are the means of triplicates with standard error.

5.2.6 Effect of external pH on the respiration rate

Rates of respiration in *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) were determined using a Clark-type oxygen electrode as described previously in section 2.8. The test was carried out to observe the effect of external pH on rate of oxygen uptake over the range of pH 7 - 12 in M9 minimal salt medium.

In general, the respiration rates of both strains at pH values tested showed that the pH pre-adapted cells had much higher respiration rates than the cells in the pH upshock experiments (Figure 5.17 and 5.18). Rates of O_2 uptake were optimum at pH 8 and 9 in pre-adapted cells of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3), respectively.

In pH pre-adapted experiment, respiration rate of *H. pacifica* (ABQ1) increased slowly from 3.79 µmoles O_2 taken up mg^{-1} cell protein h^{-1} to 5.56 µmoles O_2 taken up mg^{-1} cell protein h^{-1} when external pH increased from pH 7 and pH 8, and then declined from 5.56 µmoles O_2 taken up mg^{-1} cell protein h^{-1} to 4.71 µmoles O_2 taken up mg^{-1} cell protein h^{-1} above pH 8. In pH upshock experiment, *H. pacifica* (ABQ1) showed little difference wfrom the pre-adapted cells experiment. The optimum O_2 consumption was 5.33 µmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 8, and then decreased sharply to 2.63 µmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 10.

For pre-adapted cells experiment, O_2 uptake by *H. campisalis* (ABU3) was increased from 5.16 µmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 7 to 6.91 µmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 9. Then, a slight decrease was recorded from 6.91 µmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 9 to 6.49 µmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 10.

In the pH upshock experiment, rate of respiration of *H. campisalis* (ABU3) was measured over the range of pH values from 7 - 12. Figure 5.18 shows that a similar

effect of pH on respiration rates of *H. campisalis* (ABU3) was found in both pH pre-adapted and upshock experiments. In pH upshock experiment, the highest respiration rate was 5.88 µmoles O_2 mg^{-1} protein h^{-1} observed at pH 9 and then reduced down above pH 9 from 5.88 µmoles O_2 mg^{-1} protein h^{-1} to 0.68 µmoles O_2 mg^{-1} protein h^{-1} at pH 12. The decreased respiration rates may indicate that *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) cells are under more stress at these higher pH values (Figures 5.17 and 5.18).

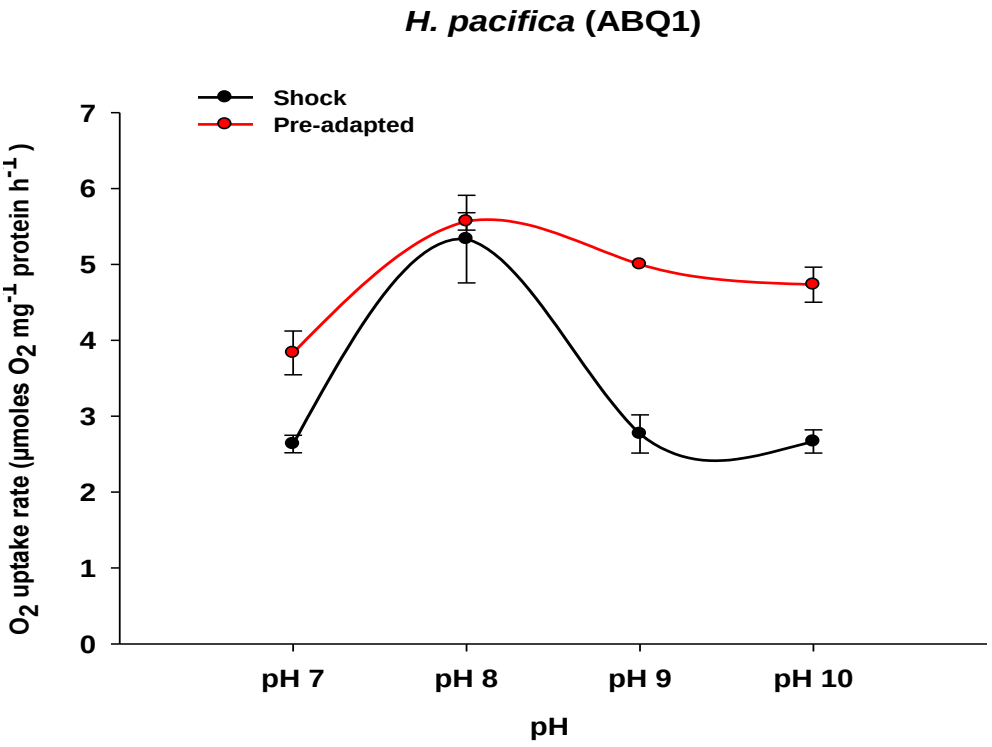


Figure 5-17 Effect of external pH on the respiration rate in *H. pacifica* (ABQ1). Pre-adapted cells were grown overnight in M9 minimal salt media over the range of pH 7 - 10, at 37°C. In the shock experiment, cells were grown overnight in M9 minimal medium at pH 9. Values represented are the means of triplicates and standard error.

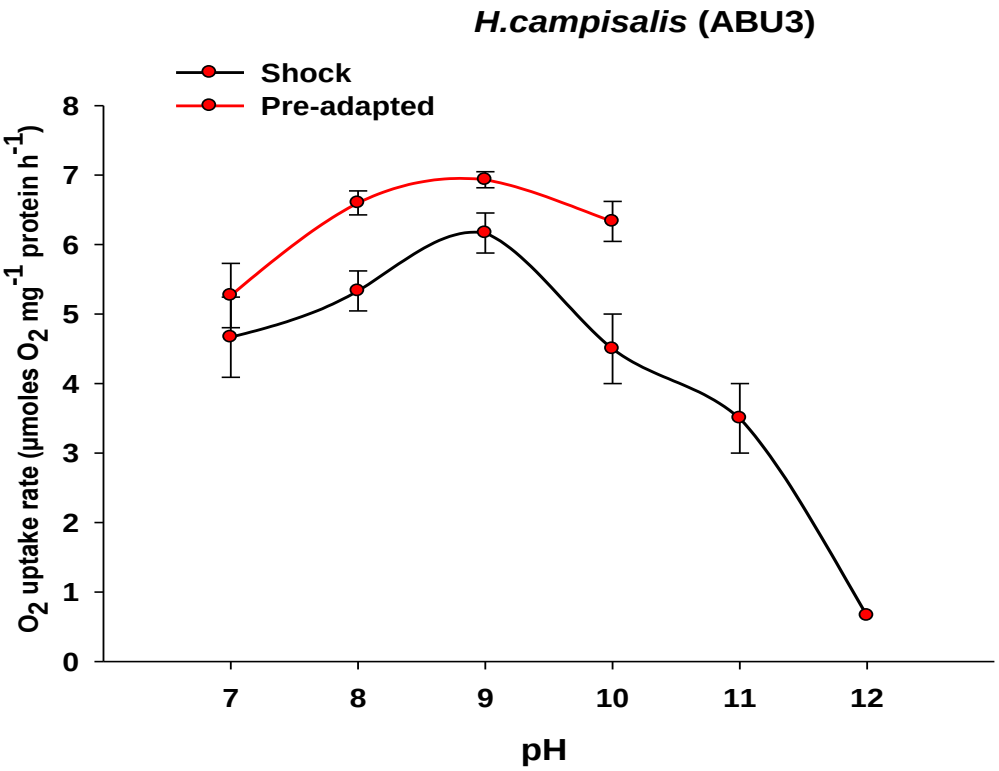


Figure 5-18 Effect of external pH on the respiration rate in *H. campisalis* (ABU3). Pre-adapted cells were grown overnight in M9 minimal salt media over the range of pH 7 - 12, at 37°C. In the shock experiment, cells were grown overnight in M9 minimal medium at pH 9. Values represented are the means of triplicates and standard error.

5.2.7 Malate dehydrogenase activity

Malate dehydrogenase (MDH) is an intracellular enzyme essential for energy metabolism, which is only found in the TCA cycle (Na, 2007). Malate dehydrogenase (EC 1.1.1.37) (MDH) catalyzes the conversion of malate into oxaloacetate (using NAD^+. In 1977, Mevarech et al. described the structure and function of malate dehydrogenase enzyme isolated from an extremely halophilic bacterium isolated from the Dead Sea.

This experiment was performed to investigate the activity of *H. campisalis* (ABU3) malate dehydrogenase at a various concentrations of NaCl. A crude cell-free extract was prepared and the speed of reaction of malate dehydrogenase was measured over the range 0.25 - 2.5 M NaCl. Malate dehydrogenase activity was assayed in the direction of oxaloacetate reduction.

Figure 5.19 shows that the specific activity of malate dehydrogenase enzyme decreases with increasing salt concentrations. The optimum activity of malate dehydrogenase in *H. campisalis* (ABU3) was recorded at 0.25 M NaCl. Then, the rate of enzyme activity decreased gradually with increasing concentrations of NaCl between 0.5 and 2.5 M. It was reported that enzyme activity decreases with increasing salt concentration in molar range for malate dehydrogenase from *Haloarcula marismortui* (*Hm* Ma1DH) (Mevarech et al., 1977). Attempts to detect malate dehydrogenase activity in *H. pacifica* (ABQ1) were unsuccessful.

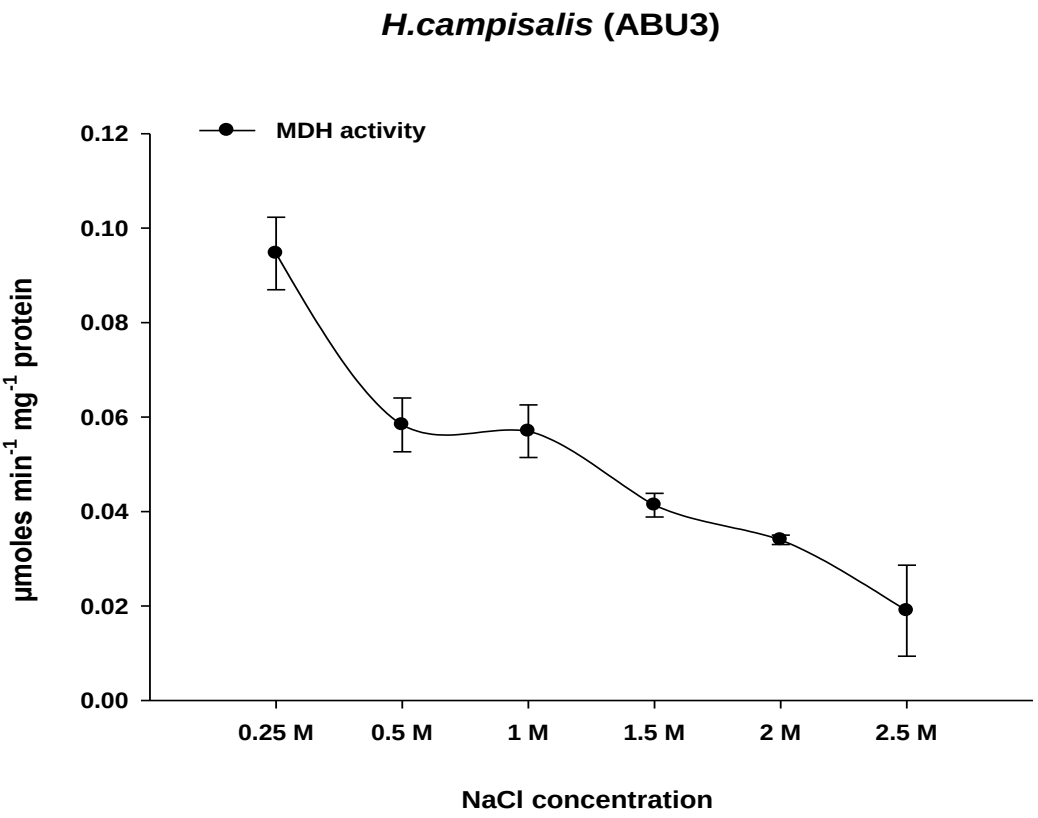


Figure 5-19 Effect of sodium chloride (NaCl) concentration on malate dehydrogenase enzyme activity in *H. campisalis* (ABU3). Data are the means of triplicate experiments with standard error.

5.2.8 NADH oxidase activity

Determination of the effect of sodium chloride (NaCl) and potassium chloride (KCl) on NADH oxidase enzyme activity was carried out using crude cell-free extract as described previously in section. The rate of reaction of NADH oxidase was measured using M9 minimal salt medium at a salt concentration range of 0.25 - 2.5 M (NaCl and KCl).

The results in Figure 5.20 show a similar effect of KCl and NaCl on activity of NADH oxidase in *H. pacifica* (ABQ1). In general, enzyme activity was higher in the presence of KCl than NaCl but the activity fell off gradually as the salt concentrations increased.

Optimal activity of the NADH oxidase enzyme in *H. pacifica* (ABQ1) was found at the same salt concentration tested (0.25 M KCl and NaCl). However, the rate of enzyme activity in *H. pacifica* (ABQ1) decreased more quickly with increasing NaCl concentration, No NADH oxidase activity was detected in crude cell-free extracts from *H. campisalis* (ABU3).

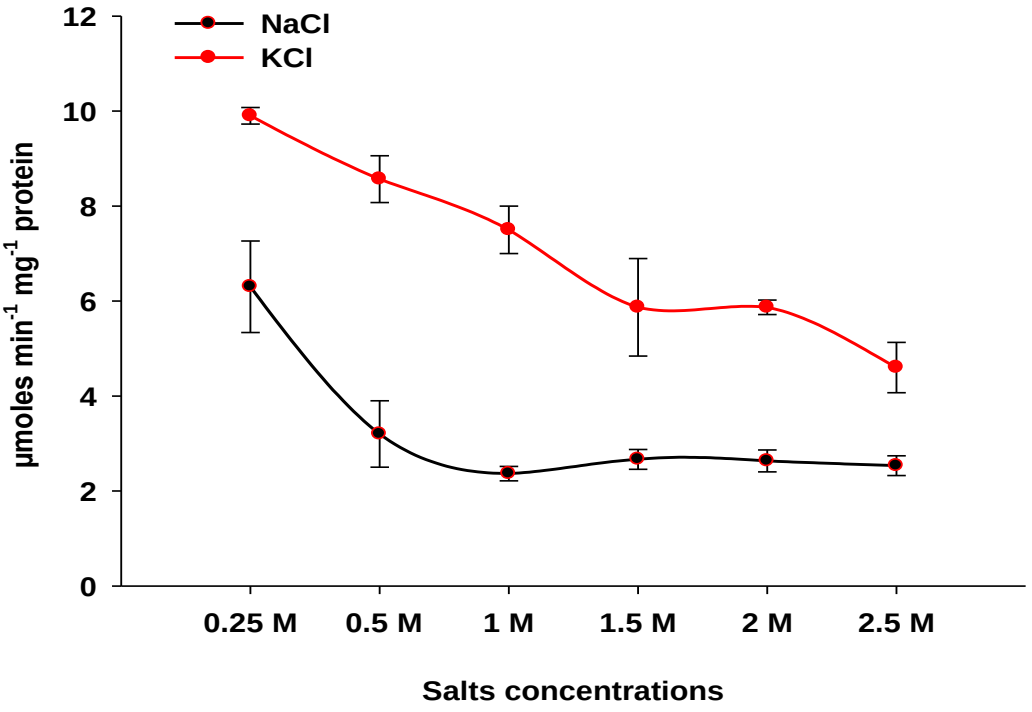


Figure 5-20 Effect of sodium chloride (NaCl) and potassium chloride (KCl) on NADH oxidase enzyme activity in *H. pacifica* (ABQ1). Data are the means of triplicate experiments with standard error.

5.2.9 Sensitivity to antibiotics

Antimicrobial susceptibility was assayed using the solid agar plate method. *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) were tested for their response to a range of antibiotics according to the recommended minimal standards for describing new taxa of the family *Halomonadaceae* by (Arahal et al., 2007). Table 5.7 shows sensitivity of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) to different antibiotics in terms of Mean ± Std.Dev. Carbenicillin and cefoxitin were the most effective against both species. In general the response to the antibiotics was similar for *H. pacifica* (ABQ1) and *H. campisalis* (ABU3), but *H. campisalis* was resistant to kanamycin.

Table 5-3 Sensitivity of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) to different antibiotics. The numbers represent the clearing zone around the antibiotic disk in mm.

