Isolation and Characterisation of Halotolerant Bacteria and Algae and their Potential for Biofuel Production

By

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DEDICATION

To my parents, my wife, my sons Mohammed, Ali, Hassan,

daughter Fatimah and to my brothers and sisters

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Abstract

The first aim of the project was to isolate, identify and characterize salt tolerant bacteria from river and pond water. This aim was achieved by the isolation of the salt tolerant bacterium *Enterococcus amnigenus* from water samples taken from Weston Park pond and by the isolation of the salt tolerant bacterium *Pseudomonas fluorescens* from a dew pond in the Derbyshire Peak District. *E. amnigenus* in common with many enterococci, is a potential pathogen, but it also has uses in industry as a producer of bacterial cellulose. *P. fluorescens* is a ubiquitous organism found in marine and soil environments and has been well characterized as an important biofilm-forming organism and as a rhizobacterium.

The second aim of the project was to isolate salt-tolerant microalgae from the fresh water Weston Park pond and this was successfully achieved by isolating and identifying two algal species - the diatom *Navicula pelliculosa* and the green alga *Chlorella* sp. Initial work measuring total lipid concentrations suggested that *Navicula* was the most promising organism for biofuel production due to having a total lipid concentration of around 20%. Further characterization of *Navicula* was undertaken to investigate its suitability for biofuel production. It was shown to grow under conditions of high pH and high salinity, making it a candidate species for growth in outdoor raceway ponds. Experiments using Nile Red fluorescence to measure neutral lipid production indicated that stress conditions (high salinity or high pH) could increase the neutral lipid accumulation by *Navicula* cells. To grow in high salinity (up to 0.8 M NaCl), *Navicula* cells must balance the external

osmotic potential by accumulating a compatible solute within the cells. NMR analysis showed that the compatible solute accumulated by *Navicula* is glucosylglycerol, which is not normally found in diatoms.

Abbreviations

bp	Base pair (s)
BSA	Bovine serum albumin
^{o}C	Centigrade
CFE	Cell free extract
Caps	3-(cyclohexylamino)-1-propanesulfonic acid
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
EB	Ethidium bromide
FAD	flavin adenine dinucleotide
g	Gram (s)
h	Hour (s)
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)
Mops	3-(N-morpholino)propanesulfonic acid
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
OAA	Oxaloacetic acid
OD	Optical density
PCR	Polymerase chain reaction
rDNA	ribosomal DNA
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minutes
SOC	Super optimal broth with catabolite repression

TAE	Tris-acetate-EDTA
Tris	(hydroxymethyl)aminomethane
V/V	Volume per unit volume
W/V	Weight per unit volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
µg	Microlitre (s)
%	Percentage
BBM	Bold,s Basal Medium
FAME	Fatty Acid Methyl Ester
GCMS	Gas chromatography–mass spectrometry

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Chapter 1

LITERATURE REVIEW

1.1 Microbial Diversity

The diversity of microorganisms can be defined as the measure of the number or relative abundance of microbial species in a particular ecological niche (Atlas, 1984; Scow *et al*., 2001). Microbial diversity can also be thought off as the distribution of genetic information in an ecosystem. Microorganisms are the often unseen part of the biota that make up an ecosystem and the domains Archaea and Bacteria often dominate ecosystems based on several billion years of evolution (Torsvik *et al*., 2002).

Whitman *et al*. (1998) reported that sediments and soils are a key ecological niche for microorganisms and may contain around 30% of the Earth's biomass emphasizing the major influence that the microbial world has on the biology of the Earth. In contrast, the microbial diversity in aquatic systems is much less than in sediments or soils (Torsvik *et al*. 2002). Nevertheless, there are a number of bacterial groups that are abundant in aquatic habitats e.g. Proteobacteria, Actinobacteria, Bacteroidetes and Verucomicrobia (Urbach and Vergin 2001; Urbach *et al*. 2007). Among the Proteobacteria, the β-Proteobacteria and γ-Proteobacteria groups were shown to be dominant in 18 freshwater lakes, making up some 75% of all the bacterial species identified (Percent *et al*., 2008). In more extreme habitats such as terrestrial hot springs in volcanic areas such as Yellowstone National Park in the US, diverse and often abundant populations of Archaea were found to be the dominant domain, reflecting their ability to grow at high temperatures and low pH (Hou *et al.,*2013).

In common with the rest of the biological sciences, microbiology sees the species as the basic unit of diversity, but the definition of a microbial species

is much less clear than the definition of a plant or animal species, which is normally based on the ability to produce fertile offspring (Torsvik *et al*., 2002). As described in section 1.5, molecular methods are now key to the phylogenetic definition of species (Rosello and Amann, 2001). Warnecke *et al* (2004) defined a microbial species "as a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity in many independent characteristic properties".

1.2 Extremophilic Microorganisms

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

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

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

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

Life in extreme environments has been studied intensively, focusing attention on the diversity of organisms and the molecular and regulatory mechanisms involved (Gomes and Steiner, 2004). Furthermore, some extremophiles are polyextremophiles that able to withstand multiple extremes such as the acidothermophiles *Sulfolobus solfataricus* and *S. acidocaldarius* that have been grown at pH 3 and 80°C (Gomes and Steiner, 2004; Irwin and Baird, 2004). Thermophilic alkalitolerant bacteria have also been isolated, such as *Anaerobranca* spp. (Engle *et al*., 1995).

Environmental parameter	Type	Characterisation	Examples
Temperature	Hyperthermophile Thermophile Mesophile Psychrophile	Grows at > 80°C 60 - 80°C 15 - 60°C < 15°C	*Pyrolobus fumarii* *Synechococcus lividis* *Homo sapiens* *Psychrobacter*, some insects
pH	Alkaliphile Acidophile	pH > 9 pH < 2	*Natronobacterium* *Bacillus firmus* OF4 *Spirulina* sp (all pH10) *Cyanidium caldarium* *Ferroplasma* sp (both pH 0)
Salinity	Halophile	Salt-loving (2-5 M NaCl)	Halobacteriacae *Dunaliella salina*
Radiation	Radioresistant		*Deinococcus radiodurans*
	Barophile Piezophile	Pressure-loving Pressure-loving	Unknown microbe,130MPa
Oxygen Tension	Anaerobe Microaerophile Aerobe	Cannot tolerate O_2 Tolerates some O_2 Requires O_2	*Methanococcus jannaschii* *Clostridium* *H. sapiens*
Chemical Extremes	Gasses Metals	Metalotolerant	*C.caldarium* *Ferroplasma acidarmanus* (Cu, As, Cd, Zn); *Ralstonia* sp. CH_4 (Zn, Co, Cd, Hg)

Table 1.1: Classification and examples of extremophiles (Reproduced and modified from Rothschild and Mancinelli, 2001; van den Burg, 2003; Irwin and Baird, 2004).

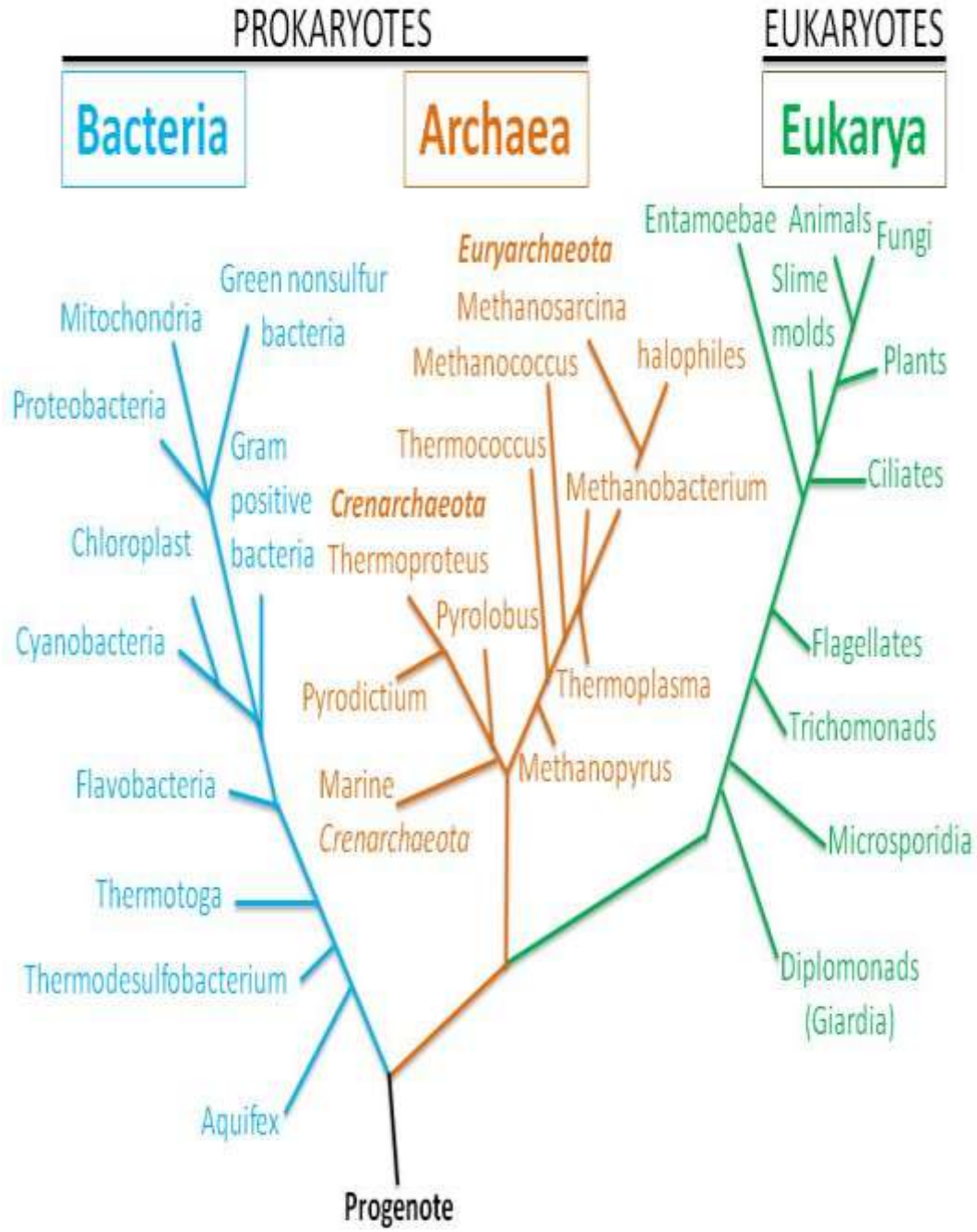

Figure 1.1: Universal phylogenetic tree as determined from comparative rRNA gene (16S or 18S RNA) sequence analysis. Adapted from Oren (2008).

1.2.1 Alkaliphilic Microorganisms

Most microorganisms (non-extremophilic) grow within the range of external pH values from 5.5 – 9, but their optimal growth normally lies within the narrow range of pH 7.4 – 7.8 (Padan *et al*., 2005). Microorganisms capable of growth outside these limits, but which show optimal growth within this range, are classified as acid- or alkali-tolerant organisms (Kroll, 1990).

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

It is worth noting that the scientific study of alkaliphilic microorganisms started fairly recently, there were only 16 published scientific papers concerning alkaliphiles prior to 1968. In ancient times in Japan, indigo was naturally reduced by bacteria that grow under highly alkaline conditions in the presence of sodium carbonate (Horikoshi, 1999). Alkaliphiles have been frequently isolated from normal pH neutral environments such as garden soil, although cell counts of the alkaliphilic bacteria are higher in alkaline

environments. Alkaliphiles can also be isolated from acidic soil samples (Horikoshi, 1996 and 1999).

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

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

Micrococcus, Paracoccus, *Pseudomonas*, *Aeromonas*, *Corynebacterium* and *Actinopolyspora,* have been isolated from neutral soils (Satyanarayana *et al.,* 2005). On the other hand, *Chimaereicella alkaliphila,* a Gram-negative bacterium, was isolated from highly alkaline groundwater at pH 11.4 (Tiago *et al*., 2006).

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

1.2.2 Halophilic and halotolerant microorganisms

A significant portion of the biosphere contains salt e.g. the oceans and seas that cover most of the Earth's surface contain about 35 g dissolved salts per litre, not all of the salt is NaCl, but it is the major sakt present. Higher salt concentrations are often found in environments such as salt lakes and

lagoons (Oren, 2006). Natural aquatic saline environments are widely distributed around the world and cover about 45% of total aquatic environments, they usually contain salinity in the range of approximately 4 - 30% (0.7 to 5.1 M NaCl) (Zahran, 1997), and have a natural bacterial flora, which may play a significant role in nutrient cycles and food webs, in addition to attenuation of pollution in these ecosystems. Extremely hypersaline environments are generally defined as those containing salt concentrations in excess of 15% NaCl and most such environments are in warm and dry climatic regions of the world (Zahran, 1997). Many hypersaline environments arise from the evaporation of sea water caused by high temperatures, low rainfall and low humidity, and are called thalassohaline environments (Schreiber, 1986; Oren, 2002).

Although NaCl is the major salt in most hypersaline waters (McGenity, 2000), the ionic composition of saline lakes can vary considerably, depending on the surrounding topography, geology, and general climatic conditions. Hypersaline ecosystems have a great variability in ionic composition, total salt concentration, and pH. High concentrations of salts exist in natural inland salt lakes such as the Great Salt Lake in Utah (USA) (thalassohaline brine), with total dissolved salts of about 332.5 g per litre. Sodium is the predominant cation in the Great Salt Lake, with a concentration of 4.6 M, whereas chloride is the predominant anion with a concentration of 5.1 M; significant levels of sulphate are also present and the pH is neutral or slightly alkaline (pH 7 to pH 8). By contrast, another hypersaline lake, the Dead Sea, Jordan, Middle East is a prime example of an athalassohaline lake (the ionic composition greatly differs from that of seawater). The Dead Sea contains

around 322.6 g dissolved salt per litre, but is relatively low in sodium (1.7 M), and contains a high level of magnesium (1.7 M) due to the abundance of magnesium minerals in the surrounding rocks. The Dead Sea also has a relatively low pH of around 5.8 to 6 (slightly acidic), (Imhoff, 1986; Ollivier *et al.,* 1994; Zahran, 1997; Yoon *et al*., 2004).

Microbes adapted to life at high salt concentrations are widespread and found in all three domains of life: Archaea, Bacteria, and Eucarya (Figure 1.2). Microorganisms can grow (not just survive) over the whole salt concentration range from near freshwater or seawater up to in excess of 300 g l^{-1} in both thalassohaline and athalassohaline environments (Margesin and Schinner, 2001; Madern and Zaccai, 2004; Ma *et al*., 2010).

Halophilic microorganisms are able to grow and survive in hypersaline environments by their ability to maintain osmotic balance. Recently, extremophiles have been isolated from habitats where they are not expected to be actively growing. Therefore, it is possible that halotolerant or even halophilic bacteria can be isolated from a wide range of environments including freshwater (Echigo *et al*., 2005). In most cases, however, each organism has a relatively limited salt concentration range enabling growth and also, the minimum, optimum and maximum salt concentrations are often found to be dependent on the medium composition and growth temperature (Ventosa *et al.,* 1998; Oren, 2008).

1.2.2.1 Diversity of halophilic and halotolerant microorganisms

Halophilic microbes can be defined as microorganisms which require at least 1.5 M (9% w/v) NaCl for growth and optimum growth is found at 3.0 M (18% w/v) NaCl or greater. Halotolerant bacteria are defined as microorganisms that can grow over a large variety of salt concentrations, however with growth rate optimum in the absence of salt (Joo and Kim, 2005; Bowers *et al*., 2009). In contrast, moderately halophilic microorganisms are defined as microorganisms that grow optimally in media containing 0.5–2.5 M (3–15%) NaCl (Ventosa *et al*., 1998; Echigo *et al*., 2005).

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

Vreeland and Huval (1991) emphasized that many strains can grow over a range of NaCl concentrations between 0 to 17% (w/v); these are the haloversatile type. Moderate halophiles were defined by these authors as growing between 2 to 20% (w/v) NaCl and extreme halophiles growing between 12 to 32% (w/v) NaCl.

Table 1.2 shows the consensus classification scheme based on the work of Kushner (1978, 1985) and influenced by the variety of authors already cited above plus Larsen (1986), Oren, (2006) and Cyplik *et al*. (2007).

Type	Properties	Examples
Non-halophilic	Grows best in media containing less than 0.2 M (1.2% w/v) NaCl	Most freshwater microorganisms
Slight halophile	Grows best in media containing 0.2-0.5 M (1.2-3% w/v) NaCl	Most marine microorganisms
Moderate halophile	Grows best in media containing 0.5-2.5 M (3-15% w/v) NaCl	*Salinivibrio costicola*
Broderline extreme halophile	Grows best in media containing 1.5-4.0 M (9-24% w/v) NaCl	*Halorhodospira halophila*
Extreme halophile	Grows best in media containing 2.5-5.2 M (15-31.2% w/v) NaCl	*Halobacterium salinarum*
Halotolerant	Non-halophile which can tolerate salt if the growth range extends above 2.5 M (15% w/v) NaCl, it may be considered extremely halotolerant or haloversatile.	*Halomonas elongata*

Table 1.2: Consensus classification scheme of microorganisms according to their responses to salt concentrations, with examples.

A fairly early report of a borderline extreme halophile was by Mullakhanbhai *et al*. (1975) who isolated a halophilic microorganism (*Haloferax volcanii*) from the bottom sediment of the Dead Sea and demonstrated that the optimum requirement for NaCl was in the range of 1.7 to 2.5 M. *H. volcanii* is an archaeon (i.e. a member of the Domain Archaea) and many extreme halophiles belong to the Archaea. Benlloch *et al*. (2002) identified and characterized Bacterial and Archaeal communities in 8%, 22% and 32% (w/v) NaCl by using molecular microbial ecology methods including 16S rDNA sequencing, denaturing gradient gel electrophoresis (DGGE), clone libraries and culturing techniques.

The majority of Bacterial sequences found in 8% NaCl were of marine origin and included the alpha-, beta-, gamma- and epsilon- subdivisions of Proteobacteria, the CFB group and Cyanobacteria. At 22% NaCl, only alpha- and gamma-Proteobacteria, Cyanobacteria and CFB were found; the majority of them are related to halophilic bacteria. Only CFB were found at 32% salt and the majority of sequences were related to *Salinibacter ruber*, an extremely halophilic bacterium. A similar decrease in species richness was also seen for the Archaea, with marine representatives being found at 8% NaCl and only the Halobacteriaceae being found in the higher salinities (Benlloch *et al*., 2002).

Jiang *et al*. (2007) studied the effects of salinity and mineralogy on microbial community composition from thalassohaline lake water fed by fresh water of Mo River in China by using cultivation methods and molecular microbiology. Those authors found that microbial composition changed with salinity gradient, and this change is related with geochemical conditions, but not by mineralogy. They also found that beta-Proteobacteria and Bacteroidetes are dominant in the hypersaline sediments at the surface while, Firmicutes and sulphate reducing bacteria are dominant in the freshwater sediments. Halophilic Archaea need 1.5 M NaCl both to grow and keep the structural integrity of the cells. The proteins of these organisms are adapted to be active in the hypersaline situation (Joo and Kim, 2005). Konstantinidis *et al.* (2007) demonstrated that the aerobic, haloalkaliphilic archaeon *Natronomonas pharaonis* could grow well in lake water of pH 11.