Sensitivity to Antibiotics: Mean ± Std.Dev (n = 3)		
Antibiotics	***H. pacifica* (ABQ1)**	***H. campisalis* (ABU3)**
Tetracycillin (50 µg)	0.60 ± 0.00	0.60 ± 0.00
Ampicillin (25 µg)	0.40 ± 0.05	0.00 ± 0.00
Carbenicillin (100 µg)	1.56 ± 0.11	1.00 ± 0.00
Kanamycin (30 µg)	0.50 ± 0.10	0.23 ± 0.05
Erythromycin (15 µg)	0.36 ± 0.57	0.53 ± 0.05
Rifampicin (30 µg)	0.63 ± 0.05	0.80 ± 0.10
Cefoxitin (30 µg)	0.76 ± 0.20	1.00 ± 0.00
Nalidixic acid (30 µg)	0.56 ± 0.11	0.43 ± 0.05

5.2.10 The effect of compatible solutes on growth of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) strains at high NaCl concentrations

The effect of compatible solutes on the growth of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) was evaluated. The experiment was conducted in highly saline M9 minimal medium media containing 1.5 M and 2 M NaCl at pH 9. The compatible solutes to be examined (betaine, ectoine and proline) were added to 50 ml of medium to give a final concentration of 1 mM. Control was medium without addition of compatible solutes.

H. pacifica (ABQ1) cells were grown at 2 M NaCl and *H. campisalis* (ABU3) cells were grown at 1.5 M NaCl. The experiment was conducted at 37°C on an orbital shaker (250 rpm), and cell growth was monitored by optical density measurements at 600 nm every hour throughout the day.

The results presented in Figures 5.20 and 5.21 show that all three compatible solutes have a positive effect on the growth of *H. pacifica* and *H. campisalis* with betaine and ectoine having a greater effect than proline. The positive effect of betaine and ectoine on the growth of halotolerant and moderately halophilic bacteria has been described in a number of previous studies (Lai and Gunsalus, 1992, Robert et al., 2000, Le Rudulier et al., 1984, Kempf and Bremer, 1998).

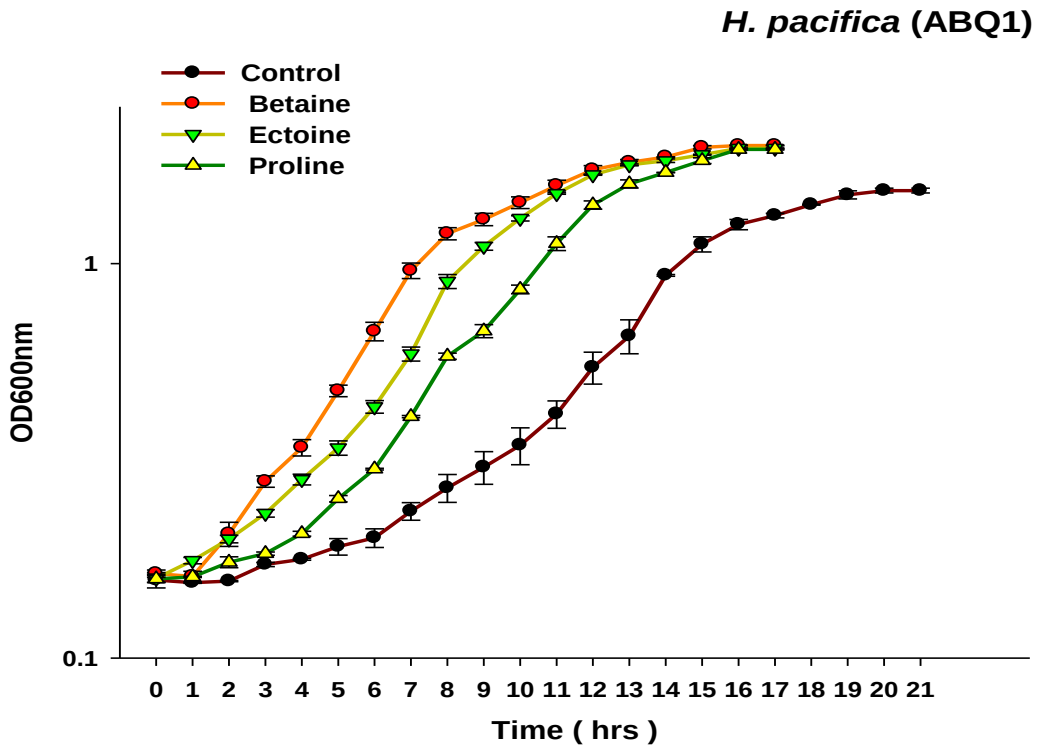


Figure 5-21 Effect of three different compatible solutes (final concentration 1 mM) on the growth of *H. pacifica* (ABQ1) on M9 minimal salt medium with 2 M NaCl. Control without addition of compatible solutes. Data represented are means of triplicate experiments with standard error. Optical density (OD_{600}) of growth curves was measured every hour during the day using spectrophotometer.

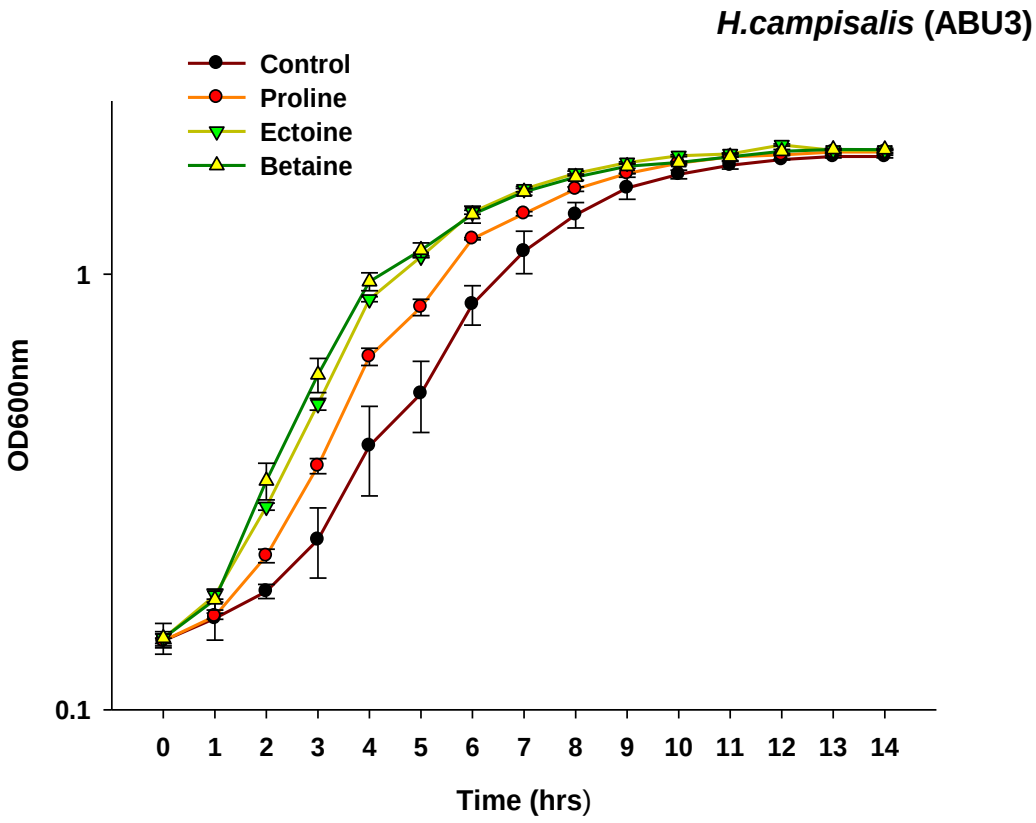


Figure 5-22 Effect of three different compatible solutes (final concentration 1 mM) on growth of *H. campisalis* (ABQ1) on M9 minimal salt medium with 1.5 M NaCl. Control without addition of compatible solutes. Data represented are means of triplicate experiments with standard error. Optical density (OD_{600}) of growth curves was measured every hour during the day using spectrophotometer.

5.2.11 Effect of compatible solutes on respiration rate

Determination of the effect of compatible solutes on respiration rates of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) was conducted on M9 media containing 1 mM betaine, ectoine and proline, at salt concentrations of 2 and 1.5 M NaCl respectively. The results in Figure 5.22 showed that a dramatic stimulation of the respiratory rate was observed with *H. pacifica* (ABQ1) when 1 mM betaine was present at asalt concentration 2 M NaCl. The effects of the addition of ectoine and proline were positive, but were much less significant than the betaine effect. The situation was slightly different for *H. campisalis* (ABU3) grown at 1.5 M NaCl (Figure 5.23). Once again betaine was highly stimulatory, but for *H. campisalis* (ABU3) ectoine was equally effective. The addition of proline gave a small positive effect on the rate of respiration.

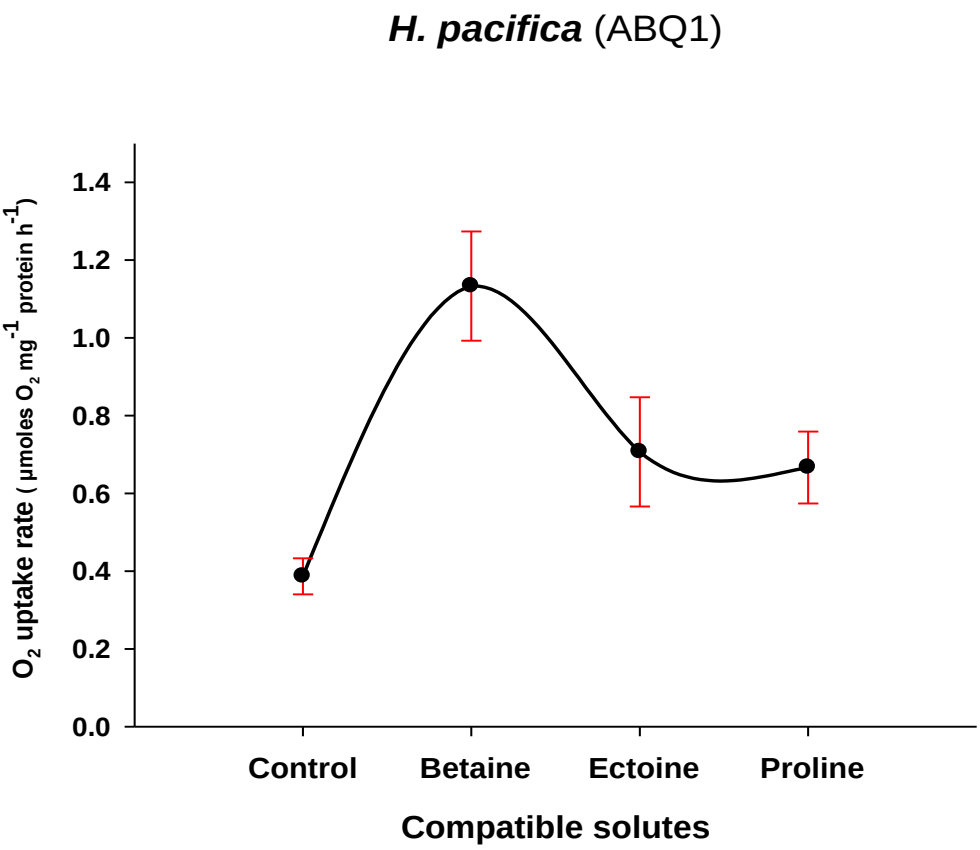


Figure 5-23 Effect of compatible solutes (1 mM) on O_2 uptake by *H. pacifica* (ABQ1) grown in M9 minimal medium with Glucose as sole source of carbon at 2 M NaCl. Control without addition of compatible solutes. Data represented are means of triplicate experiments with standard error.

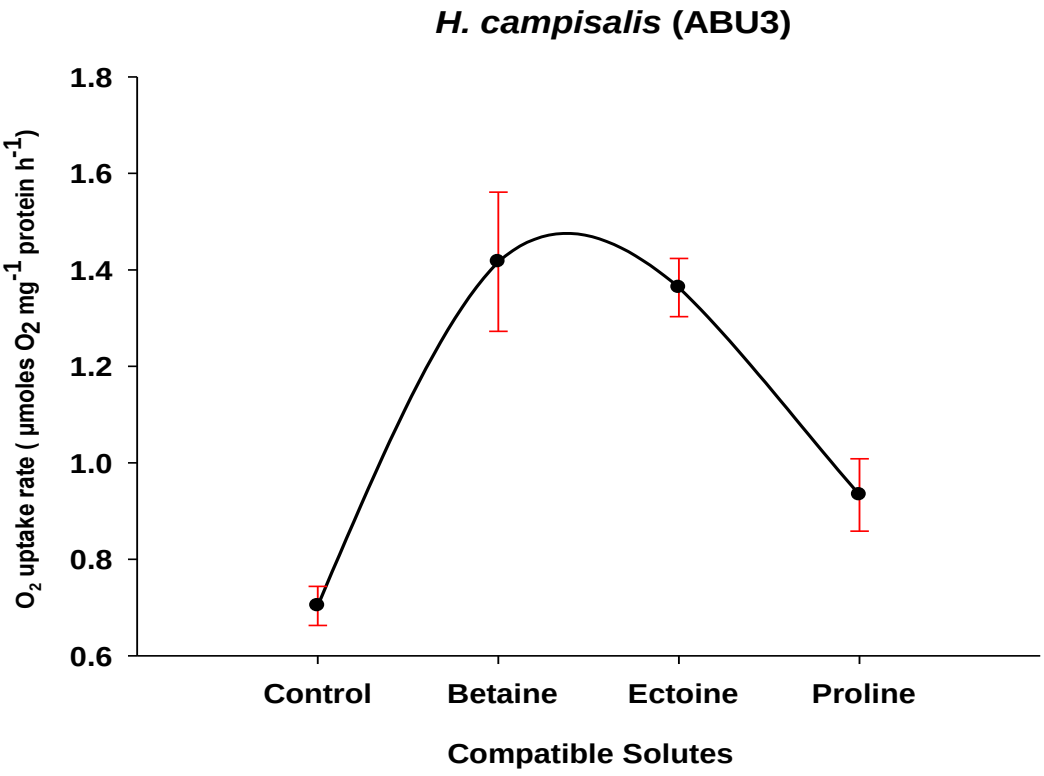


Figure 5-24 Effects of compatible solutes (1 mM) on O2 uptake by *H. campisalis* (ABU3) grown in M9 minimal medium with glucose as sole source of carbon at 1.5 M NaCl NaCl. Control without addition of compatible solutes. Data represented are means of triplicate experiments with standard error.

5.2.12 Compatible solutes analysis using NMR

Nuclear magnetic resonance spectroscopy (NMR) has become a routine analytical method used to identify the organic compatible solutes accumulated by halotolerant and halophilic microorganisms. The aim of this experiment was to investigate the accumulation of intracellular compatible solutes of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3), and to monitor the influence of different NaCl concentrations on the accumulation of the compatible solutes in LB and M9 minimal salt medium. The experimental method was described in section 2.18.1.

The results in Figures 5.25 and 5.27 showed that the intracellular content of betaine was detected at low concentrations of external NaCl in both *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) grown in rich LB medium, and increased with increasing salinity up to 2.5 M NaCl. However, ectoine was only detected in the cell extracts of *H. pacifica and H. campisalis* cells grown under high salt conditions in LB medium (2.5 M NaCl).

Figures 5.26 and 5.28 showed that ectoine was the predominant compatible solute accumulated when both strains were grown in M9 minimal medium. There is a clear relationship between the salt concentration in M9 medium and the intracellular content of ectoine in the cells. The amount of ectoine increased significantly with increasing salinity of the medium to a maximum at 2.5 M. *H. pacifica* and *H. campisalis* also accumulated hydroxyectoine when the cells were grown in M9 medium containing 2 and 2.5 M NaCl. The accumulation of hydroxyectoine was delayed when compared with that of ectoine.