Studies on hypersaline environments have shown that halophilic members of the domain Archaea tend to dominate, whereas members of the domain

Bacteria are relatively minor components (Oren 1994; Rothschild and Mancinelli 2001; Ochsenreiter *et al*., 2002). Other studies, based on phylogenetic techniques and fluorescence *in situ* hybridization, come to a different conclusion suggesting that members of the domain Bacteria play an important role in the microbial diversity of hypersaline environments (Anton *et al*., 1999, 2000). It is certainly true that halophilic Bacteria inhabit a wide range of saline environments whereas the halophilic Archaea are more restricted to the most extreme salt habitats (Rodriguez-Valera 1986; Ventosa *et al*., 1998).

Moderately halophilic Bacteria are not a homogeneous group of microorganisms, instead they that belong to many different genera (Ventosa *et al.*, 1998). Examples of moderate halophiles are *Staphylococcus aureus*, *Salinivibrio costicola, Nesterenkonia halobius, Halomonas halodenitrificans, Halomonas halmephilum, Marinococcus halophilus, and Spirochaeta halophila* (Ventosa *et al.*, 1998).

1.3 Microalgae

Algae are generally considered to be “aquatic, oxygen-evolving photosynthetic autotrophs that are unicellular, colonial or are constructed of filaments or composed of simple tissues” (Guiry, 2012). The original classification of algal groups based on pigment composition and cell morphology led to the recognition of 12 phyla as shown in Table 1.3 (Round, 1973). One phylum, Cyanophyta, is composed of prokaryotic organisms, sometimes called blue-green algae. All the other phyla are eukaryotic organisms with the Phaeophyta (brown algae) being composed of macroscopic seaweeds, as is part of the Rhodophyta phylum (red algae). The other phyla make up the group of microalgae (Table 1.3). More recent phylogenetic based 18S rDNA sequencing has changed the groupings, in particular a new phylum the Ochrophyta has been created to include the diatoms, seaweeds and several other groups. The major groups of green algae (Chlorophyta), red algae (Rhodophyta) and the euglenoids are unchanged in the new classification (Table 1.3).

The number of algal species extant on Earth is unknown, since many await isolation and identification. As of 2012, around 44,000 algal species had been described in the scientific literature and a conservative estimate would suggest that at least another 38,500 species remain unknown (Guiry, 2012). This overall figure of 72,500 is almost certainly an underestimate.

Algal Phyla (Round, 1973)	Comments	Algal Phyla (Guiry, 2012)
Cyanophyta	Prokaryotic, blue-green algae	Cyanobacteria
	Prokaryotic	Prochlorophyta
Euglenophyta	Euglenoid flagellates	Euglenozoa
Chlorophyta	Green algae	Chlorophyta
Charophyta		Charophyta
Haptophyta		Haptophyta
		Ochrophyta
Xanthophyta		Xanthophyceae
Bacillariophyta	Diatoms	Bacillariophyceae
Chrysophyta		Chrysophyceae
Phaeophyta	Brown algae, macroscopic	Phaeophyceae
		Eustigmatophyceae
		Myzozoan
Dinophyta	Dinoflagellates	Dinophyceae
Rhodophyta	Red Algae, part macroscopic	Rhodophyta
Cryptophyta		Cryptophyta
		Glaucophyta

Table 1.3: Summary of the major groups of algae and how the groupings have changed between 1973 and 2012. Data from Round (1973) and Guiry (2012).

1.3.1 Green Algae (Chlorophyta)

The green algae and higher plants form a monophyletic group (i.e. they evolved from a common ancestor) (Becker, 2013). The group of plants and green algae are divided into two groups the Chlorophyta and Streptophyta, the latter containing all the land plants and the Charophyta. Therefore, the closest algal relatives of land plants are the charophycean algae and not the Chlorophyta (Becker, 2012),

In addition to chlorophyll a, which is present in all algae, the major pigments present in green algae are chlorophyll b, beta-carotene and zeaxanthin. The chloroplast envelope is a double membrane and the thylakoids within the chloroplast are grouped to form lamellae stacks. Pyrenoids are found embedded in the chloroplast and the storage polysaccharide is starch, which is found as grains surrounding the pyrenoid (van den Hoek *et al*., 1995). If present, the cell wall of green algae is composed of cellulose. The major genera found in the Chlorophyta phylum include *Chlamydomonas, Chlorella* and *Dunaliella.*

1.3.2 Diatoms (Bacillariophyceae)

Kooistra and Medlin (1996) and Schieber *et. al*. (2000) suggested that the diatom group originated about 185 million years ago based on recent genetic and sedimentary evidence. Diatoms are the main primary producers within

the phytoplankton and are major contributors to the production of sedimentary organic matter (Killops and Killops, 2005). Therefore, diatoms can be considered also as a sustainable source of oil. The knowledge about diatoms is increasing rapidly because they are central to a new direction in nanotechnology which is involved in growing and harvesting their hard silica cell walls (Gordon *et al.*, 2005, 2009). The silica in diatom cell walls is present as a pair of frustules and a variable number of girdle bands which constrains the size of the oil droplets within the cell (Round *et al.*, 1990; Cox, 1996; De Stefano, 2007).

In addition to chlorophyll a, diatoms contain chlorophyll c (never chlorophyll b) and their principal accessory pigment is fucoxanthin. In addition to the double membrane of the chloroplast envelope, the chloroplast is also enclosed by a fold of the endoplasmic reticulum. Thylakoids are stacked into lamellae and the main storage compound is chrysolaminarin (β-1,3-glucan) (van den Hoek *et al.,* 1995). As noted above, the cell wall consists of silica. The major genera found within the diatoms include *Phaeodactylum, Navicula* and *Nitzschia*.

Diatoms produce natural products which are widely used in foodstuffs, pharmaceuticals, cosmetic chemicals, and biofuels (Hung *et al*., 2013). Therefore, both biotechnology engineers and marine ecologists are keen to improve their knowledge about the physiological responses of diatoms to environmental and cultivation conditions. Phosphorus (P) is an essential nutrient to maintain their population growth as a component of nucleotides and phospholipids which are necessary for the integrity of diatom cells. It is

considered in many marine ecosystems as a limiting factor for the growth of diatoms (Chung *et al*., 2011).

1.4 Biofuels from Microalgae

1.4.1 History

Borowitzka and Moheimani (2012) affirmed that farming of plants has thousands of years of history while isolation of microalgae is only about 140 years old, and the commercial microalgae farm is not as much as 60 years old. Simple inorganic media were the early choice to try to culture microalgae (e.g. Cohn (1850) cultivated the chlorophyte *Haematococcus pluvialis* in such media). However, modern microalgae culture began with the experiments of Beijerinck who cultured *Chlorella vulgaris* (Beijerinck, 1890) and the culturing of diatoms by Miquel (1892).

Harder and von Witsch (1942a, b) proposed that microalgae such as diatoms could be appropriate sources for lipid production and Milner (1951) also considered that algae can produce oils via photosynthesis. However, Spoehr and Milner (1948, 1949) and Geoghegan (1951) recognized that the actual lipid productivity from microalgae was low although individual cells can accumulate very high levels of lipids. The analysis of the engineering requirements for algal production systems and work on larger-scale cultures started at the Stanford Research Institute, USA in 1948-1950 (Cook 1950; Burlew 1953a, b).

1.4.2 Biofuels

The use of fossil fuels as a long term source of energy is unsustainable because it is a finite resource and the release of CO_2 into the atmosphere is linked with global warming (Friedlingstein and Solomon, 2005). Liquid biofuels such as ethanol can potentially replace fossil fuels currently used for transport. The use of food crops as the source of ethanol (an example of first generation biofuels) is controversial because of the potential impact on food availability and doubts about sustainability of these fuels in terms of CO_2 emissions and energy balance i.e. energy stored in ethanol/energy used to produce ethanol (Pimentel and Patzek, 2005; Kikuchi *et al.,* 2009). Sousa *et al*. (2009) stated that the food versus fuel debate has limited the ability of first generation feed stocks to meet the demand for the production of biofuels.

Second generation biofuels are based on so-called '' energy crops'' (*Miscanthus* or Switchgrass), which can grow on marginal land. The main problem with second generation feedstock is the difficulty in extracting the lignocellosic substrates, which make up the bulk of the carbon sources in grasses and agricultural waste like straw. Nevertheless, cheap lignoccellulosic substrates should allow second generation biofuels to meet some of the global demand for fuel (Metzger and Hüttermann, 2009; Taylor, 2008; Kim and Dale, 2004).

There is growing interest in third generation biofuels using microalgae as the feedstock. Microalgae can be grown in saline water or waste water and do not compete for arable land and precious freshwater (Gilmour and Zimmerman, 2012). Algae are very efficient at taking up CO_2 from the atmosphere and converting it into organic compounds through the process of

photosynthesis. In fact, microalgae can be used to utilize the CO_2 directly from flue (waste) gases from steelworks or other industries (Zimmerman *et al.*, 2011). Gunstone *et al*. (2007) reported that algal lipids consist of two main kinds: polar lipids such as phosphoglycerides, glycosylglycerides, and non-polar lipids e.g. triacylglycerols, sterols, free (non-esterified) fatty acids, hydrocarbons, wax and steryl esters. Algal biomass can be used in a number of ways to produce biofuels, but the most likely possibility is using microalgae that produce high levels of neutral lipids i.e. triacylglycerol (TAG) as a basis for biodiesel production (Chisti, 2008). Many microalgae, including *Dunaliella*, *Chlorella*, *Nannochloropsis* and *Tetraselmis* can produce high levels of neutral lipids and can be grown in saline media (Chisti, 2008). The key breakthroughs required to make algal biodiesel a commercial reality are: a) finding a highly productive strain that will produce high levels of neutral lipid during growth and not just in stationary phase, b) finding a good method to harvest small microalgal cells efficiently and c) efficient recovery of the lipids from the algal cells (Gilmour and Zimmerman, 2012). One key idea is the formation of ‘’an algal biorefinery‘’ which will utilize microalgal biomass to produce biodiesel, protein for animal feed, health supplements and fertilizer (Chisti, 2008).

In existing biotechnological applications (e.g. health food production), microalgae are grown in large outdoor ponds. To avoid contamination by other algae or bacteria, extremophile algal species are used that grow best at high salinity and/or high pH e.g. the halotolerant microalgae *Dunaliella sp.* (Li and Qi, 1997). An extremophile will thrive in an environment that is inhibitory to contaminating species of microorganisms. The triacylglycerides (TAGs)

produced by the microalgae are extracted and transesterified to form fatty acid methyl esters (FAMEs) which are equivalent to diesel fuel or kerosene depending on the chain length of the fatty acids. However, many practical challenges need to be overcome before this system is commercially viable.

The major breakthrough required is the discovery or of a strain of microalgae that shows as many as possible of the following characteristics: tolerance of high light intensities, tolerance to changes in the composition of the growth medium in the outdoor pond (e.g. changes in salinity caused by evaporation and rainfall), high growth productivity and high yields of TAGs. It is preferable to isolate this ideal algal species from a natural habitat near to the production site, since there are limitations on the use of foreign organisms in many countries. Also, a local species should be pre-adapted to the local climate.

1.5 Molecular Techniques

Nucleic acid sequence information (16S rRNA gene for prokaryotes or 18S rRNA gene for eukaryotes) is used to determine the degree of similarity between groups of organisms and their relationships with other microorganisms found in the universal tree of life. Therefore, the large genetic sequence database of known species can be used to identify newly isolated organisms. It is generall accepted that only around 1% of microbial species can be described based on traditional cultivation methods (Amann *et al*., 1997).

Torsvik *et al*. (2002) provided evidence that there may be hundreds of thousands or even millions of bacterial species, but only about 4000 - 5000

species have been isolated and described by conventional cultivation methods. Therefore, traditional microbiology methods based on phenotypic characters such as morphology, physiology, and metabolism are being replaced by molecular-based analysis (Scow *et al*., 2001). Amann *et al*. (1997) suggested that nucleic acid probes are very useful in microbial ecology by allowing the detection of microorganisms *in situ*. The same authors produced a very useful diagram describing the methods required to identify microorganisms from natural habitats (Figure 1.2). In the modern context, using phenotypic information alone to identify species in microbiology is insufficient. The true composition of microbial communities is described better by the molecular methods.

Using molecular identification techniques has an impact on the definition of a speies (Roszak and Colwell, 1987). Rosello and Amann (2001) described the phylogenetic definition of species as “a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity in many independent characteristic properties”.

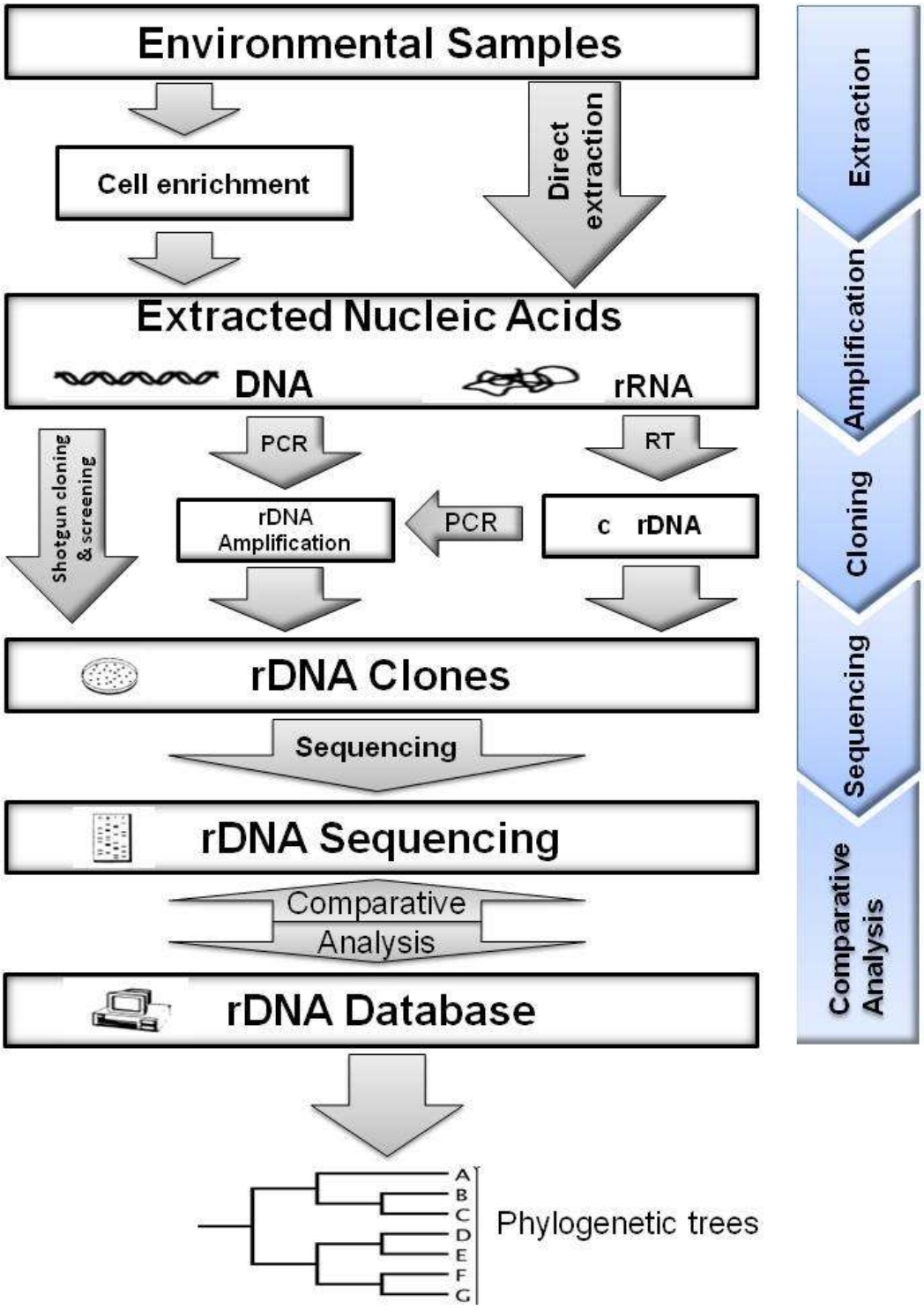

Figure 1.2: Flow diagram of the different stages in the molecular methods used to identify microorganisms from environmental samples. Adapted from Amann *et al*. (1997).

Since the 1980s, 16S rRNA gene sequencing has been used to detect possible phylogenetic relationships between different bacteria to produce a molecular based classification of bacteria from various sources, such as environmental or clinical speciments (Cai *et al*., 2003; Clarridge, 2004; Mignard and Flandrois, 2006). Carl Woese, during the 1970s and 1980s, developed the idea that molecular sequences of highly conserved molecules could be used to detect possible phylogenetic relationships between bacterial groups (Pace, 1997). Olsen *et al.* (1986) noted that three types of rRNA molecules are found in the microbial ribosomes including, 5S rRNA, 16S rRNA (~1500 nucleotides) and 23S rRNA (~3000 nucleotides). The first attempts to use rRNA to characterize microbes were carried out by extracting the 5S rRNA molecules. However, the information content in the approximately 120 nucleotide long molecule is relatively small and therefore it was abandoned in favour of the 1,500 nucleotide long 16S rRNA gene.16S rRNA gene sequence is composed of both variable and conserved region which often contain a high degree of species specificity. The gene is large enough, with statistically relevant sequence information.

As described in Saiki *et al.* (1985), Kary Mullis was the first to invent the polymerase chain reaction in 1983. Polymerase chain reaction (PCR) is a powerful tool in molecular biology to exponentially amplify specific DNA sequences (Lexa *et al*., 2001; Fenollar *et al*., 2006; Yeung *et al*., 2009); a large amount of target DNA sequences can be amplified from tiny amounts of DNA. However, the success of PCR analysis is highly dependent on the selection of appropriate primers (Lexa *et al*., 2001). A useful primer must have various properties; it must be specific to the region that needs to be

replicated, it must join with enough energy to support the experimental conditions and it must not allow the formation of structures that might weaken the reaction. A number of cycles of amplification are performed in the same vial so that enough copies of the desired sequence are reproduced for analysis.

Many modern molecular approaches such as ribosomal DNA restriction analysis, restriction fragment length polymorphism and fluorescent *in situ* hybridization, are now being widely used to identify non-culturable bacteria in a range of habitats including soil, potable water and other aquatic environments (Bockelmann *et al.,* 2000). Often sequencing the 16S rRNA gene is most suitable way to define microbial diversity in novel environments (Pernthaler and Amann, 2005). Many experiments involve the isolation of DNA from natural environments and the use of specific 16 or 18S primers to amplify the rDNA using PCR. If required, the subsequent PCR products can be ligated into commercially available vectors and then transferred into *E. coli* (Amann *et al*., 1997).

A good example of the application of these molecular techniques to microbial ecology was demonstrated by Crump *et al.* (1999). This study involved particle-attached and free-living bacteria in the Columbia River and showed that almost 75% of the particle associated bacteria were members of the Proteobacteria. In contrast the free living bacterial clones correlated with ubiquitous freshwater bacteria (β-Proteobacteria, Gram-positive bacteria, and *Verrucomicrobium spp*).

Romano *et al.* (2007) isolated an alkalitolerant and halotolerant bacterium from a salt lake within Ras Muhammad in Egypt. 16S rRNA sequencing was used to identify the bacterium and the results indicate that the strain was a member of the genus *Halomonas.* Yoon *et al.* (2003) isolated and identified *Halobacillus* as a new species by using molecular methods. Inagaki *et al.* (2002) used a molecular ecological approach to detect and identify microorganisms in a deep sea extreme environment, with molecular phylogenetic analyses of RNA and PCR amplification of 16S rDNA being used.