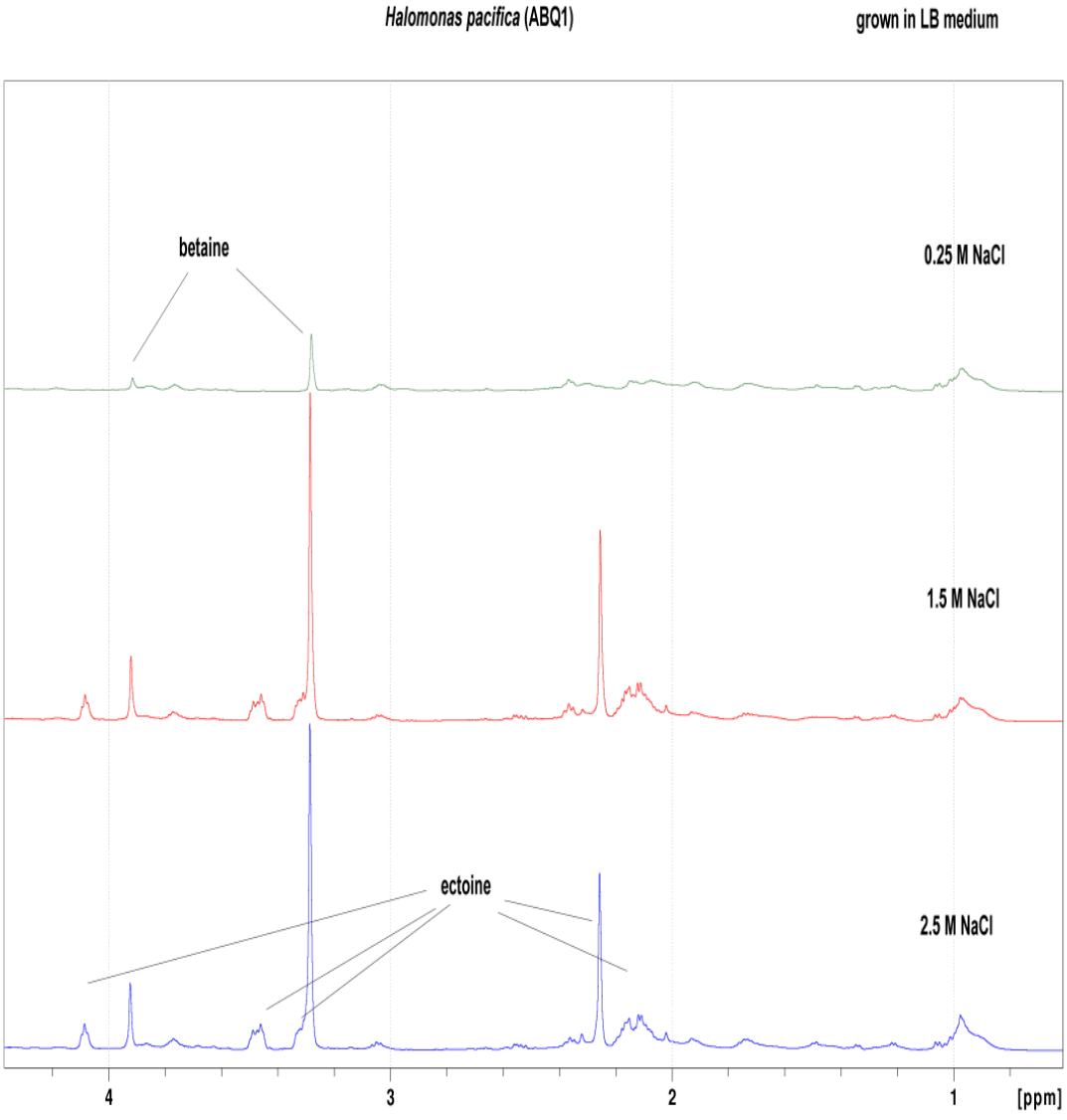


Figure 5-25 ^{13}C – NMR spectra of *Halomonas pacifica* (ABQ1) grown in LB medium at different concentrations of NaCl showing presence of betaine and ectoine.

In contrast, when *H. pacifica* is grown in M9 minimal medium, no betaine is accumulated with increasing salinity. Instead, ectoine is the dominant compatible solute and above 2 M NaCl, hydroxyectoine is also present (Figure 5.26)

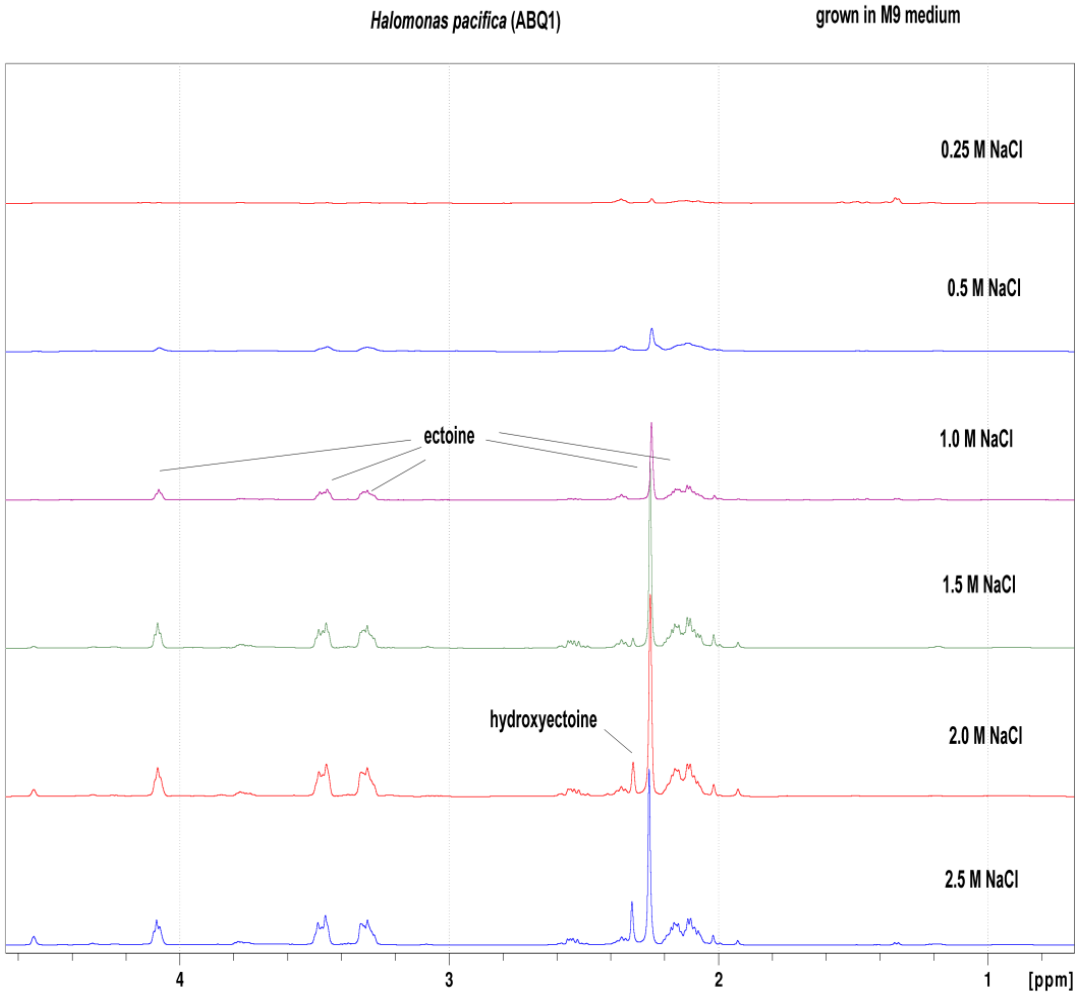


Figure 5-26 ^{13}C – NMR spectra of *Halomonas pacifica* (ABQ1) grown in M9 minimal salt medium at different concentrations of NaCl showing presence of ectoine and hydroxyectoine.

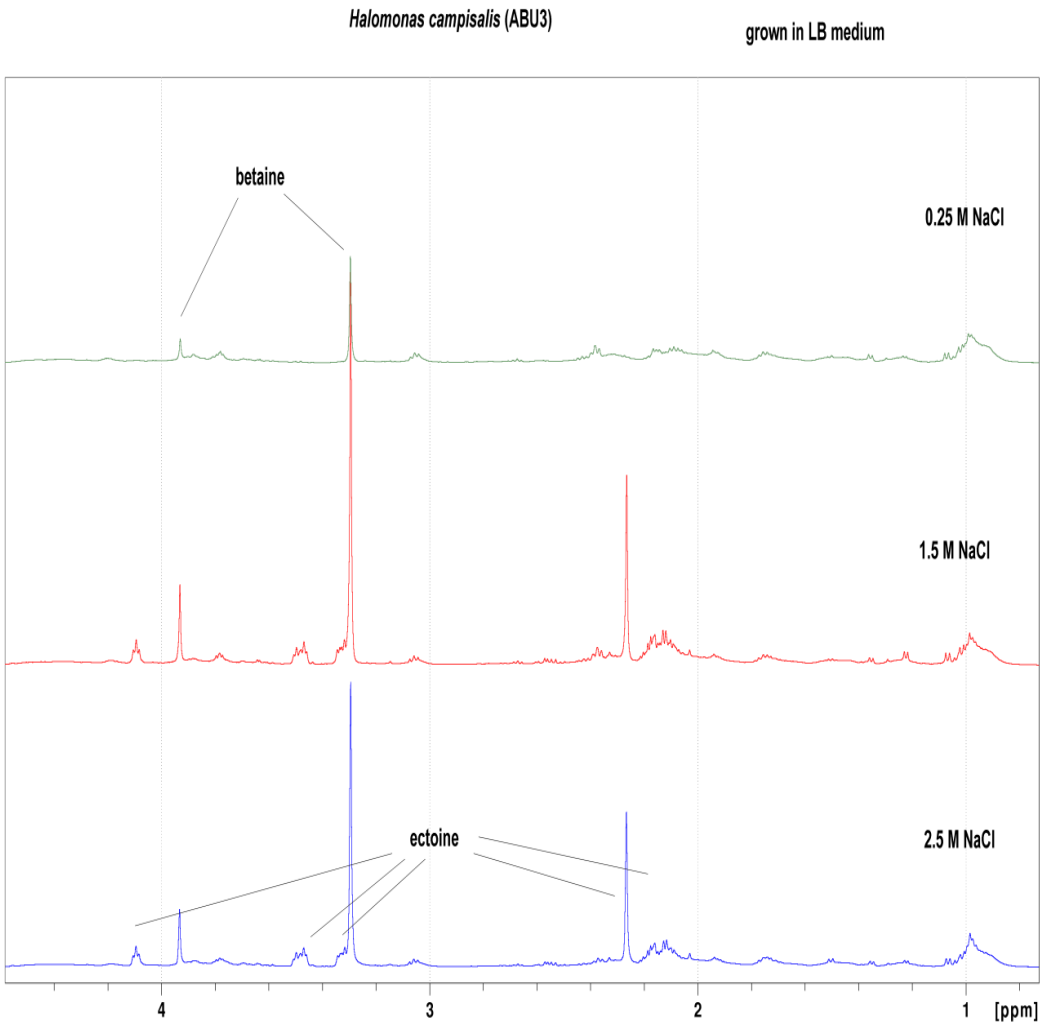


Figure 5-27 ^{13}C – NMR spectra of *Halomonas campisalis* (ABU3) grown in LB medium at different concentrations of NaCl showing presence of betaine and ectoine.

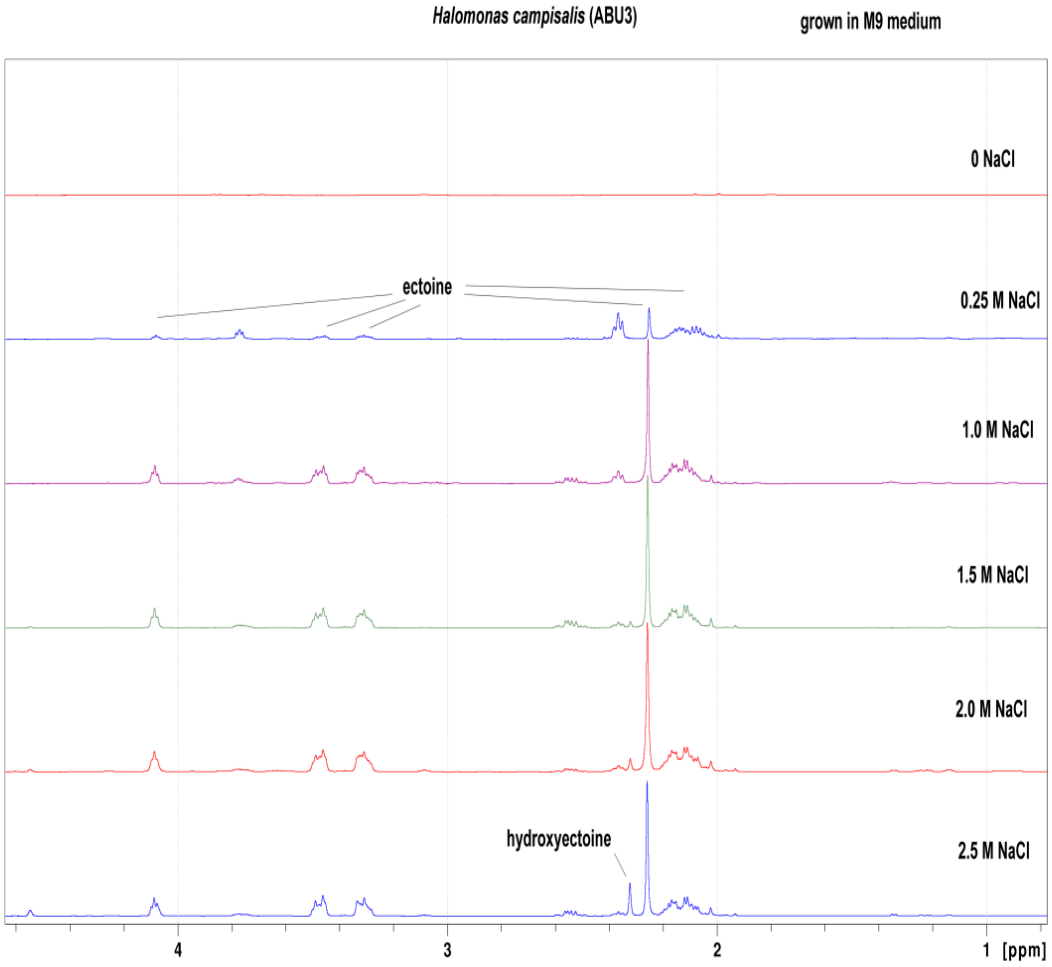


Figure 5-28 ^{13}C – NMR spectra of *Halomonas campisalis* (ABU3) grown in M9 minimal salt medium at different concentrations of NaCl showing presence of ectoine and hydroxyectoine.

5.2.13 Determination of compatible solutes in betaine grown cells

^{13}C - NMR spectroscopy was used to determine which compatible solutes are being accumulated in the cytoplasm of betaine grown cells at 0.5 and 2 M NaCl. *H. pacifica* (ABQ1) and *H.campisalis* (ABU3) were pre-adapted for two days in M9 minimal medium containing 17 mM betaine as sole source of carbon.

It is clear from the data in Figures 5.28 and 5.29 that both betaine and ectoine were accumulated *when H. pacifica* (ABQ1) *and H. campisalis* (ABU3) were grown at 0.5 and 2 M NaCl with betaine as the sole carbon source. The relative amounts of ectoine production compared to betaine production were calculated from these results. For *H. pacifica* (ABQ1) 8.5% and 43% ectoine were accumulated at 0.5 and 2 M NaCl, respectively. The amounts of ectoine accumulated by *H. campisalis* (ABU3) were 33% and 36% at 0.5 and 2 M, respectively. Thus for *H. campisalis* (ABU3), the relative amounts of ectoine and betaine do not change depending on salt concentration. However for *H. pacifica* (ABQ1), at low salt it uses betaine almost exclusively as the osmolyte, but at higher salt it uses an increasing proportion of ectoine.