Drancourt and Raoult (2005) showed that under some circumstances the 16S rRNA gene sequence is not sufficeient for characterization of a new bacterial species. They found that knowledge of the key phenotypic characters such as colony morphology, motility and cell morphology was also essential. Biochemical tests such as oxidase and catalase tests and the capability to metabolize major carbohydrates are still employed to complete the full description of a new species.

1.6 Aims of the Project

The following are the main aims of the work described in this thesis.

- To isolate, identify and characterize salt tolerant bacteria from freshwater samples of river and pond water (Chapters 3 and 4).
- To isolate, identify and characterize salt tolerant microalgal species from pond water (Chapter 5).
- To examine the potential for biofuel production from a species of halotolerant diatom (*Navicula*) isolated from pond water (Chapter 6).

Chapter 2

MATERIALS AND METHODS

2.1 Isolation of Bacteria

The first sample was approximately 45 ml of water taken from Weston Park pond (WP), Sheffield, during January 2009 by collection of the surface water with a sterile 50 ml Falcon tube. The second set of samples was collected from a Derbyshire dew pond (DP) near Tideswell (SK172809) during March 2009 in three separate 50 ml sterile Falcon tubes. Water depth of the dew pond was 2-3 cm. The samples were transported to the laboratory and stored at 4^{o}C for later analysis.

Isolation of microbes from WP samples was either in nutrient medium (broth and agar) or minimal M9 medium (broth and agar) which contained the ingredients shown in section 2.2 with different NaCl concentrations (Table 2.1). The influence of temperature on growth was studied using WP samples only by incubation of inoculated minimal medium and nutrient medium (liquid and solid) at 25^{o}C and 37^{o}C. Initial cultivation was carried out by taking 5 ml of the sample and adding to 50 ml of minimal media and nutrient medium separately in 9 x 250 ml autoclaved flasks. All flasks were incubated with shaking (250rpm) at 25^{o}C and 37^{o}C overnight. The inoculation step was repeated three times.

Three plates of both minimal medium and nutrient medium were streaked from each flask of both samples (WP1 and WP2) and incubated at 25^{o}C and 37^{o}C overnight. The plating step was repeated three times for each isolate to ensure purity and the isolates were given strain numbers.

On the other hand DP samples were incubated at 25^{o}C only, and all DP samples were grown in M9 minimal medium.

2.1.1 Media for Bacterial Growth

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

NaCl concentrations		
Weight (g l^{-1})	Molar (M)	Percentage (% w/v)
14.61	0.25	1.45
29.22	0.5	2.9
43.83	0.75	4.4

Table 2.1: Concentrations of NaCl used in both LB medium and M9 minimal medium.

2.1.1.1 Minimal M9 medium

M9 minimal medium was prepared as shown in Table 2.2. All solutions were autoclaved separately and the glucose, NH_4Cl, $MgSO_4$, and $CaCl_2$ were added to the minimal solution after cooling. The composition of the M9 minimal salts is shown in Table 2.3. To prepare solid medium, 15 g l^{-1} Agar No 1 (Oxoid) was added to the minimal salts before autoclaving.

Ingredient	Weight (w/v)
M9 minimal salts (Sigma M-6030)	11.3 g in 980 ml of distilled water
Glucose	9 ml of (15 g in 45 ml)
NH_4Cl	9 ml of (5 g in 45 ml)
$MgSO_4$	1ml of 1 M $MgSO_4$
$CaCl_2$	1 ml $CaCl_2$ (100 mM)

Table 2.2: Composition of M9 minimal medium.

Compounds	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

Table 2.3: Composition of M9 minimal salts (Sigma M-6030).

2.1.1.2 Nutrient medium

The recipes for nutrient broth and nutrient agar are shown in Tables 2.4 and 2.5 below.

Ingredient	**g/litre**
Lab-Lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0

Table 2.4: Composition of Nutrient Broth (Oxoid CM0001).

Ingredient	**g/litre**
Lab-Lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15

Table 2.5: Composition of Nutrient Agar (Oxoid CM0003).

2.1.2 Phenotypic Characterization of Bacterial Isolates

2.1.2.1 Gram stain

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

2.1.2.2 Motility

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

2.1.2.3 Growth under anaerobic conditions

Effect of oxygen on the growth of the strains was examined in both broth and plate minimal M9 medium using Gas-Pak Anaerobic System to generate an oxygen free environment for growth of the strains. 15 ml sterile tubes

containing minimal M9 medium were inoculated with 1 ml overnight culture and also, streaked plates were placed inside the chamber, and anaerobic conditions were made by adding water to a gas generator envelope (containing, sodium borohydride and sodium bicarbonate) in order to produce hydrogen gas and carbon dioxide. The reaction was catalyzed by the wire palladium, which was found in the bottom of the cover of the jar. Anaerobic conditions inside the chamber were confirmed by using a colour indicator.

2.1.2.4 Optimum temperature for growth

The influence of temperature on growth was studied by incubation of inoculated minimal M9 and nutrient media (liquid and solid) at 25ºC and 37ºC, with shaking (250rpm) for liquid cultures.

2.1.3 Measurement of Respiration and Photosynthesis of Bacterial Isolates using an Oxygen Electrode

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

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

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

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

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

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

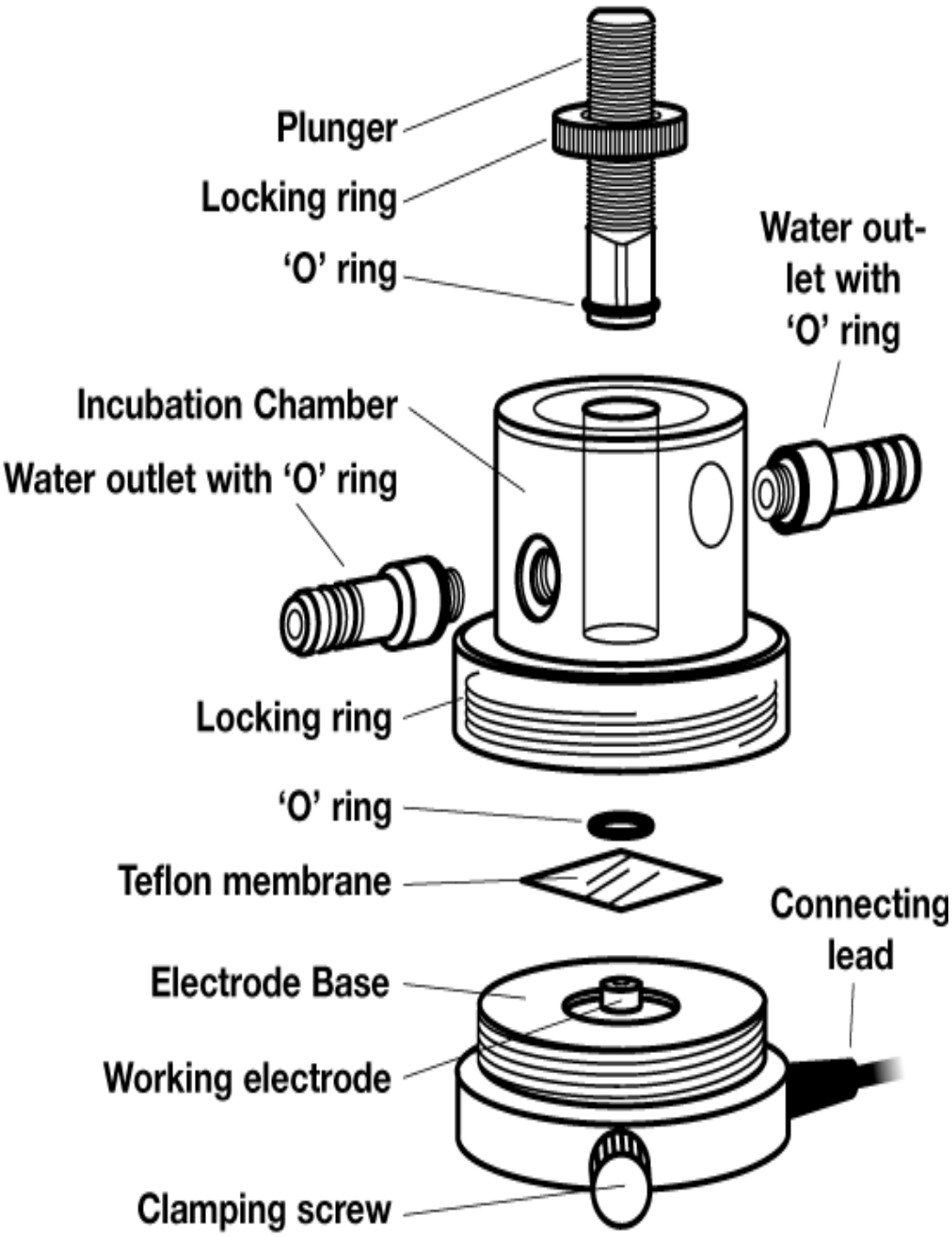

Figure 2.1: Schematic diagram illustrating the key components of an oxygen electrode. Figure from http:// www.rankbrothers.co.uk/prod lexp.htm.

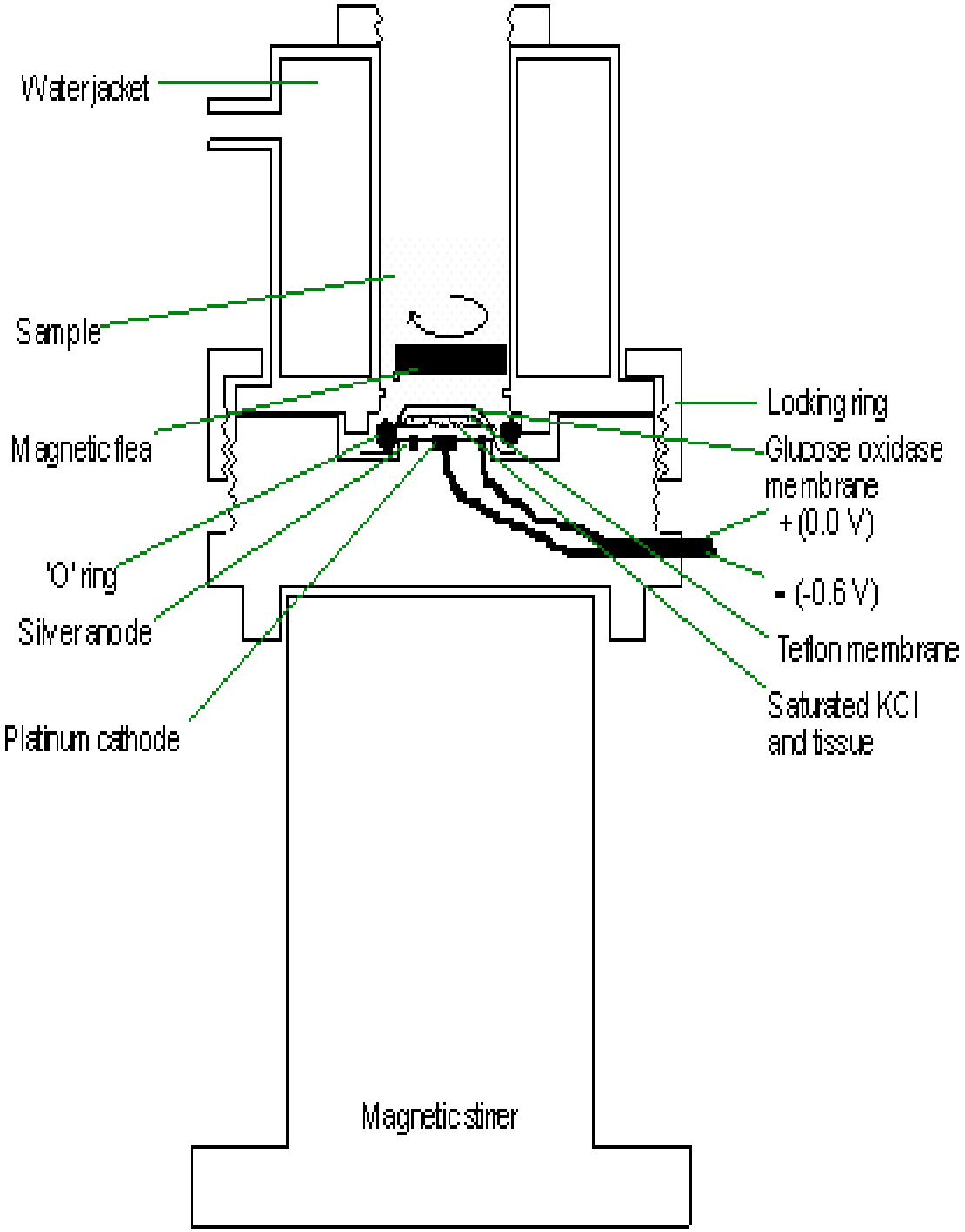

Figure 2.2: A diagram of the oxygen electrode showing more details of the electrode components. Only a Teflon membrane was used in the current work and the tissue was cigarette paper.

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

To calculate the respiration rate we need to know the concentration of O_2 in air saturated water at 25°C and the protein content of the bacterial suspension (see section 2.7). Then the following equation is used:

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

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

- **Standard:** Amount of oxygen solubility in 2 ml medium (sample) = 0.660 µmoles ml^{-1} at 30°C or 0.722 µmoles ml^{-1} at 25°C
- **Range:** Units taken from calibration (0 – 100%)
- **Number of units:** Number of units are read directly from the chart recorder, it is normal to draw best fit straight line over 5 min
- **Time:** The length of time in minutes for which the sample was measured
- **60:** This converts the time from minutes to hours
- **Protein present in sample (mg):** this relates to amount of protein in a sample of 2 ml of cells from Bradford assay (see section 2.2).

To calculate the photosynthesis rate of an algal cell suspension, the same equation is used to calculate both oxygen evolution in the light and oxygen uptake in the dark and OD_{600} is measured instead of protein content.

2.1.4 Effect of Salinity on Respiration Rate

Effect of salinity on the rate of oxygen uptake by WP and DP bacterial isolates was assayed using an oxygen electrode in two types of experiments,

immediately after salinity shock and after incubation in the new salinity. To prepare cells for measurements in the oxygen electrode, the OD_{600} of overnight cultures was measured and 5 ml cells from each sample grown at 0.0 M, 0.25 M, 0.5 M and 0.75 M NaCl in minimal M9 medium were centrifuged at 3000 *g* in the bench centrifuge for 10 minutes and then each pellet was re-suspended in 2 ml of minimal M9 medium of the same salinity. The respiration rate of 2 ml of each sample was measured three times in the oxygen electrode with distilled water washes between each sample. The temperature of reaction chamber was controlled at 25^{o}C, 30^{o}C and 37^{o}C. The oxygen electrode was calibrated using sodium dithionite to remove all the oxygen from air saturated water. The protein content of both strains was estimated by the Bradford (1976) assay (section 2.2). The respiration rate was calculated using the equation in section 2.1.3.

2.2 Determination of Protein Content of Bacterial isolates

2.2.1 Determination of Standard Curve

A standard curve was produced using Bovine serum albumin (BSA) in the range of 0 - 100 µg by dissolving 250 mg of BSA in 50 ml of distilled water. The final concentration of protein in the stock solution was 5 mg ml^{-1} (i.e. 5 µg $µl^{-1}$). The stock solution was used to make a range of protein concentrations by a series of dilutions as shown in Table 2.7.

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

Table 2.6: Components in test tubes which were needed to make a standard protein curve.

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

2.2.2 Determination of Sample Protein

Soluble protein in bacterial cells was measured using the method described by Bradford (1976). Bovine serum albumin (BSA) was used as the standard as described in section 2.2.1.

Samples were prepared for protein determination as follows:

- 0.1 ml from the same concentrated cells which were prepared for respiration rate measurements (see section 2.1.3) was placed into a clean test tube and then 0.9 ml of 1M NaOH was added. The sample was then thoroughly mixed and heated at 90°C for 10 minutes.
- The test tube was cooled on ice for 5 minutes and dried outside and then centrifuged for 10 minutes in bench centrifuge at full speed (3000 *g*).
- 0.3 ml of the resulting supernatant was added to 3 ml of Bradford's reagent in a fresh test tube and the contents were thoroughly mixed. After 5 minutes incubation at room temperature the optical density was measured in the Unicam Helisα spectrophotometer against 0.1 ml water plus 3 ml of Bradford's reagent (blank) at 595 nm.

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

2.3 Determination of Carbon Source Utilization by BIOLOG Tests

DP and WP strains were grown in M9 minimal medium overnight, then the cells were washed with minimal medium without glucose three times with a bench centrifuge step between each washing. The OD_{600} was adjusted to 0.2 by diluting with fresh M9 medium without any carbon source.150 µl of bacterial suspension were pipetted into 96 wells of Gramnegative (GN) plate which was incubated overnight at 25^{o}C and red in the Viktor plate reader.

A1 Water	A2 a-Cyclodextrin	A3 Dextrin	A4 Glycogen	A5 Tween 40	A6 Tween 80	A7 N-Acetyl-Dgalactosamine	A8 N-Acetyl-Dglucosamine	A9 Adonitol	A10 L-Arabinose	A11 D-Arabitol	A12 D-Cellobiose
B1 i-Erythritol	B2 D-Fructose	B3 L-Fucose	B4 D-Galactose	B5 Gentiobiose	B6 α-D-Glucose	B7 m-Inositol	B8 α-D-Lactose	B9 Lactulose	B10 Maltose	B11 D-Mannitol	B12 D-Mannose
C1 D-Melibiose	C2 β-Methyl-D-Glucoside	C3 D-Psicose	C5 L-Rhamnose	C5 L-Rhamnose	C6 D-Sorbitol	C7 Sucrose	C8 D-Trehalose	C9 Turanose	C10 Xylitol	C11 Pyruvate Acid Methyl Ester	C12 Succinate Acid Mono-Methyl-Ester
D1 Acetic acid	D2 Cis-Aconitic Acid	D3 Citric Acid	D4 Formic Acid	D5 D-Galactonic Acid Lactone	D6 D-Galacturonic Acid	D7 D-Gluconic Acid	D8 D-Glucosaminic Acid	D9 D-Glucuronic Acid	D10 α-Hydroxy Butyric Acid	D11 β-Hydroxy Butyric Acid	D12 Y-Hydroxy Butyric Acid
E1 p-Hydroxy Phenylacetic Acid	E2 Itaconic Acid	E3 α-Keto Butyric Acid	E4 α-Keto Glutaric Acid	E5 α-Keto Valeric Acid	E6 D,L-Lactic Acid	E7 Malonic Acid	E8 Propionic Acid	E9 Quinic Acid	E10 D-Saccharic Acid	E11 Sebacic Acid	E12 Succinic Acid
F1 Bromo Succinic Acid	F2 Succinamic Acid	F3 Glucuronamide	F4 L-Alaninamide	F5 D-Alanine	F6 L-Alanine	F7 L-Alanylglycine	F8 L-Asparagine	F9 L-Aspartic Acid	F10 L-Glutamic Acid	F11 Glycyl-LAspartic Acid	F12 Glycyl-LGlutamic Acid
G1 L-Histidine	G2 Hydroxy-LProline	G3 L-Leucine	G4 L-Ornithine	G5 LPhenylalanine	G6 L-Proline	G7 L-Pyroglutamic Acid	G8 D-Serine	G9 L-Serine	G10 L-Threonine	G11 D,L-Carnitine	G12 Y-Amino Butyric Acid
H1 Urocanic Acid	H2 Inosine	H3 Uridine	H4 Thymidine	H5 Phenyethylamine	H6 Putrescine	H7 2-Aminoethanol	H8 2,3-Butanediol	H9 Glycerol	H10 D,L-α-Glycerol Phosphate	H11 Glucose-1-Phosphate	H12 Glucose-6-Phosphate

Table 2.7: Carbon Sources in GN MicroPlate.