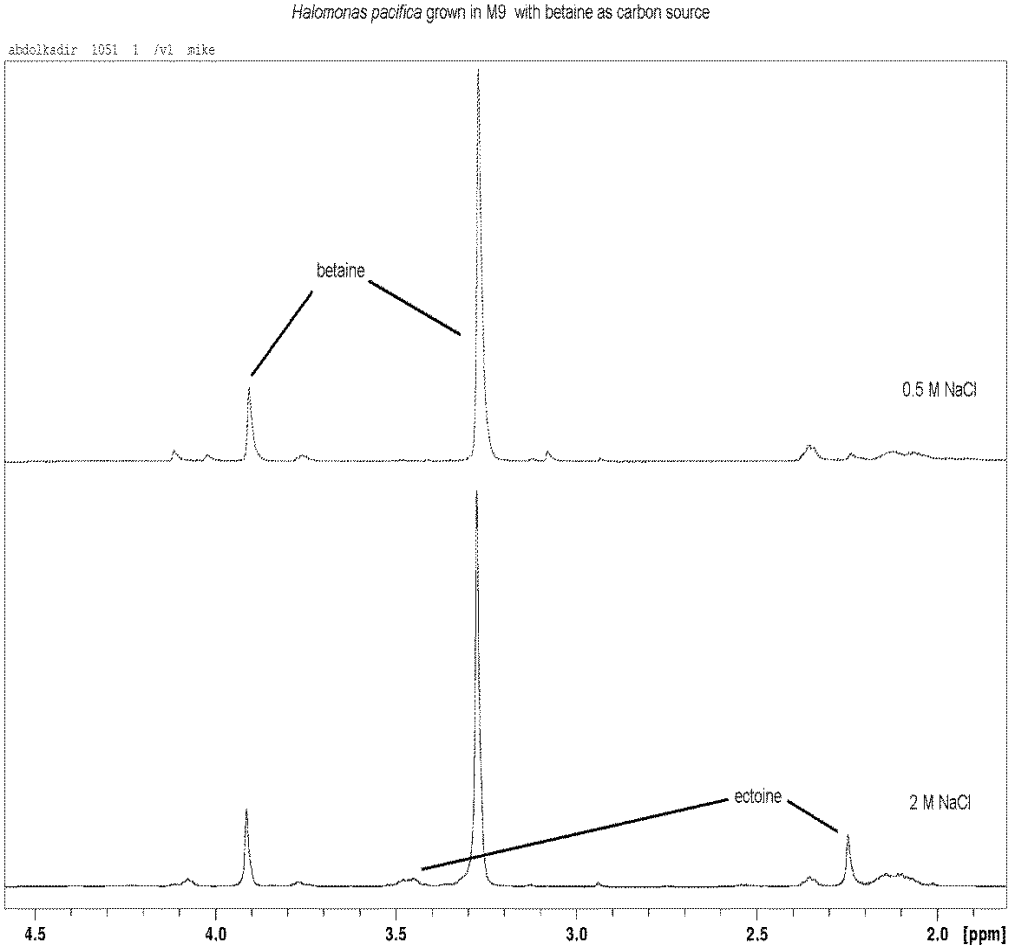


Figure 5-29 ^{13}C - NMR spectra of *Halomonas pacifica* (ABQ1) grown in M9 minimal salt medium at 0.5 and 2 M NaCl with 17 mM betaine as sole carbon source showing presence of betaine and ectoine.

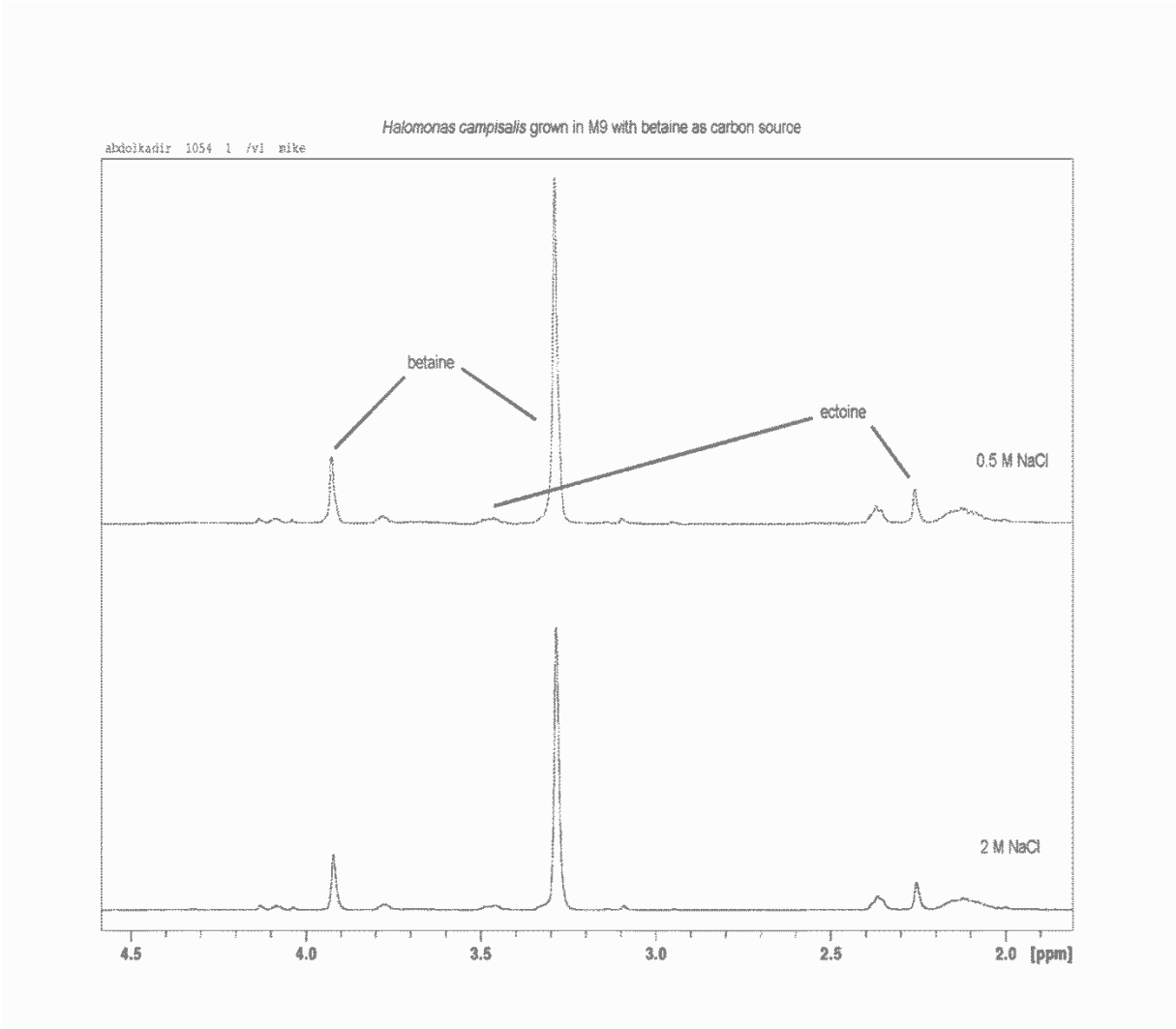


Figure 5-30 ^{13}C - NMR spectra of *Halomonas campisalis* (ABU3) grown in M9 minimal salt medium at 0.5 and 2.5 M NaCl with 17 mM betaine as sole carbon source showing presence of betaine and ectoine.

5.3 Conclusions

H. pacifica and *H. campisalis* were chosen for further study because neither has been well characterized. In particular, *H. pacifica* has only been mentioned in one publication in the title of Suzuki et al. (1991). All physiological characterization experiments (except some of the NMR studies) of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) were performed in M9 minimal salt medium.

The data in Figure 5.5 shows that *H. pacifica* (ABQ1) is a moderate halophile with optimum growth at 0.5 M NaCl in M9 minimal medium (Ventosa et al., 1998b, Kushner, 1978). In contrast, *H. campisalis* (ABU3) is not a halophile, growing well in the absence of NaCl with optimum growth between 0.25 and 0.5 M NaCl, it also has a lower maximum salinity for growth than *H. pacifica* (ABQ1) (Figure 5.5*).* Previous studies have been reported that *H. campisalis* (ABU3) grew aerobically over a wide range of salinities from 0 - 4.4 M NaCl, with optimum growth observed at 0.3 M NaCl (Aston and Peyton, 2007).

H. pacifica (ABQ1) and *H. campisalis* (ABU3) are facultative anaerobes able to grow at temperatures from 25 - 37°C with optimum growth at 37°C (Figures 5.7 and 5.8). *H. pacifica* (ABQ1) showed a good growth in the pH range from 7 to 8, with optimal pH for growth at 9 (Figure 5.6). The major characteristic of *H. campisalis* is the ability to grow well at high pH values (Figure 5.6). The growth rate initially increased with pH, forming a stable growth rate from pH 7 to 10 for *H. campisalis* (ABU3). The alkaliphilic nature of *H. campisalis* was described by (Mormile et al., 1999) and my results are in agreement. The fact that *H. pacifica* also grows well at pH 9 indicates that both species are well adapted to grow in the Qabar-oun and Um-alma lakes.

Results of Biolog GN2 96 well microplate tests showed that *H. pacifica* (ABQ1) and *H. campisalis* (ABU3 that isolated from Qabar-oun and Um-Alma lakes were able to utilize a wide range of carbon substrates. This is a well-known characteristic of the genus *Halomonas* and helps confirm that, these strains belong to the genus *Halomonas.* Table 5.4 and 5.5 show the comparison between the Halomonas strains isolated in the present study (*H. pacifica* (ABQ1) and *H. campisalis* (ABU3)) with previously published data from Mata et al. (2002). It is clear that the strains are every closely to the previously published strains and any differences in the Biolog tests are probably due to the different minimal/basal media used.

Table 5.4 Phenotypic characteristics of type strains of species of the genus *Halomonas,* (A) *Halomanse pacifica* (ABQ1) NC000366; (B) *Halomonas pacifica* ATCC 27122; *(C) Halomonas campisalis (ABU3)* 17956 ;*(D) Halomonas campisalis* ATCC 700597.

Characteristics	A	B	C	D
Morphology	Rod	Rod	Rod	Rod
pigmentation	cream	cream	white	White
Motility	+	+	+	+
Facultative anaerobic	+	-	+	-
Growth temperature (°C)	25 - 37	4 - 45	25 - 37	4 - 50
pH range	5.5 - 9	5 -10	5.5 - 10	8 -11
NaCl Salt range (M)	0.05 - 2.5	0 -3.4	0 - 2.5	0.08 - 2.5
Susceptibility to:				
Tetracycillin (50 µg)	+	ND	+	ND
Ampicillin (25 µg)	+	+	-	+
Carbenicillin (100 µg)	+	+	+	+
Kanamycin (30 µg)	+	+	-	-
Erythromycin (15 µg)	-	-	+	+
Rifampicin (30 µg)	+	+	+	+
Cefoxitin (30 µg)	+	+	+	+
Nalidixic acid (30 µg)	+	ND	-	ND

- Halomonas pacifica (ABQ1) isolated from Qaber-oun lake, and *Halmonas campisalis* (ABU3) isolated from Um-Alma lake, south Libyan sahara
- ND indicates that the test not determined.
- M9 minimal salt medium was used for phenotypic test for *Halomonas pacifica* (ABQ1), and *Halomanse campisalis* (ABU3).
- Basal medium (minimal medium) was used for phenotypic tests of *Halomonas pacifica* ATCC 27122, and *Halomonas campisalis* ATCC 700597.

Table 5.5 Phenotypic characteristics (BIOLOG system) of type strains of species of the genus *Halomonas*: 1, *H. pacifica* (ABQ1); 2, *H. pacifica* ATCC 27122; 3, *H. campisalis* (ABU3); 4, *H. campisalis* ATCC 700597.*H. pacifica* (ABQ1) and *H. campisalis* (ABU3) score > 1= (+), score > **0.5 – 1= (+/-),** score ≤ **0.5 = (-).** In contrast**,** *H. pacifica* ATCC 27122 and *H. campisalis* ATCC 700597 were characterized as score ≥ 0.5 = (+).

Characteristics	**1**	**2**	**3**	**4**
Water	-	-	-	-
α -Cyclodextrin	-	+	-	-
Dextrin	+	+	+	-
Glycogen	-/+	+	-/+	-
Tween 40	-/+	+	-/+	-
Tween 80	-/+	+	-/+	-
N-Acetyl-D-Galactosamine	-	-	-	-
N-acetyl-D-Glucosamine	+	+	+	-
Adonitol	-	-	-	-
L-Arabinose	-	-	-	-
D-Arabitol	-	-	-	-
D-Cellobiose	-	-	-	-
i-Erythritol	-	-	-	-
D-Fructose	+	+	+	-
L-Fucose	-	-	-	-
D-Galactose	-	-	-	-
Gentiobiose	-	+	-	-
α- D-Glucose	+	+	+	-
m-Inositol	-	-	-	-
α- D-Lactose	-	-	-	-
Lactulose	-	-	-	-
Maltose	+	+	+	-
D-Mannitol	-	-	-	-
D-Mannose	-	+	-	-

Table 5.5 Continued.

Characteristics	1	2	3	4
D-Melibiose	-	-	-	-
β-methyl-D-Glucoside	-	-	-	-
D-Psicose	+	+	+	-
D-Raffinose	-	+	-	-
L-Rhamnose	-	+	-	-
D-Sorbitol	-	-	-	-
Sucrose	+	+	+	-
D-Trehalose	+	+	+	-
Turanose	+	+	+	-
Xylitol	-	-	-	-
Pyruvic Acid Methyl Ester	+	+	+	-
Succinic Acid Mono-Methyl-Ester	-/+	+	-/+	-
Acetic Acid	+	+	+	-
Cie-Aconitic Acid	+	+	+	-
Citric Acid	+	+	+	-
Formic Acid	-	-	-	-
D-Galactonic Acid Lactone	-	-	-	-
D-Galacturonic Acid	-	-	-	-
D-Gluconic Acid	+	+	+	-
D-Glucosaminic Acid	-	-	-	-
D-Glucuronic Acid	-	-	-	-
α-Hydroxybutyric Acid	-	+	-	-
β-Hydroxybutyric Acid	+	ND	+	ND
y-Hydroxybutyric Acid	-	-	-	-

Table 5.5 Continued.

Characteristics	1	2	3	4
p-Hydroxy Phenylacetic Acid	+	-	+	-
Itaconic Acid	-/+	+	-	-
α-Keto Butyric Acid	-	ND	-	ND
α-Keto Glutaric Acid	+	-	+	-
α-Keto Valeric Acid	-		-	
D,L-Lactic Acid	+	+	+	-
Malonic Acid	-	+	-	-
Propionic Acid	+	-	+	-
Quinic Acid	-	+	-	-
D-Saccharic Acid	-	+	-	-
Sebacic Acid	-	+	-	-
Succinic Acid	+	-	+	-
Bromosuccinic acid	+	+	+	-
Succinamic Acid	-/+	+	-/+	-
Glucuronamide	-	+	-	-
L-Alaninamide	-/+	-	-/+	-
D-Alanine	+	-	+	-
L-Alanine	+	+	+	-
L-Alanyl-glycine	-/+	+	-/+	-
L-Asparagine	+	+	+	-
L-Aspartic Acid	-/+	-	-/+	-
L-Glutamic Acid	+	+	+	-
Glycyl-L-Aspartic Acid	-	-	-	-
Glycyl-L-Glutamic Acid	-/+	-	-/+	-

Table 5.5 Continued.

Characteristics	1	2	3	4
L-Histidine	+	+	+	-
Hydroxy-L-Proline	-	+	-	-
L-Leucine	-/+	-	-/+	-
L-Omithine	-/+	-	-/+	-
L-Phenylalanine	+	+	+	-
L-Proline	+	+	+	-
L-Pyroglutamic Acid	+	+	+	-
D-Serine	-	-	-	-
L-Serine	+	+	+	-
L-Threonine	-	+	-	-
D,L-Carnitine	-	-	-	-
y-Amino Butyric Acid	-/+	+	-/+	-
Urocanic acid	+	+	+	-
Inosine	+	+	+	-
Uridine	+	+	+	-
Thymidine	-	-	-	-
Phenyethyl-amine	+	+	+	-
Putrescine	+	+	+	-
2-Aminoethanol	-	-	-	-
2,3-Butanediol	-/+	+	-/+	-
Glycerol	+	+	+	-
D,L-α-Glycerol Phosphate	-/+	-	-/+	-
α-D-Glucose-1-Phosphate	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-

The data in Figures 5.23 and 5.24 show that the addition of betaine and stimulated the respiration rates of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) cells grown at high salt concentrations (1.5 and 2 M NaCl, respectively). Thus it seems likely that these well-known compatible solutes were involved in the physiological mechanisms that allow *H. pacifica* (ABQ1) and *H. campisalis* (ABU3). To test this theory, NMR analysis experiments were used to investigate the accumulation of compatible solutes by *H. pacifica* (ABQ1) and *H. campisalis* (ABU3), and to monitor the influence of NaCl concentrations of accumulation of compatible solutes in LB rich medium and M9 minimal medium. Figures 5.25 and 5.27 show accumulation of compatible solutes by *H. pacifica* (ABQ1) and *H.campisalis* (ABU3), respectively, grown in LB medium. The NMR spectra shows accumulation of intercellular betaine at low salinity and the concentration of betaine increased with increasing salinity to a maximum 2.5 M NaCl. Intracellular ectoine was only detected at high salinity (2.5 M NaCl) in LB medium for both strains.