2.4 Determination of Growth Curves

Bacterial cultures were inoculated from overnight grown cultures in the same medium and optical density (OD) was measured with wavelength of 600 nm for four replicate 250 ml flasks (50ml of media) the inoculum was 3 ml of overnight culture to give an OD_{600} between 0.1 and 0.2. The OD_{600} was measured every hour throughout the day using un-inoculated medium as a blank.

The effect of changing salinity (NaCl) on the growth of bacteria was determined using overnight cultures in minimal M9 medium. 1 ml of active inoculum from each strain was inoculated into minimal M9 medium containing 0.25 M, 0.5 M and 0.75 M NaCl and the optical density (OD) at 600 nm was measured using the Unicam Helisα spectrophotometer using 1 ml plastic cuvettes. M9 minimal medium containing the appropriate NaCl concentration was used as the blank.

To produce a growth curve 4 X 250 ml conical flasks containing 50 ml of each salinity value minimal M9 medium were each inoculated with 1 ml of the same salinity adapted cells from an overnight culture. The OD_{600} was measured against same salinity value minimal M9 medium blank immediately after inoculation then every hour over an incubation period at 25ºC on a rotary shaker at 250 rpm. The growth curves were plotted against time of incubation.

2.5 Isolation of Microalgae

Approximately 45 ml of water was sampled on 01/06/2010 from Weston Park pond (Sheffield, UK) in three locations (the SE corner, the N corner and the SW corner) by collection of the surface water with sterile 50 ml Falcon tubes. 10 ml of each of these samples were transferred by pipette into 6 separate conical flasks containing 50 ml of 0.4 M NaCl *Dunaliella* medium (Table 2.11). These flasks were gently mixed before being placed in the growth room with 3 of each set of flasks stationary on the shelf and 3 of each set shaken at 250 rpm. After a fortnight in the growth room, microalgal growth was observed in all stationary samples, but no growth was observed in flasks that were shaken. Microalgae were maintained in an active growth phase by regular sub-culturing approximately every 15 days, into *Dunaliella* medium with a NaCl concentration of 1.5 M.

Other samples were collected on 01/09/2010 as described above. 10 ml of each of these samples were transferred by pipette into 6 separate conical flasks containing 50 ml of Basal Bold's medium (BBM) see Table 2.9. These flasks were gently mixed before being placed in the growth room stationary on the shelf at 25^0C. After three weeks in the growth room, microalgal growth was observed in all samples. 5 ml of each culture were transferred in to 250 conical flasks containing 0.2 M NaCl BBM and 250 conical flasks containing f/2 artificial sea water medium (Table 2.8) and incubated without being shaken. After a week in the growth room, microalgal growth was observed in all samples. The cultures taken from the samples were then subcultured approximately every 10 days using the method described above, which is 5 ml of culture into 50 ml of fresh medium. Cultures which showed no growth

were discarded. All of the samples were eventually sub-cultured into 0.4 M NaCl BBM and f/2 medium and then finally by a number of sub-cultures after approximately six months, These had been sub-cultured into 0.6 M NaCl BBM and 0.6 M f/2 medium.

2.5.1 Media for Microalgal Growth

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

2.5.1.1 f/2 medium

33.6 g of Ultramarine Synthetica Sea Salt were dissolved in to 950 ml of dH_2O and made to 1 litre, then the components listed in Table 2.8 were added to 950 ml of the artificial sea water.

Component	**Stock Solution g l^{-1} dH_2O**	**Quantity Used**
$NaNO_3$	75	1 ml
$NaH_2PO_4. H_2O$	5	1 ml
$Na_2SiO_3. 9H_2O$	30	1 ml
Trace Metals solution	-	1 ml
Vitamins solution	-	0.5 ml

Table 2.8: Composition of f/2 medium nutrients.

2.5.1.2 Bold's Basal Medium (BBM)

The ingredients in Table 2.9 were added to 900 ml of distilled water in a 1 litre Duran bottle and the pH was adjusted to 7, then made up to 1 litre and autoclaved.

Ingredient	**Stock Solution**	**Quantity Used**
$NaNo_3$	25.00	10 ml
$CaCl_2\,2H_2O$	2.50	10 ml
$MgSO_4\,7H_2O$	7.50	10 ml
K_2HPO_4	7.50	10 ml
KH_2PO_4	17.50	10 ml
NaCl	2.50	10 ml
Boran Solution	11.42	1 ml
Trace Metals solution	-	1 ml
Vitamins solution	-	0.5 ml

Table 2.9: Composition of Bold's basal medium (BBM).

NaCl Concentrations		
Weight ($g\ l^{-1}$)	Molar (M)	Percentage (% w/v)
11.68	0.2	1.2
23.36	0.4	2.3
35.04	0.6	3.5
46.72	0.8	4.7

Table 2.10: Concentrations of NaCl used in both f/2 and BBM medium.

2.5.1.3 *Dunaliella* medium

Dunaliella medium was prepared as described by Hard (1991). Stock solutions were prepared using distilled water and stored at room temperature. All chemicals were "Analar" grade and obtained from different companies mainly Sigma.

Stock Solution	Volume (ml)	Final Concentration (mM)
KCl	5.0	10
$MgCl_2$	10.0	20
$CaCl_2$	10.0	10
$MgSO_4$	10.0	24
$NaNO_3$	1.25	5
Na_2SO_4	48.0	24
NaH_2PO_4	1.0	0.1
FeEDTA	1.0	0.0015
Micronutrients	1.0	-
Tris-HCl	20.0	20

Table 2.11: Composition of *Dunaliella* medium.

NaCl Concentrations		
Weight (g l^{-1})	Molar (M)	Percentage (% w/v)
29.2	0.5	2.9
58.4	1.0	5.8
87.6	1.5	8.8
116.8	2.0	11.7
146	2.5	14.6
175.2	3.0	17.5

Table 2.12: Concentrations of NaCl used in *Dunaliella* medium.

2.6 Growth Curve of Algal Strains

2.6.1 Adaptation and Growth Curves for Algal Strains Adapted to Different Salinities

Algal cultures were inoculated from stationary phase cultures in the same medium and optical density (OD) was measured with wavelength of 600 nm. For 250 ml flasks (50ml of media), the inoculum was 5 ml of stationary phase culture. The OD_{600} was measured every day using un-inoculated medium as a blank.

The effect of changing salinity (NaCl) on the growth of algae was determined using stationary phase cultures grown in BBM and f/2 media. 5 ml of viable cells from a BBM culture were inoculated into BBM medium containing 0.4 M, 0.6 M, 0.8 M and 1 M NaCl. 5 ml of viable cells from a f/2 culture were inoculated into f/2 medium containing 0.4 M, 0.6 M and 0.8 M NaCl. The OD_{600} was measured using the Unicam Helisα spectrophotometer using 1 ml

plastic cuvettes. BBM and f/2 media containing the appropriate NaCl concentration were used as the blank.

To produce a growth curve, 250 ml conical flasks containing 50 ml of each medium containing different salinity values were each inoculated with 5 ml of the same salinity adapted cells from a stationary phase cultures. The OD_{600} was measured against the same salinity value of each medium blank immediately after inoculation then every day over an incubation period at 25ºC. The growth curves were plotted against time of incubation.

2.6.2 Adaptation and Growth curves of Algal Strains Adapted to Different pH Values

The effect of changing pH on the growth of algae was determined using stationary phase cultures in BBM and f/2 media. 5 ml of viable cells from a BBM culture were inoculated into BBM medium with pH 7, 9 and 11. 5 ml of viable cells from a f/2 culture were inoculated into f/2 medium with pH 7.6, 8.5, 9.25 and 10. The OD_{600} was measured using the Unicam Helisα spectrophotometer using 1 ml plastic cuvettes. BBM and f/2 media with the appropriate pH concentration were used as the blank.

To produce a growth curve, 250 ml conical flasks containing 50 ml of each medium containing different pH values were each inoculated with 5 ml of the same pH adapted cells from a stationary phase culture. The OD_{600} was measured against pH value of each medium blank immediately after inoculation then every day over an incubation period at 25ºC. The growth curves were plotted against time of incubation.

2.6.3 Adaptation and Growth Curves of Algal Strain to Different Concentrations of Silica

The effect of changing concentration of silica on the growth of algae was determined using stationary phase cultures in BBM and f/2 media. 5 ml of viable cells from a BBM culture were inoculated into BBM medium containing 25%, 50% and 100% silica. 5 ml of viable cells from a f/2 culture were inoculated into f/2 medium containing 25%, 50%, 100% and 200% silica. The OD_{600} was measured using the Unicam Helisα spectrophotometer using 1 ml plastic cuvettes. BBM and f/2 media containing the appropriate silica concentration were used as the blank.

To produce a growth curve, 250 ml conical flasks containing 50 ml of each medium containing different concentrations of silica were each inoculated with 5 ml of the same silica concentration adapted cells from a stationary phase culture. The OD_{600} was measured against the same silica concentration value of each medium blank immediately after inoculation and then every day over an incubation period at 25ºC. The growth curves were plotted against time of incubation.