Figures 5.26 and 5.28 represent accumulation of compatible solutes in both strains grown in M9 minimal medium, it is clear that there was no betaine detected in M9 medium, but, there was a clear relationship between accumulation of ectoine and NaCl concentration in M9 medium. Figure 5.26 revealed that intracellular ectoine in *H. pacifica* was detected at 1 M NaCl and increased significantly with increasing salinity to a maximum at 2.5 M NaCl, whereas *H. campisalis* (ABU3) accumulated ectoine at 0.25 M NaCl and it increased with increasing salinity. *H .pacifica* (ABQ1*)* and *H.campisalis* (ABU3) also accumulated hydroxyectoine when grown in M9 medium at 2 M and 2.5 M, respectively, but there was no accumulation of hydroxyectoine at lower salinities (Figures 5.26 and

5.28). In previous work, *H. campisalis* was observed to accumulate ectoine and glycine betaine at low and moderate salinity (Boltyanskaya et al., 2005). However, as shown by the results of the current work, accumulation of compatible solutes by microorganisms normally depends on the medium composition (Severin et al., 1992, Mojica et al., 1997).

The results in Figures 5.29 and 5.30 show that betaine can be utilized by *H. pacifica* (ABQ1) and *H.campisalis* (ABU3) as the sole source of carbon and energy. This provides the cells with a problem to solve when grow at high salinity with betaine as the sole source of carbon. Some of the betaine must be oxidised to provide energy and metabolic precursors, but some of the betaine needs to be used to balance the external osmotic potential. The results described here show that both *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) can solve this problem, but the relative amounts of ectoine produced by the two species varied wnhen they were grown using betaine a sthe sole carbon souirce. This suggests that the two species regulate their production of compatible solutes differently and this would be interesting to explore in further work.

CHAPTER 6

Further characterization of *H. campisalis* (ABU3)

6 Further characterization of *H. campisalis* (ABU3)

6.1 Introduction

Microorganisms can be found practically everywhere on the surface of the earth, and over a wide range of extreme environments (Cantrell et al., 2006). Alkaliphiles, halophiles and haloalkaliphile microorganisms have been commonly isolated from alkaline habitats and saline or hypersaline lakes (Sahay et al., 2011, Oren, 2002a, Horikoshi, 2004). In high salinity environments, halophilic microorganisms must balance the high osmotic potential across their membrane in order to thrive in such conditions (Aston and Peyton, 2007). The ability of halophilic and alkaliphilic bacteria to survive in high salt and high pH environments makes them candidates for industrial biotechnology and bioremediation applications (Margesin and Schinner, 2001b, Prabhu et al., 2004, Ventosa and Nieto, 1995, Detkova and Boltyanskaya, 2007). As a result of industrial activities and urban water effluents, saline and hypersaline environments are often contaminated with organic compounds (García et al., 2004). Furthermore, it is well known that industrial wastes containing high concentrations of nitrate create environmental problems to the water quality and human health (Foglar et al., 2005, Glass and Silverstein, 1999).

Remediation of contaminated natural water sources and hypersaline wastes by physical process is an urgent and costly issue (Okeke et al., 2002). Thus, bioremediation process considered as an attractive option because the technology is environmentally friendly and cost-effective (Okeke et al., 2002).

Bioremediation processes to remove nitrate have become widely used methods in the treatment of domestic and industrial wastewater (Delanghe, 1994, Dong and Tollner, 2003, Kesserű et al., 2003).

In addition, the identification of moderate halophilic bacteria is of great interest in the context of assessing acceleration of the environmental repair process for bioremediation in contaminated saline habitats (García et al., 2004). *Halomonas* species are well investigated and they are of interest in the decontamination of pollutants in saline environments (Berendes et al., 1996, García et al., 2004, Oren, 2002b, Yang et al., 2010). For example, *Halomonas campisalis* isolated from alkaline saline lake, Washington, U.S., was previously characterized as haloalkaliphile capable of surviving in denitrifying conditions (Mormile et al., 1999). Further studies have been reported that *H. campisalis* was shown to reduce nitrate with three carbon electron donors: lactate, acetate and glycerol, and to degrade toxic and other aromatic compounds under high pH and high salinity conditions (Peyton et al., 2001). Due to its ability to grow under denitrifying conditions by utilizing a wide range of carbon sources, and its high salinity and pH tolerance, this strain holds promise for the treatment of saline alkaline waste (Mormile et al., 1999, Oie et al., 2007).

Soda lakes contain dense populations of alkaliphilic bacteria and archaea (Jones et al., 1998). However, some organisms are unique to soda lakes such as the haloalkaliphiles found in Lake Magadi (Jones et al., 1998). Haloalkaliphilic organisms require both high pH value (pH 9 and above) and high salinity for growth. They are able to tolerate up to 4 M NaCl, but grow best from 0.5 – 3 M NaCl (Ventosa et al., 1998b, Horikoshi, 1999).

The purpose of this chapter was to further characterize the haloalkaliphic *H. campisalis* by growing the strain in minimal M9 medium containing different concentrations of nitrate and urea. In addition, the internal pH of the cells was measured under a range of growth conditions.

6.2 Results and Discussion

6.2.1 Effect of urea and nitrate concentrations on growth of *H. campisalis* (ABU3)

The effect of urea [CO $(NH_2)_2$] or nitrate (NO_3) as the sole source of nitrogen N was examined on the growth of *H. campisalis* (ABU3) using the methods described in section 2.19.1. Tolerance of this strain to urea and nitrate (in the form of $NaNO_3$) was assayed by determining the growth of cultures in NH_4 - free M9 minimal salt medium (Table 2.5) The range of concentrations of urea and nitrate used was from 2 mM to 16 mM.

Figure 6.1 show that *H. campisalis* (ABU3) was able to grow well aerobically in M9 minimal medium with up to 16 mM of urea or nitrate. The higher final biomass levels after overnight growth were observed with urea as the sole source of N, especially at the lower concentrations tested. There was also evidence that the final biomass decreased slightly at nitrate levels above 8 mM. However, when urea was the N source, the biomass levels were unaffected at high concentrations of urea (Figure 6.1). It should be kept in mind when interpreting these results that urea is also a source of carbon for cell growth.

Nitrate is considered to be the most abundant form of nitrogen in the biosphere after atmospheric dinitrogen, thus, a wide range of living organisms including higher plants algae, fungi and bacteria use it as source of N (Guerrero, 1981, Stewart, 1988). However, in biological treatment plants, high concentrations of nitrate usually inhibited cell growth because of the futile reduction of nitrate (NO_3) to nitrite (NO_2) (Krishnamachari, 1993).

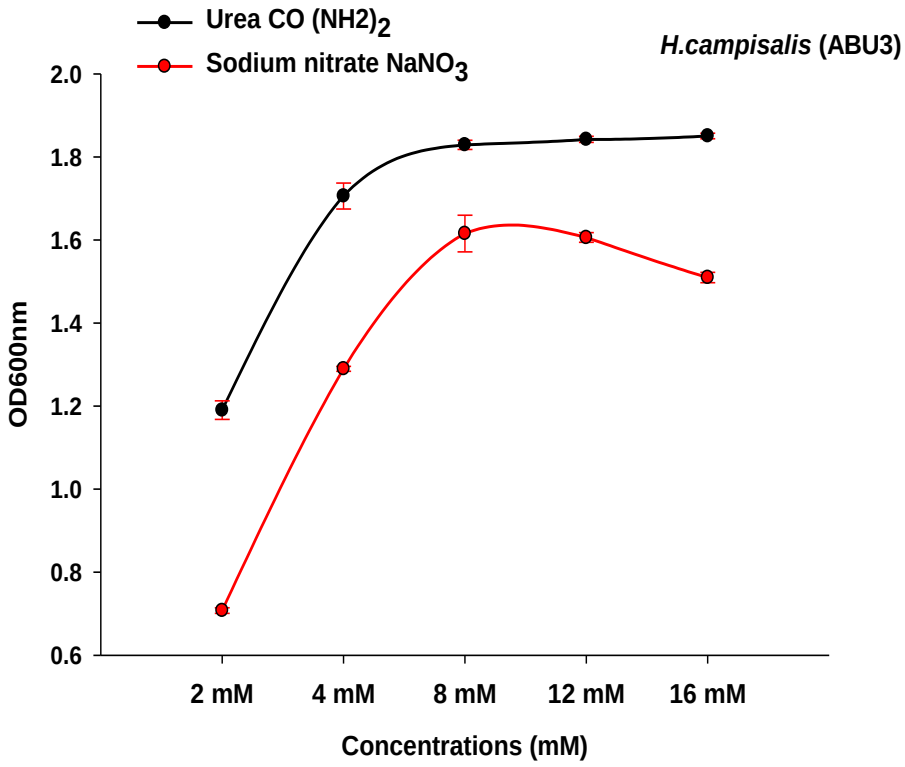


Figure 6-1 Growth curves of *H. campisalis* at different concentrations of urea [$CO(NH_2)_2$] and sodium nitrate ($NaNO_3$). Cells were grown in 0.5 M NaCl M9 minimal medium at pH 9, incubated in a 37°C constant temperature room on an orbital shaker at 250 rpm. The OD_{600} was measured after overnight growth. Data points are the means of triplicates and standard error.

6.2.2 Growth curves for *H. campisalis* (ABU3) with 8 mM sodium nitrate or 8 mM urea as the sole source of nitrogen

To further investigate the roles of urea and nitrate in the growth of *H. campisalis* (ABU3), growth curves were constructed for each N source at a concentration of 8 mM. Figure 6.2 shows that the growth rate was faster in the presence of urea, but the final OD_{600} was similar for the two nitrogen sources after 26 hours incubation. These data suggest that the main stimulatory effect of urea is as a good source of N. If urea was acting as an additional source of carbon, the final OD_{600} value would be expected to be higher in the presence of urea.

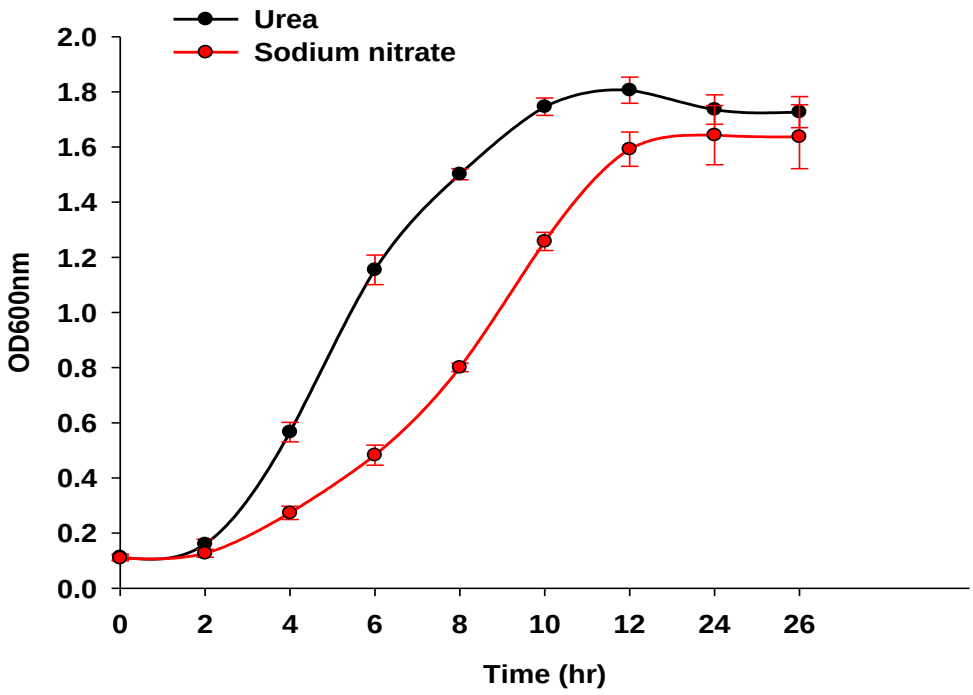


Figure 6-2 Growth curves for *H. campisalis* (ABU3) at 8 mM nitrate or 8mM urea using M9 medium containing 1.5 M NaCl, pH 9, at 37°C. The OD for growth was measured at 600 nm every two hours during the day. Data represent the means of triplicates and standard error.

6.2.3 Aerobic nitrate assimilation by *H. campisalis* (ABU3)

Experiments were conducted to investigate rate of aerobic nitrate removal from the medium by assimilation into the biomass of *H. campisalis* cells. M9 minimal salt medium (Table 2.5) was used with three different carbon sources; glucose, sodium lactate and sodium acetate as described in section 2.19.3. Note that the cells were incubated aerobically on a shaker for these experiments in line with previous growth experiments. Growth curve of *H. campisalis* in this experiment was measured using OD at 600 nm, and nitrate reduction was determined at 232 nm.

It can be seen from Figures 6.3 and 6.4 that glucose and lactate showed good nitrate uptake activity and biomass growth. Figure 6.5 indicates that when acetate was used as the carbon source the rate of nitrate uptake was slower. In all cases an increase in growth results in a decrease in nitrate concentration in the media, but for glucose and lactate grown cells, the nitrate level was close to zero after 14 and 24 hours respectively (Figures 6.3 and 6.4). For acetate grown cells, it was about 36 hours before the nitrate in the medium approached zero (Figure 6.5). .

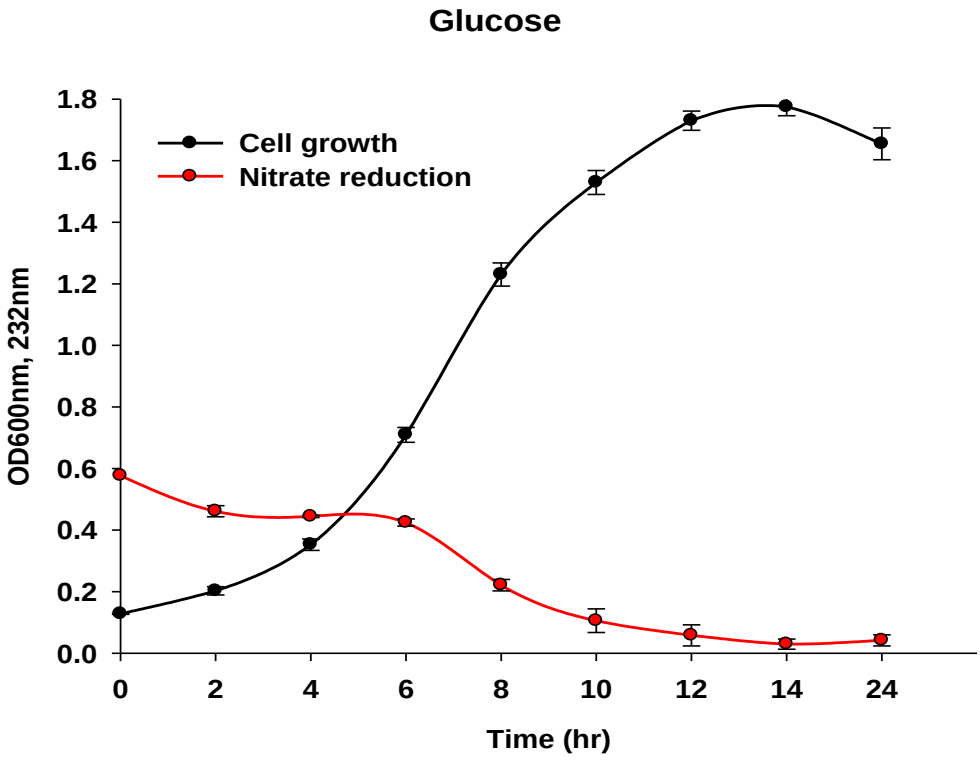


Figure 6-3 Growth curve and nitrate removal curve shown by *H. campisalis* (ABU3) using glucose as carbon source. Cells were grown in M9 minimal medium containing 8 mM $NaNO_3$, 1.5 M NaCl, at pH 9. The growth curve was measured every two hours under these conditions, and the disappearance of nitrate from the medium was measured in supernatant (OD at 232 nm) by spinning down cells after measuring growth rate (OD at 600 nm). Data show the means and standard error of triplicates.

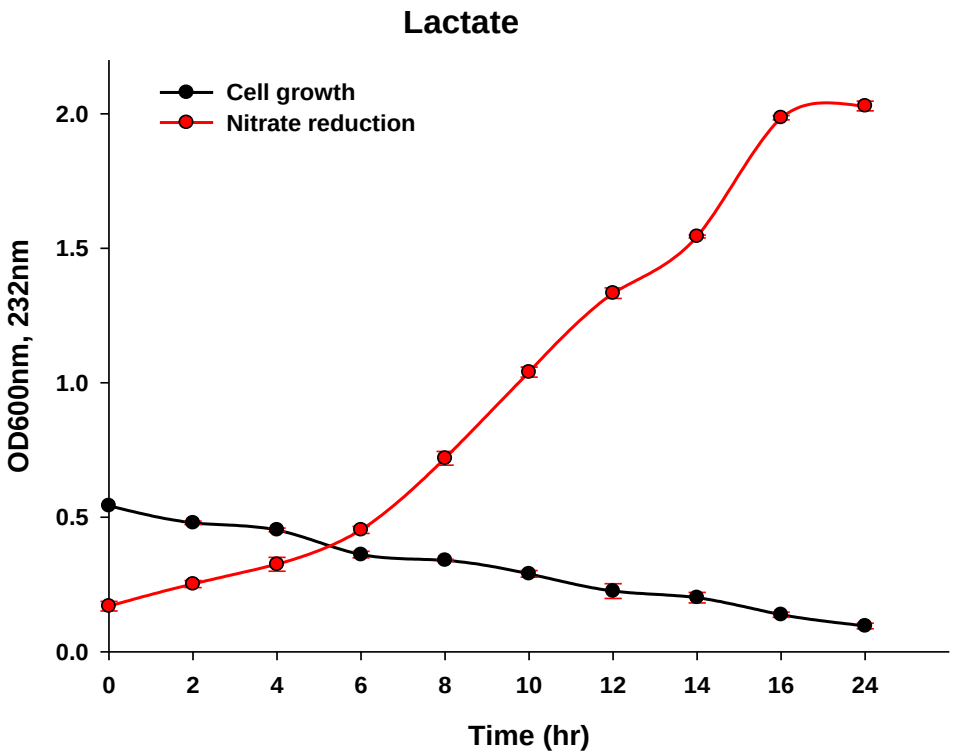


Figure 6-4 Growth curve and nitrate removal curve shown by *H. campisalis* (ABU3) using lactate as carbon source. Cells were grown in M9 minimal medium containing 8 mM NO_3, 1.5M NaCl, at pH 9. The growth curve was measured every two hours under these conditions, and the disappearance of nitrate from the medium was measured in supernatant **(**OD at 232 nm**)** by spinning down cells after measuring growth rate (OD at 600 nm). Data show the means and standard error of triplicates.

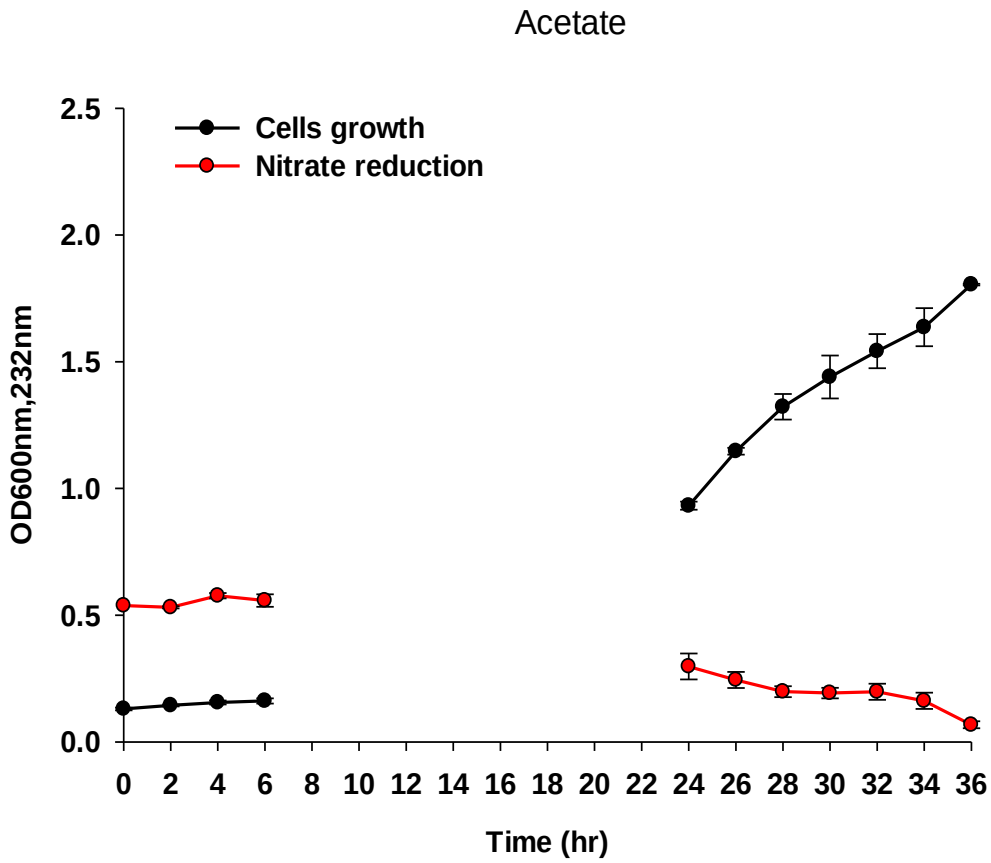


Figure 6-5 Growth curve and nitrate removal curve shown by *H. campisalis* (ABU3) using acetate as carbon source. Cells were grown in M9 minimal medium by containing 8 mM NO_3, 1.5M NaCl, at pH 9. Measurements were made for the first six hours after inoculation and between 24 and 36 hours after inoculation. Data represent the means of triplicates and standard error.

6.2.4 Intracellular volume of *H. campisalis* (ABU3)

Since *H. campisalis* is a haloalkaliphile, it is interesting to measure the internal pH and membrane potential of the cells when grown at high pH levels. The first step in this process is to measure the intracellular volume of the cells. Determination of intracellular volume of bacterial cells was measured by centrifugation through silicon oil as described in methods section 2.20. The presence of the silicone oil is used to completely separate the cell pellets from their external media. Determination of both the total pellet volume (PV) using tritiated water (^3H_2O) and extracellular volume in the pellet (ECV) using ^{14}C - dextran allows the calculation of the intracellular volume of the pellet (ICV) using the equations given in Hard and Gilmour (1996) – see section 2.20.1. This information can then be combined with other radioactive probes to determine the membrane potential (^3H-TPP) and internal pH level (^{14}C - methylamine). More details are provided in sections 2.20.3 and 2.20.4.

In order to measure intracellular volume based on the distribution of isotopes, a time course experiment must first be performed for ^{14}C - dextran uptake (using *H. campisalis* 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 an incubation period of up to 10 minutes was suitable for measuring the pellet volume (PV), extracellular volume (ECV) and determination of the subsequent intracellular volume (ICV) (Figures 6.7 and 6.8).

After 20 minutes incubation, there was evidence of active further uptake of ^{14}C - dextran, which is not desirable. The measurement of intracellular volume (ICV µl mg^{-1} protein) of *H. campisalis* which was grown in M9 minimal salt medium at 0.5 M NaCl and pH 9 was found to be 1.08 ± 0.5 µl mg^{-1} proteins. The results represent means plus/minus standard error for three replicates.

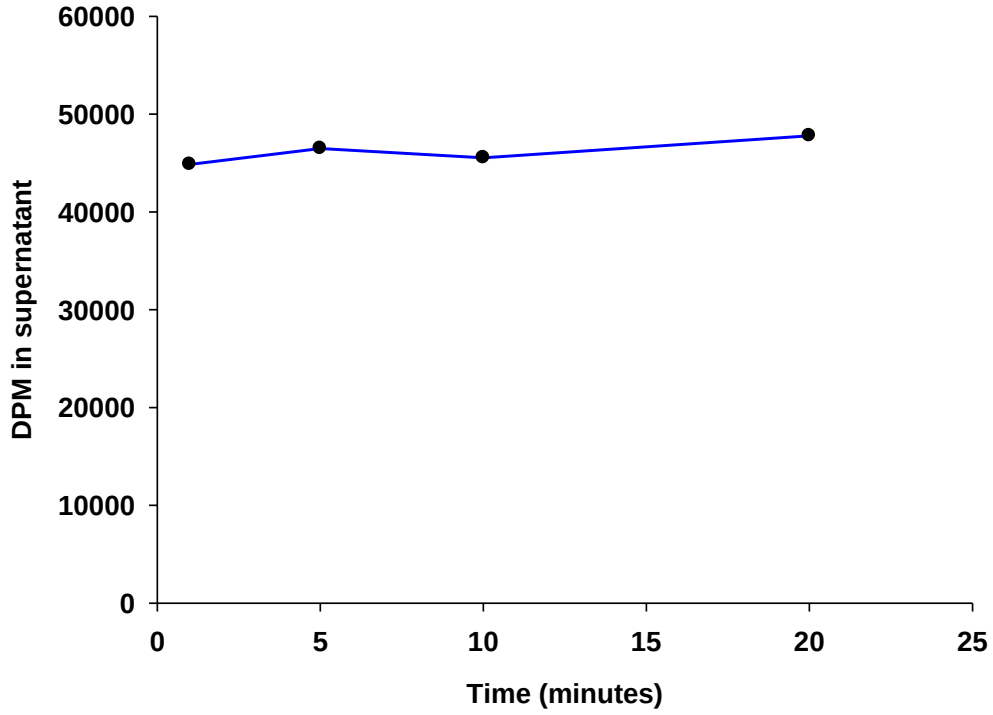


Figure 6-6 Time course of ^{14}C - dextran uptake by *H. campisalis* (ABU3). DPM in supernatant was measured after 1, 5, 10 and 20 minutes incubation periods. Cells were grown overnight in M9 minimal salt medium at 0.5 M NaCl and pH 9. Data represent means of three replicates and standard error.

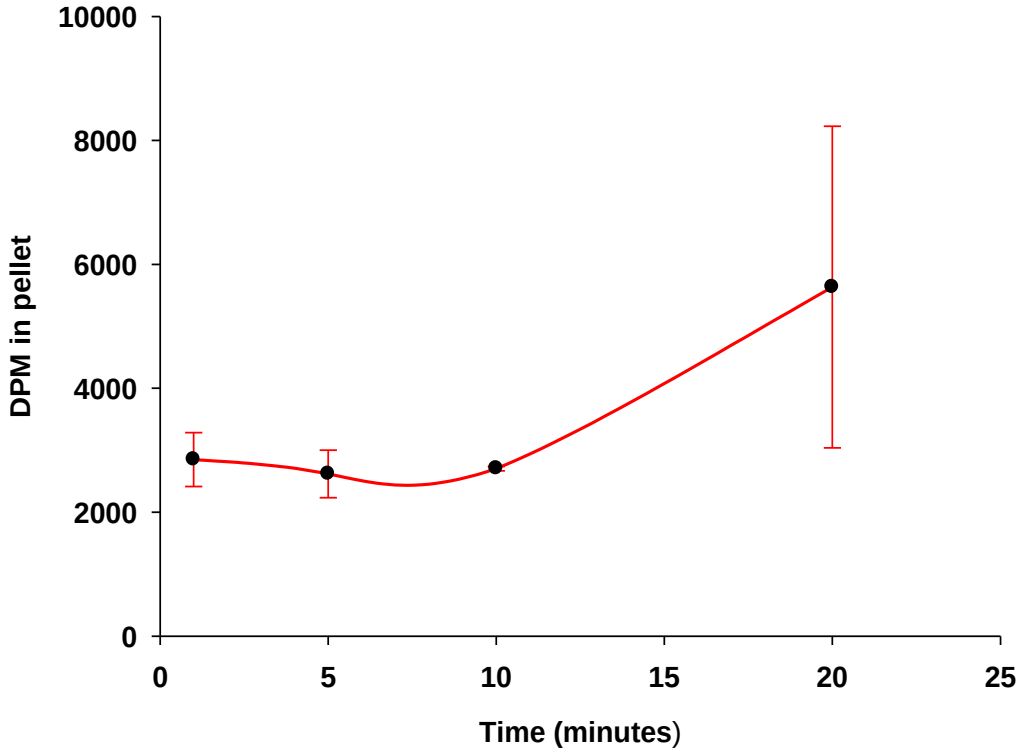


Figure 6-7 Time course of ^{14}C - dextran uptake by *H. campisalis* (ABU3). DPM in pellet was measured after 1, 5, 10 and 20 minutes incubation period. Cells were grown overnight in M9 minimal salt medium at 0.5 M NaCl and pH 9. Data represent means of three replicates and standard error.

6.2.5 Determination of membrane potential (ΔΨ) of *H. campisalis* (ABU3)

Membrane potential ΔΨ (inside negative) is defined as the electrical potential difference that would be measured between two identical reference electrodes positioned on opposite sides of a cell membrane. Bacterial cells when exposed to an external stress need to maintain the internal osmotic balance of their cytoplasm. Therefore, it is very important to study the membrane potential and internal pH of cells in relation to change in external pH.

Determination of membrane potential ΔΨ (inside negative) of *H. campisalis* was performed using an indirect method involving ^{3}H-TPP (tetraphenylphosphonium) as a probe and for calculation of (ΔΨ), using the silicone oil centrifugation technique as described by (Rottenberg, 1979, Rottenberg, 1989). An incubation period of 5 minutes was suitable and used for membrane potential determination.

The measurement of (ΔΨ) was carried out as a function of the external pH for *H. campisalis* (ABU3) cells grown at pH 9 in M9 minimal salt medium. It was found that *H. campisalis* (ABU3) showed a membrane potential of -123.3 mV at pH 9.

6.2.6 Determination of internal pH (pH_i) and Δ pH of *H.campisalis* (ABU3)

Determination of internal pH (cytoplasmic pH) was performed using the silicone oil centrifugation method as described by (Rottenberg, 1979) (section 2.20) to separate cell pellets from their external media allowing the measurement of both intracellular and extracellular volumes of cells. In this experiment, 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 when the cytoplasmic pH is lower than the external pH (section 2.20.4). In this study, the labelled radioisotope probe ^{14}C - methylamine, with a pK of 10.6, was used to measure internal pH of cells of *H. campisalis* (ABU3) grown in M9 minimal salt medium at pH 9.

The time course of ^{14}C - methylamine probe uptake was carried out using *H. campisalis* (ABU3) cells in order to ensure that there was no active uptake or efflux of isotope after the initial distribution between cells and medium. The results indicate that a 5 minute incubation period was suitable for determination of cytoplasmic pH (Figures 6.9 and 6.10).

For *H. campisalis* (ABU3) cells grown in M9 minimal medium at pH 9 the internal pH (pH_i) was found to be 8.79 ± 0.01 (means and standard error for three replicates), thus the ΔpH was minus 0.21. The results indicate that the cells of *H. campisalis* (ABU3) exhibited an internal pH value very close to the external pH.

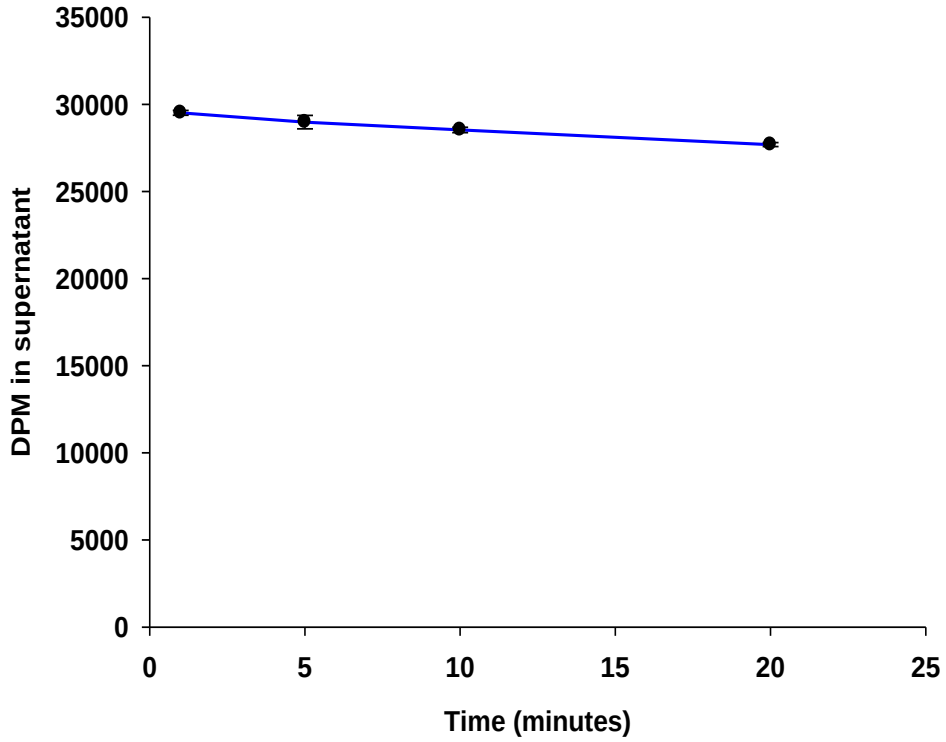


Figure 6-8 Time course of ^{14}C - methylamine uptake by *H. campisalis* (ABU3). DPM in supernatant was measured after 1, 5, 10 and 20 minutes incubation periods. Cells were grown overnight in M9 minimal salt medium at 0.5 M NaCl and pH 9. Data represent means of three replicates and standard error.

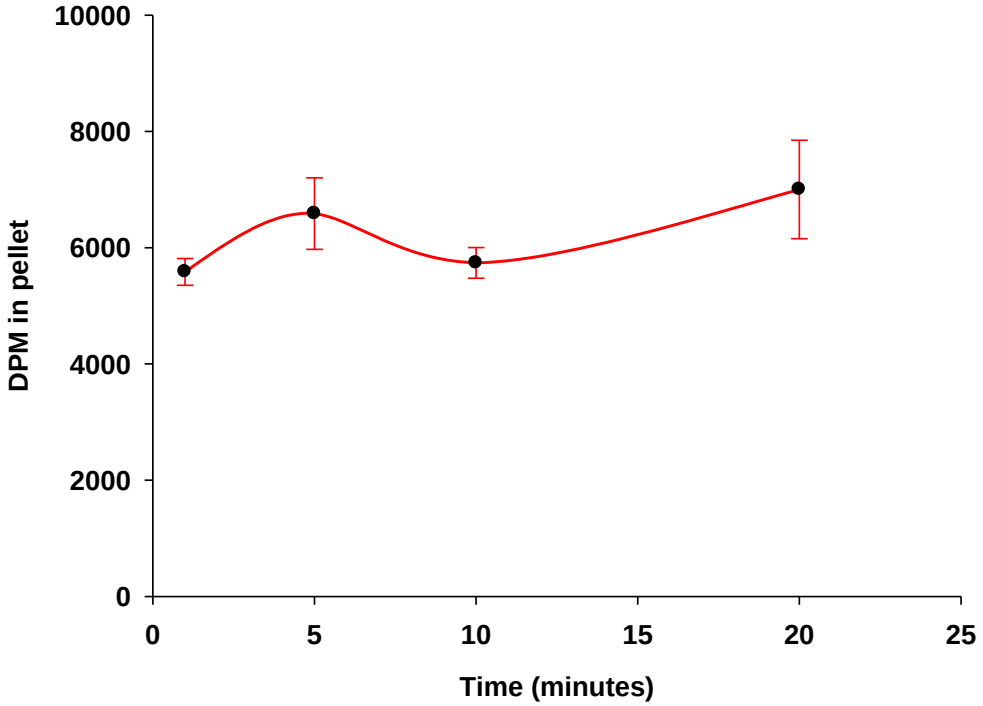


Figure 6-9 Time course of ^{14}C - methylamine uptake by *H. campisalis* (ABU3). DPM in pellet was measured after 1, 5, 10 and 20 minutes incubation periods. Cells were grown overnight in M9 minimal salt medium at 0.5 M NaCl and pH 9. Data represent means of three replicates and standard error.

6.3 Conclusions

Halomonas species inhabit a wide range of environments and play an important role in the microbial diversity of saline and alkaline habitats (Duckworth et al., 2000, Mata et al., 2002, Dobson and Franzmann, 1996). *H. campisalis* was described by (Mormile et al., 1999, Romano et al., 2005) as moderately halophilic and alkaliphilic/or a haloalkaliphilic bacterium. *H. campisalis* are Gram–negative rod shaped cells 3 to 5 µm long and 1 µm wide. The temperature range for growth is between 4°C and 50°C with optimal growth occur at 30°C. The strain is able to grow in the range of pH values between 6 -11, with an optimal pH of 9.5. Growth occurred in the salinity range 0.2 to 4.5 M NaCl, and the optimal salinity was 1.5 M NaCl.

Bioremediation process has been studied widely using different groups of bacteria, and only a few studied have reported the use of halophilic bacteria for the degradation of pollutants in saline environments (Margesin and Schinner, 2001a, García et al., 2004). Thus, the main aim of this chapter was to investigate the aerobic removal of nitrate with *H. campisalis* (ABU3). *H. campisalis* has already been identified as a denitrifying bacterium under anaerobic conditions when NO_3 is utilized as the electron acceptor instead of oxygen (Peyton et al., 2001). Denitrification took place in high salinity (2.1 M NaCl) and high pH (pH 9) (Peyton et al., 2001). In the current work, it can be seen that *H. campisalis* (ABU3) was able to grow well at a wide range of nitrate and urea concentrations as the sole source of nitrogen (Figure 6.1). However, the growth was decreased slightly at 16 mM nitrate. Figure 6.2 shows the growth curves of *H. campisalis* (ABU3) in M9 medium and in the presence of 8 mM nitrate and urea at 1.5 M NaCl. It can be seen in Figure 6.2 that *H. campisalis* (ABU3) was able to grow faster in M9 medium containing 8 mM urea than 8 mM nitrate, but after 26 hours, the biomass levels were similar. Simultaneous measurements of nitrate concentration in the medium and OD_{600} of *H. campisalis*

(ABU3) cells in 1.5 M NaCl minimal salts medium at pH 9, in the presence of three different carbon sources are shown in Figures 6.3, 6.4 and 6.5. The overall results show that *H. campisalis* (ABU3) was able to decrease the amount of nitrate in M9 minimal salt tested media by taking it as a source of cellular nitrogen. The highest nitrate removal was found with glucose as the carbon source, followed by lactate second and acetate was least effective. According to these features, it can be suggested that *H. campisalis* (ABU3) could be useful in environmental bioremediation.

The effect of pH of the culture medium on the bioenergetics of growth of *H. campisalis* (ABU3), the ICV, $\Delta\psi$ and cytoplasmic pH (pHi) of cells were all investigated. Using radiolabelled isotopes in the silicon oil experiments described in the current study produced acceptable results. The use of the isotope method allows the calculation of a volume for a pellet of cells through the ratio of tritiated water ($^{3}H_2O$) and ^{14}C - dextran.

The cell volume of *H. campisalis* (ABU3) bacteria was measured in cells grown at pH 9. The intracellular volume of *H. campisalis* (ABU3) was found to be 1.08 ± 0.5 µl mg^{-1} protein. This value is smaller than the ICV measurements made by (Miguelez and Gilmour, 2008) on *H. elongata* (2 - 2.5 µl mg^{-1} protein), which suggests that *H. campisalis* cells are smaller than *H. elongata* cells. Furthermore, ^{3}H-TPP^{+} (tetraphenylphosphonium) was used to determine membrane potential of cells. aA external pH 9, the membrane potential of the cells was found -123.3 mV.

The cytoplasmic pH (pHi) of cells was determined by the distribution of ^{14}C - methylamine between the cell and the surrounding medium. At external pH 9, the internal pH value was found to be close to the external pH, with the cytoplasmic pH found to be 8.79, while external

pH was 9. This result is in line with expectation - most alkaliphilic cells keep their internal pH below 9.

Alkaliphile bacteria uses one such Na^+/H^+ antiporters system in pH regulation, and to maintain a relatively acidified internal pH (pHi) two or more unit below the external pH, when grow above pH 9.5 (Krulwich, 1995). This mechanism depends on the presence of sodium ions in the surrounding environment, which is essential for transporting solutes through the cell membrane of the cells. In the presence of sodium, H^+ is exchange with Na^+ by a Na^+/H^+ antiporter system and thus generates a motive force which transport substrates accompanied by Na^+ across the cell membrane (Horikoshi, 1999). Haloalkaliphilic growth is an energetically favourable way for cells to grow at high pH.

CHAPTER 7

General Discussion and Future Work

7 General Discussion and Future Work

7.1 General Discussion

Five strains of Gram-negative bacteria were isolated from Qabar - oun (ABQ1 and ABQ2) and Um - Alma (ABU1, ABU2 and ABU3) lakes situated in the Libyan Sahara (Chapter 3). Both lakes are saline lakes which are also alkaline (pH 9.2 to 9.8) making them harsh environments even for microbial cells. The main criterion for selection of the strains was the ability to grow at a range of salinities in M9 minimal medium. Initial characterization of the isolates showed them to be morphologically similar. Therefore, to identify the strains, molecular methods were used, specifically 16S rDNA sequencing (Chapter 4). 16S rDNA sequencing is acknowledged as an excellent way of identifying new isolates to the genus (and often) species level (Stackebrandt and Fox, 1985).

Phylogenetic analysis based on the 16S rDNA sequences obtained from the five isolates placed them all in the genus *Halomonas*. It is perhaps surprising that all the isolates fell within the one bacterial genus, but the lakes are a highly specialised habitat and the genus *Halomonas* contains a large number of species (between 50 and 60), which are found throughout saline habitats worldwide (Arahal and Ventosa, 2006). It is generally accepted that greater than 97% similarity is required between two 16S rDNA sequences to allow them to be classed as the same species (Arahal et al., 2002). On this basis, four of the five isolates were identified to species level: ABQ1 = *H. pacifica*, ABU1 = *H. salifodinae*, ABU2 = *H. elongata* and ABU3 = *H. campisalis*. Only ABQ2 was identified to the genus level only.

At this stage of the work, it was decided to fully characterize only two of the five strains, since they were all members of the *Halomonas* genus. *H. pacifica* (ABQ1) was chosen for further study, because it has not been well characterized to date. *H. campisalis* (ABU3) was chosen for further work, because it was a haloalkaliphilic strain most suited to the conditions

in the Um-Alma lake. It also meant that one strain from each lake would be fully characterized. The physiological data described in Chapter 5 showed that *H. pacifica* ABQ1 is a moderate halophile (i.e. it has an absolute requirement for NaCl in the growth medium). In contrast *H. campisalis* is classified as a Halotolerant strain, because it could grow in the absence of NaCl. The other clear difference between the strains was the ability of *H. campisalis* (ABU3) to grow at high pH levels (up to pH 10), which was not seen with *H. pacifica* (ABQ1). Both strains were shown to grow on a wide range of carbon sources – this is a characteristic of the *Halomonas* genus (Arahal and Ventosa, 2006).

A significant part of the work described in Chapter 5 involved the role of compatible solutes in the ability of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3). In the first set of growth experiments, betaine and ectoine were shown to stimulate the growth of both strains at high salinities. A lesser stimulatory effect was seen with proline. NMR analysis was used to identify both betaine and ectoine in cell extracts of *H. pacifica* (ABQ1) and *H. campisalis* (ABU3). The pattern of compatible solute accumulation followed a pattern seen already in *Halomonas* species (Severin et al., 1992). In rich LB medium, betaine is the predominant compatible solute, because it can be easily formed from choline present in the yeast extract found in LB medium. However, in minimal medium ectoine was the predominant compatible solute and hydroxyectoine was also detected at higher salinities in cells grown in minimal medium. In line with previous work (Cummings and Gilmour, 1995), both strains were challenged with growing at high salinity with betaine as the sole source of carbon. The cells need to use some of the compatible solute for carbon and energy and potentially the rest can be used to balance the external salinity. Both *H. pacifica* (ABQ1) and *H. campisalis* (ABU3) could grow under these conditions and both produced ectoine as an additional compatible solute, showing that complex interactions were taking place between using betaine for growth and betaine as a compatible solute.

The final results chapter (Chapter 6) concentrated on *H. campisalis* (ABU3) and examined two aspects of this organism. Firstly, it was demonstrated that *H. campisalis* (ABU3) can take up nitrate or urea as the sole source of nitrogen while growing at 1.5 M NaCl on minimal medium. In parallel with the ability of *H. campisalis* to act as a denitrifying bacterium under anaerobic conditions, it appears that it is a good candidate for bioremediation studies. The second aspect examined was the measurement of the intracellular pH of *H. campisalis* (ABU3) when grown at pH 9. The value found for the intracellular pH was 8.79, which is only 0.21 less than the external pH. However, the the membrane potential was found to be 123.3 mV inside negative. This means that although the ΔpH is small, when added to the substantial memberane potential, it is sufficient to drive ATP synthesis.

7.2 Future Work

The work described in this thesis has started the process of investigating the microbial diversity of the Qabar-oun and Um-Alma lakes. The next step is use cultivation-independent methods to examine the biodiversity of the lakes without relying on isolating and growing the organisms. DNA can be extracted directly from the lake water and techniques such as denaturing gradient gel electrophoresis (DGGE) and restriction fragment length polymorphism (RFLP) to characterize the organisms present. The number and type of organisms found via DGGE and RFLP can then be compared to the organisms isolated and purified in pure cultures such as the *Halomonas* strains described in this thesis.

The molecular identification technique using 16S rDNA sequencing employed in the current work served well to identify the unknown isolates. However, the amount of information required to validly publish new strains of *Halomonas* and related microorganisms is increasing year on year (Arahal et al., 2002; de la Haba et al., 2010). It would be a good idea to sequence the 23S rRNA gene from isolates to complement the 16S rDNA studies. Future

work on the isolates from the lakes will need to meet these stringent new rules to ensure the strains can be properly documented and published.

The bioremediation part of the current work needs to be expanded to look at the range of metabolic activities of *Halomonas* species that can be potentially exploited to clean up polluted environments. The ability of *H. campisalis* (ABU3) to grow at high salinities and high pH levels both aerobically and anaerobically utilizing a variety of carbon and nitrogen sources makes it a very good candidate for future bioremediation studies.

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Appendixes

Appendix A - Genomic DNA extraction protocol

- All steps are to be carried out at room temperature unless stated otherwise.
- Wash Buffer (concentrate) has to be diluted with absolute ethanol before use.
- Please refer to Reconstitution of Solutions.
- If precipitation forms in **Buffer BG**, incubate at 55^0 C – 65^0 C with occasional mixing until complete dissolved.

Pre-set waterbath to 37^0 C and the second waterbath to 65^0 C.

Pre-heat **Elution Buffer** to 65^0 C (optional)

1. Centrifugation

Pellet 1-3 ml of bacteria culture grown overnight or culture grown to log phase by centrifugation at 6,000 × g for 2 min at room temperature. Decant the supernatant completely. *Thorough removal of supernatant is essential as residual culture media may affect both yield and purity.*

2. Resuspension of pellet

Add 100µl Buffer R1 to the pellet and resuspend the cells completely by pipetting up and down. (*Ensure complete cell resuspension. Lysis will not occur if clumps of bacteria remain following an inefficient resuspension procedure*).

3. Lysozyme treatment

For gram – negative bacteria strains, add 10µl lysozyme (50mg/ml) into the cell suspension.

For gram – positive bacteria strains, add 20µl lysozyme (50mg/ml) into the cell suspension. Mix thoroughly and incubate at 37^0C for 20 min. (*Some bacterial strains may required longer incubation time in lysozyme*).

4. Centrifugation

Pellet digested cells by centrifugation at 10,000 × g for 3 min. Decant the supernatant completely.

5. Protein denaturation

Resuspend pellet in 180µl of **Buffer R2** and add 20 µl of **Proteinase K**. Mix thoroughly. Incubate at 65^0C for 20 min in a shaking water bath or with occasional mixing every 5 min. (*Lysate should be clear at the end of incubation or else extend the incubation time to 30 min*) **(Optional).**

Removal of RNA

If RNA – free DNA is required, add 20 µl (or 4µl of 100mg/ml) of RNase A (DNase –free, 20 mg/ml). Mix and incubate at 37^0 for 5 min (*Residual RNA fragments will be removal during column washin*).

6. Homogenization

Add 2 volume (~ 400 µl without RNase A treatment, ~ 440µl with RNase A treatment) of Buffer BG and mix thoroughly by inverting tube several times until a homogeneous solution is obtained. Incubate for 10 at 65^0C.

7. Addition Ethanol

Add 200 µl of absolute ethanol. Mix immediately and thoroughly (*Mix immediately to prevent precipitation of nucleic acid due to high local ethanol concentrations*).

8. Loading to column

Transfer the sample into a column assembled in a clean collection tube (provided). Centrifuge at 10,000 × g for 1 min. Discard flow through (*If column clogs, add 200 µl Buffer BG into column and centrifuge as above*).

9. Column washing

Wash the column with 750 µl of wash Buffer and centrifuge at 10,000 × g for 1 min. Discard flow through (*Ensure that ethanol has been added into the wash Buffer before use (refer to Reconstitution of Solutions*).

10. Column drying

Centrifuge the column at 10.000 × g for 1 min to remove residual ethanol (*This step has to be carried out to remove all traces of ethanol as residual can affect the quality of DNA and may subsequently inhibit enzymatic reactions*).

11. DNA elution

Place the column into a clean micro centrifuge tube. Add 50 - 100 µl preheated Elution Buffer, TE buffer or sterile water directly onto column membrane and stand for 2 min. Centrifuge at 10,000 × g for 1 min to elute DNA (*Store DNA at 4^0C, Or store DNA at – 20^0C as DNA may degrade in the absence of a buffering agent*).

Appendix B - List of solutions used

50X TAE

242 g Tris base, 57.1 ml Acetic acid, and 0.5 M in 100 ml EDTA (18.6 g in 100 ml) are added to 900 ml dH_2O before adjusting the final volume to 1 litre with additional dH_2O, and pH adjusted to 8.5. This solution is diluted 1 in 50 to produce 1 X TAE suitable for use as an electrophoresis buffer. In order to prepare 1X TAE, pouring 50X TEA just below the first marker on the 1L flask and added distilled water to the 1L. This can be added to the gel tank until the buffer fills both end wells and covers the gel completely.

Table 1 Buffers used to maintain pH level in M9 minimal medium

50 mM Buffers			
Buffer name	Working pH level	g^{-1}	Molecular formula
MES	5.5 – 6.5	9.76 g	$C_6H_{13}NO_4S$
Trisma	7.5 - 8.5	6.05 g	$C_4H_{11}NO_3$
CAPS	9.5 – 10.5	11.06 g	$C_9H_{19}NO_3S$

Appendix C.

Table 2 List primers used

primers	Sequence **(5'→ 3')**	Tm (C^0)
16S rDNA Bac8f	AGRGTTTGATCCTGGCTCAG (20)	58.3
16S rDNA bac 1492r	CGGCTACCTTGTTACGACTT (20)	57.3
16S rDNA arc8f	TCCGGTTGATCCTGCC (16)	54.3
16S rDNA arc1492r	GGCTACCTTGTTACGACTT (19)	54.5

Appendix D: Optical density (OD) at 595 nm for utilization carbon substrates

Table 3 Initial optical density (OD) of *H.pacifica* (ABQ1) bacterial cells inoculated into Biolog GN2 Microplate wells containing different carbon sources.

0.186	0.213	0.263	0.178	0.259	0.277	0.216	0.182	0.186	0.210	0.189	0.192
0.176	0.213	0.194	0.175	0.173	0.174	0.194	0.179	0.185	0.177	0.190	0.210
0.282	0.230	0.240	0.300	0.240	0.320	0.329	0.292	0.296	0.273	0.288	0.355
0.359	0.299	0.261	0.325	0.290	0.331	0.321	0.319	0.329	0.333	0.292	0.192
0.313	0.269	0.203	0.298	0.292	0.279	0.296	0.305	0.238	0.249	0.325	0.243
0.213	0.249	0.219	0.237	0.233	0.250	0.246	0.206	0.230	0.216	0.217	0.211
0.197	0.197	0.511	0.210	0.295	0.201	0.224	0.212	0.233	0.225	0.242	0.232
0.306	0.310	0.260	0.321	0.299	0.294	0.264	0.314	0.336	0.275	0.252	0.277

Table 4 Initial optical density (OD) of *H.campisalis* (ABU3) bacterial cells inoculated into Biolog GN2 Microplate wells containing different carbon sources.

0.255	0.292	0.339	0.239	0.288	0.303	0.264	0.281	0.233	0.279	0.257	0.280
0.312	0.345	0.339	0.285	0.272	0.218	0.247	0.336	0.279	0.260	0.272	0.310
0.222	0.255	0.316	0.317	0.306	0.307	0.271	0.269	0.342	0.298	0.296	0.391
0.326	0.281	0.236	0.306	0.306	0.267	0.262	0.237	0.280	0.344	0.382	0.460
0.316	0.311	0.325	0.269	0.363	0.289	0.382	0.351	0.262	0.264	0.342	0.283
0.245	0.356	0.322	0.311	0.331	0.305	0.303	0.316	0.371	0.328	0.337	0.323
0.319	0.305	0.505	0.319	0.280	0.293	0.267	0.262	0.271	0.265	0.289	0.309
0.369	0.335	0.338	0.323	0.361	0.338	0.286	0.301	0.335	0.313	0.349	0.282

Table 5 Overnight optical density (OD) of *H. pacifica* (ABQ1) utilized different carbon sources into Biolog GN2 Microplate.

0.477	0.505	1.282	0.608	0.605	0.924	0.441	1.383	0.470	0.404	0.405	0.418
0.407	1.438	0.411	0.358	0.377	1.557	0.382	0.401	0.396	1.589	0.429	0.514
0.422	0.395	1.191	0.459	0.449	0.433	1.528	1.557	1.540	0.427	1.531	0.842
1.062	1.438	1.556	0.502	0.301	0.387	1.639	0.400	0.423	0.608	1.447	0.436
1.209	0.588	0.410	1.466	0.344	1.411	0.527	1.255	0.388	0.421	0.471	1.207
1.459	0.674	0.420	0.558	1.529	1.636	0.638	1.535	0.739	1.432	0.551	0.568
1.499	0.519	0.678	0.616	1.175	1.656	1.112	0.389	1.651	0.524	0.520	0.553
1.690	1.462	1.344	0.524	1.323	1.701	0.457	0.995	1.655	0.628	0.500	0.474

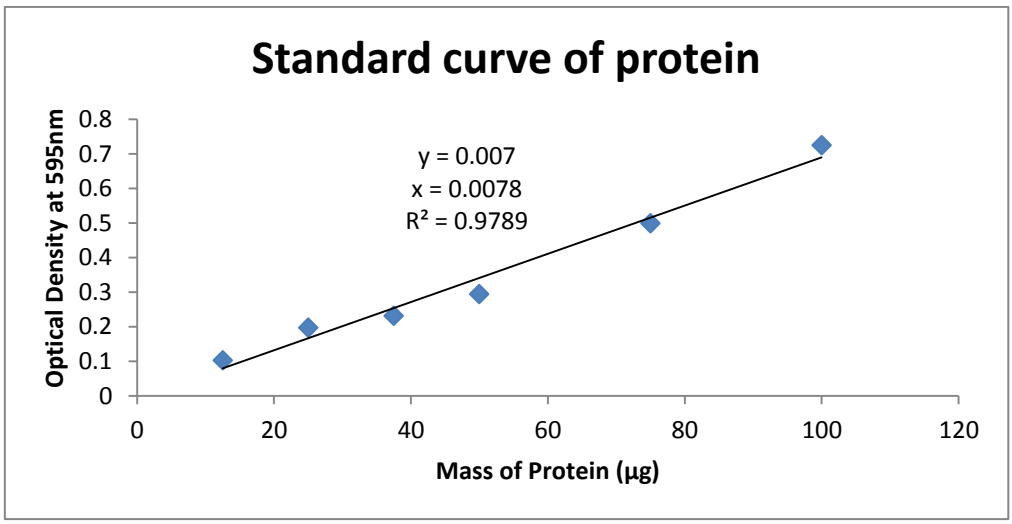
Table 6 Overnight optical density (OD) of *H. campisalis* (ABU3) utilized different carbon sources into Biolog GN2 Microplate.

0.457	0.465	1.337	0.621	0.619	0.941	0.425	1.370	0.484	0.424	0.406	0.430
0.406	1.430	0.410	0.365	0.387	1.676	0.389	0.405	0.415	1.600	0.442	0.523
0.427	0.399	1.164	0.458	0.461	0.447	1.556	1.645	1.551	0.435	1.544	0.858
1.015	1.445	1.601	0.481	0.309	0.407	1.681	0.401	0.419	0.584	1.427	0.434
1.221	0.492	0.404	1.499	0.344	1.399	0.522	1.276	0.405	0.443	0.471	1.197
1.472	0.649	0.412	0.557	1.523	1.665	0.630	1.528	0.767	1.446	0.548	0.584
1.497	0.502	0.663	0.607	1.187	1.678	1.126	0.393	1.654	0.526	0.524	0.556
1.688	1.477	1.275	0.512	1.322	1.717	0.454	0.980	1.660	0.628	0.494	0.475

Appendix E: Standard curve of protein

Table 7 Components in the test tubes which were needed to make a standard curve of protein.

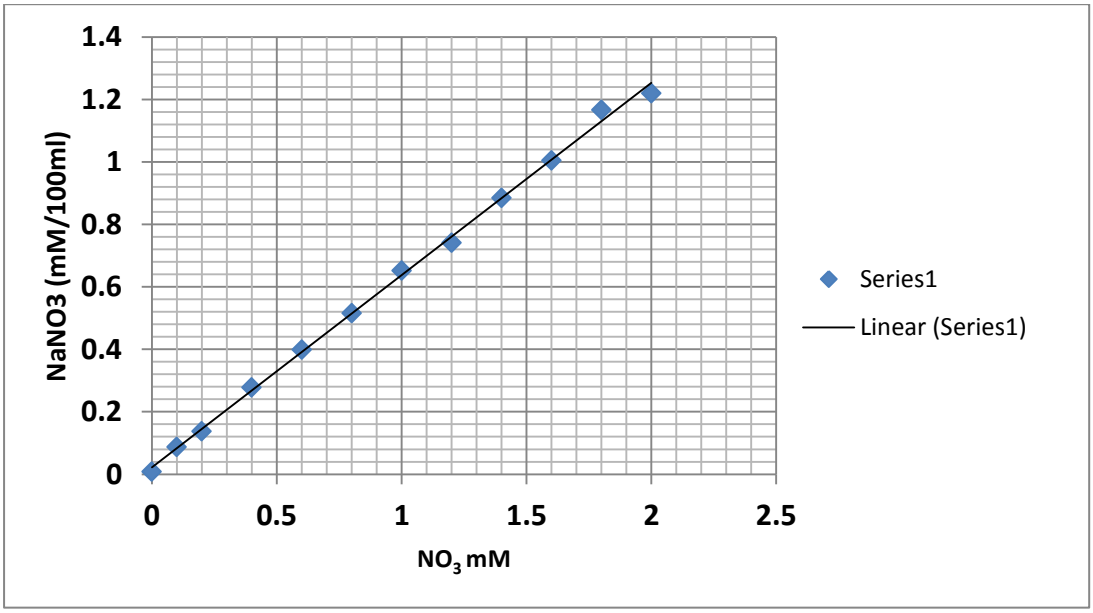
Tube number	BSA (5 mg ml^{-1}) Stock solution (µl)	Distilled Water (µl)	Total volume in each tube (µl)	$OD_{595\ nm}$	Mass of protein (µg)
1	0	100	100	0.000	0 (Blank)
2	2.5	97.5	100	0.102	12.5
3	5	95	100	0.196	25
4	7.5	92.5	100	0.231	37.5
5	10	90	100	0.294	50
6	15	85	100	0.498	75
7	20	80	100	0.724	100



Appendix F: $NaNO_3$ concentrations

Table 8 Different concentrations of $NaNO_3$ in the test tubes which were needed to make a standard curve.

$NaNO_3$ (mM/100 ml)	$NaNO_3$ Stock Solution (ml)	M9 minimal medium (ml)	OD_{600nm}
0.0	0.0	100	0.008
0.1	0.5	99.5	0.087
0.2	1	99	0.137
0.4	2	98	0.278
0.6	3	97	0.399
0.8	4	96	0.516
1	5	95	0.652
1.2	6	94	0.741
1.4	7	93	0.885
1.6	8	92	1.005
1.8	9	91	1.167
2	10	90	1.22



Standard Curve of $NaNO_3$ in M9 minimal salt medium (free of NH_4Cl)

Appendix G: Optical density of *H. campisalis* (ABU3) growth against OD of Nitrate reduction.

Table 9 Optical density (OD) with Glucose as sole source of carbon.

Time (h)	**Bacteria growth OD_{600nm}**			**Nitrate OD_{232nm}**		
	A	B	C	A	B	C
0	0.1260	0.1280	0.1300	0.577	0.577	0.577
2	0.1970	0.2180	0.1930	0.458	0.481	0.445
4	0.3550	0.3700	0.3330	0.446	0.447	0.440
6	0.7280	0.7180	0.6820	0.411	0.432	0.430
8	1.2560	1.2480	1.1870	0.205	0.241	0.217
10	1.5570	1.5460	1.4850	0.111	0.141	0.065
12	1.7340	1.7590	1.6970	0.095	0.027	0.052
14	1.7550	1.8080	1.7610	0.025	0.016	0.048
24	1.6260	1.7140	1.6240	0.028	0.035	0.062

Table 10 Growth (Mean ± Std.Dev) and Nitrate reduction (Mean ± Std.Dev) of *H. campisalis* (ABU3) in M9 medium using Glucose as carbon source.

Time (hr)	**Cells growth Mean ± Std.Dev**	**Nitrate reduction Mean ± Std.Dev**
0	0.12 ± 2.00	0.57 ± 0.00
2	0.20 ± 0.01	0.46 ± 0.01
4	0.35 ± 0.01	0.44 ± 3.78
6	0.70 ± 0.02	0.42 ± 0.01
8	1.23 ± 0.03	0.22 ± 0.01
10	1.52 ± 0.03	0.10 ± 0.03
12	1.73 ± 0.03	0.05 ± 0.03
14	1.77 ± 0.02	0.02 ± 0.01
24	1.65 ± 0.05	0.04 ± 0.01

Table 11 Optical density (OD) with Sodium lactate as sole source of carbon.

Time (h)	**Bacteria growth OD_{600nm}**			**Nitrate OD_{232nm}**		
	A	B	C	A	B	C
0	0.1570	0.1900	0.1620	0.5430	0.5430	0.5430
2	0.2660	0.2490	0.2400	0.4670	0.4890	0.4820
4	0.3550	0.3140	0.3070	0.4570	0.4400	0.4620
6	0.4670	0.4500	0.4420	0.3390	0.3640	0.3810
8	0.7260	0.7410	0.6910	0.3330	0.3460	0.3410
10	1.0600	1.0360	1.0230	0.2930	0.3090	0.2670
12	1.3470	1.3420	1.3110	0.2420	0.1720	0.2630
14	1.5400	1.5410	1.5500	0.2370	0.1700	0.1970
16	1.9840	1.9940	1.9780	0.1320	0.1250	0.1570
24	2.0440	2.0340	2.0090	0.0760	0.1010	0.1110

Table 12 Growth (Mean ± Std.Dev) against Nitrate reduction (Mean ± Std.Dev) of *H. campisalis* (ABU3) grown in M9 medium using sodium lactate as carbon source.

Time (hr)	**Cells growth Mean ± Std.Dev**	**Nitrate reduction Mean ± Std.Dev**
0	0.16 ±0.01	0.54 ± 0.00
2	0.25 ± 0.01	0.47 ± 6.48
4	0.32 ± 0.02	0.45 ± 6.65
6	0.45 ± 0.01	0.36 ± 0.01
8	0.71 ± 0.02	0.34 ± 3.78
10	1.03 ± 0.01	0.28 ± 0.01
12	1.33 ± 0.01	0.22 ± 0.02
14	1.54 ± 5.50	0.20 ± 0.01
16	1.98 ± 8.08	0.13 ± 9.71
24	2.02 ± 0.01	0.09 ± 0.01

Table 13 Optical density (OD) with Sodium acetate as sole source of carbon.

Time (h)	Bacteria growth OD_{600nm}			Nitrate OD_{232nm}		
	A	B	C	A	B	C
0	0.1260	0.1360	0.1280	0.5380	0.5380	0.5380
2	0.1430	0.1480	0.1410	0.5340	0.5260	0.5330
4	0.1530	0.1640	0.1490	0.5670	0.5760	0.5880
6	0.1610	0.1720	0.1520	0.5420	0.5860	0.5450
24	0.9470	0.9340	0.9150	0.3220	0.2390	0.3320
26	1.1450	1.1350	1.1610	0.2660	0.208	0.2590
28	1.2690	1.3700	1.3280	0.2180	0.1750	0.2220
30	1.3580	1.5270	1.4350	0.2130	0.1710	0.2020
32	1.4690	1.6020	1.5540	0.2100	0.1620	0.1940
34	1.5520	1.6970	1.6600	0.1790	0.1250	0.1820
36	1.8010	1.8060	1.8060	0.0820	0.0550	0.0660

Table 14 Growth (Mean ± Std.Dev) against Nitrate reduction (Mean ± Std.Dev) of *H. campisalis* (ABU3) grown in M9 medium using Sodium acetate as carbon source.

Time (hr)	**Cells growth Mean ± Std.Dev**	**Nitrate reduction Mean ± Std.Dev**
0	0.13 ± 5.29	0.53 ± 0.00
2	0.14 ± 3.60	0.53 ± 4.35
4	0.15 ± 7.76	0.57 ± 0.01
6	0.16 ± 0.01	0.55 ± 0.02
24	0.93 ± 0.01	0.29 ± 0.05
26	1.14 ± 0.01	0.24 ± 0.03
28	1.32 ± 0.05	0.19 ± 0.02
30	1.44 ± 0.08	0.19 ± 0.02
32	1.54 ± 0.06	0.19 ± 0.03
34	1.63 ± 0.07	0.16 ± 0.03
36	1.80 ± 2.88	0.06 ± 0.01