2.7 Determination of Algal Cell Number

900 µl of algal culture were added with 100 µl of Gram's Iodine to an Eppendorf tube and the solution mixed. An aliquot (20 µl) of the stained algal culture was placed in the centre of the counting chamber of a Helber slide. The slide was positioned on the microscope under the x40 objective. The algal cells were counted in four large squares – one hundred small squares in total, the cell count was repeated ten times for each sample. Using the

mean number of cells per small square, the number of cells in the original culture was calculated as follows:

$$Depth = 0.02mm; area\ of\ small\ square = {}^{1}/_{400}\, mm^2 = 2.5 \times 10^{-3} mm^2$$

$$\therefore volume\ of\ small\ square = 2.5 \times 10^{-3} \times 0.02 = 5.0 \times 10^{-5} mm^3 = 5.0 \times 10^{-8} cm^3 (\equiv mL)$$

$$Cell\ no.per\ small\ square \times \frac{1}{5 \times 10^{-8}} = cells\ mL^{-1} sample$$

$$\frac{cells\ mL\ sample}{9} \times 10 = cells\ mL^{-1} original\ culture$$

2.8 Lipid Determination of Algal Strains by Gravimetric/Colorimetric Methods

To determine the lipid content of algal strains, 3 x 15 ml of a well grown algal culture were centrifuged for 10 minutes at 3000 *g*, supernatant was discarded. Each pellet was re-suspended in 5 ml of distilled water and centrifuged for 5 minutes at 3000*g*. The step was repeated twice and then each pellet were re-suspended in 1 ml of distilled water. Three Eppendorf tubes were labelled and weighed on the fine balance and then 1 ml samples were transferred to the three pre-weighed Eppendorf tubes.

The lids of another three Eppendorf tubes were cut off and a hole was made in the lids using a dissecting needle or scissors. These lids were put on the three Eppendorf tubes containing the samples. The samples were frozen at minus 80°C overnight and then freeze dried (lyophilized) for 24 hours. The Eppendorfs were then re-weighed to estimate the weight of biomass.

500 µl of methanol/chloroform (2:1 v/v) were added and whirlimixed. Then 250 µl of the solution were transferred to another Eppendorf tube and

labelled as ‘A’ and ‘B’. Each tube was sonicated on ice for 1 min total, using 20 second bursts and each tube was then centrifuged in the microfuge at full speed for 5 mins and transferred to a fresh Eppendorf to estimate volume of supernatant. 67 µl methanol, 233 µl chloroform and 200 µl 1% NaCl were added to each sample to give a 2:2:1 ratio of methanol:chloroform:1% NaCl.

Samples were centrifuged in a microfuge for 2 minutes at full speed and fresh Eppendorfs were labelled and weighed and the chloroform phase (green phase) was transferred to the pre-weighed Eppendorfs. The chloroform was evaporated from the sample slowly on a heat block set to 80°C for 20-30mins (all liquid was evaporated before carrying on, with heat on higher than 80°C to avoid spitting). Eppendorfs were then reweighed to estimate lipid weight.

0.1ml of concentrated sulphuric acid was added to each sample (directly added to the bottom of the Eppendorf), and vortexed thoroughly (quickly microcentrifuge the Eppendorfs if necessary to bring all the acid and lipid to the bottom, this cycle is repeated until all lipids look dissolved). The samples were then placed in the heat block at 100°C for 15 mins and allowed to cool to room temperature. A 15ml Falcon tube for each sample was prepared containing 2.4 ml vanillin reagent (protected from light). The spectrophotometer was blanked with 100% acetone at 490nm. Samples were tested in cycles of five, 10 µl of acid-lipid sample were added to the 2.4 ml vanillin tubes and vortexed thoroughly, with waiting for one minute before adding the next 10 µl sample to the next vanillin tube etc. Exactly 5 min after vortexing every vanillin-lipid sample; 1 ml of the contents was pipetted into a cuvette and measured the OD490 (Van Handel, 1985. Bligh and Dryer, 1959)

2.9 Lipid Determination by Nile Red Fluorescence Method

2.9.1 Cell count and OD_{595} Method

30 ml of well grown algal culture were taken and adjusted to an OD of 1 at 595 nm then dilutions were made from 5% to 100% of adjusted culture in 11 autoclaved 1.5 ml Eppendorf tube. 8 X 200 µl were transferred to a 96 well microplate. The prepared 96 well microplate was placed in the plate reader and the data were saved on a memory stick. 900 µL of algal culture were added with 100 µL of Gram's Iodine to an Eppendorf tube and the solution mixed. An aliquot 20 µL of the stained algal culture was placed in the centre of the counting chamber of a Helber slide. The slide positioned on the microscope at x40 objective. The algal cells were counted in four large squares – one hundred small squares in total, the cell count was repeated ten times for each sample (Madigan *et al.*, 2003; Reed, 1998; Skoog *et al.*, 2007)

2.9.2 Nile Red Peak Fluorescence

10ml of stationary phase culture was removed from a growth vessel and the OD was adjusted to 1 at 595 nm (appropriate medium was used to blank). The adjusted culture was centrifuged for 5 minutes at 3000 *g* and the supernatant was discarded immediately. The supernatant was replaced with an equivalent volume of fresh media and mixed until algal pellet is re-suspended. The culture was pipetted using the following concentrations into 2ml Eppendorf tubes:

Percentage:	100	87.5	75	62.5	50	37.5	25	12.5	Total (ml)
Culture	2000	1750	1500	1250	1000	750	500	250	9
Medium	0	250	500	750	1000	1250	1500	1750	7

The culture was vortexed, and then 4 x 200 µl were removed from each 2 ml Eppendorf tube and added to the plate as the 'unstained cells. An additional 200 µl were discarded from theEppendorf tube to make the remaining volume 1 ml. The plate was placed in the plate reader and a reading at OD_{595} was taken, and the results were saved. The timer was started, and then 20 µl of the 15.9 µg ml^{-1} Nile red stock solution were added to each Eppendorf tube quickly. Each tube was whirlimixed then the contents were transferred to a row of the reagent reservoir. Stained samples were transferred to appropriate wells using a 200 µl multichannel pipette. The plate was placed in the plate reader, the timer was stopped when the start button was clicked and a note of the time was made. The data were saved on a memory stick (Chen *et al*., 2009; Alonzo and Mayzaud, 1999; Pick and Rachutin-Zalogin, 2012; Gardner *et al*., 2011; Cooksey, *et al*. 1987; Elsey *et al*., 2007; Bertozzini *et al.,* 2011)

2.9.3 Nile Red Concentration Test

10ml of stationary phase culture was removed from the growth vessel and the OD was adjusted to 1 at 595 nm (appropriate medium was used for the blank). The adjusted culture was centrifuged for 5 minutes at 3000 *g* and the supernatant was discarded immediately. The supernatant was replaced with an equivalent volume of fresh media and mixed until the algal pellet was re-

suspended. Stationary phase culture was pipetted into 5 separate 2 ml Eppendorf tubes, one for each Nile Red concentration 0.25 - 3 µmoles ml^{-1}.

The culture was vortexed, and then 4 x 200 µl aliquots were removed from each 2 ml Eppendorf tube and added to the plate as the 'unstained cells. An additional 200 µl were discarded from the Eppendorf tube to make the remaining volume 1 ml. The plate was placed in the plate reader and a reading was taken at OD_{595}. The results were saved.

The timer was started, and then 20 µl of the 15.9 µg ml^{-1} Nile Red stock solution were added to each Eppendorf tube quickly. Each tube was whirlimixed and then the contents were transferred to a row of the reagent reservoir. Stained samples were transferred to appropriate wells using a 200 µl multichannel pipette. The plate was placed in the plate reader, the timer was stopped when the start button was clicked and a note of the time was made. The data were saved to a memory stick (Chen *et al*., 2009; Alonzo and Mayzaud, 1999; Pick and Rachutin-Zalogin, 2012; Gardner *et al*., 2011; Cooksey *et al*., 1987; Elsey *et al*., 2007; Bertozzini *et al.,* 2011)

2.9.4 Nile Red Triolein Concentration Test

16 ml of culture were removed from the growth vessel and the OD adjusted to 1 at 595 nm (appropriate medium was used to blank). The adjusted culture was centrifuged for 5 minutes at 3000 *g* and the supernatant was discarded immediately. The supernatant was replaced with an equivalent volume of fresh media and mixed until the algal pellet was re-suspended. The culture was vortex, then 4 x 200 µl were removed from each 2 ml Eppendorf tube

and added to the plate as the 'unstained cells. An additional 200 µl were discarded from the Eppendorf tube to make the remaining volume 1ml.

The plates was placed in the plate reader and a reading was taken at OD_{595}, and the results were saved. The timer was started, and then 20 µl of the 15.9 µg ml^{-1} Nile red stock was added. Each tube was whirlimixed and then the contents were transferred to a row of the reagent reservoir. Stained samples were transferred to appropriate wells using a 200 µl multichannel pipette. The plate was placed in the plate reader, the timer was stopped when the start button was clicked and a note of the time was made. The data were saved to a memory stick (Chen *et al*., 2009; Alonzo and Mayzaud, 1999; Pick and Rachutin-Zalogin, 2012; Gardner *et al*., 2011; Cooksey *et al*., 1987; Elsey *et al*., 2007; Bertozzini *et al.,* 2011)

2.9.5 Nile Red Sample Measurement Test

7 ml from each culture vessel were aseptically removed; 2 ml were added to a 2 ml Eppendorf tube for the Nile Red measurement and 5 ml to a 15 ml Falcon tube for the dry weight calculation.

1 ml of culture was taken from the 2 ml tube and the OD at 595 nm was measured using a 1 ml plastic cuvette, diluting with native medium where appropriate if the OD is higher than 1. When finished, the sample was pipetted back into the 2 ml tube. The 2 ml tube was microfuged for 10 minutes at 14,000 *g* and then the supernatant was discarded immediately. The pellet in the 2 ml tube was re-suspended with fresh medium (the medium

used for re-suspension is the same as used from the previous optimisation tests, regardless if a strain was grown in a different medium).

The 2 ml of culture were adjusted to the optimal cell concentration using OD at 595 nm by transferring between the 2 ml tube and a 1 ml plastic cuvette, diluting with media where appropriate. When finished, Transferred was back to the 2 ml Eppendorf tube. The 2 ml of culture were vortexed, then 3 x 200 µl were removed from each 2 ml Eppendorf tube and added to the plate as the 'unstained cells' at the relevant sample number. An additional 400 µl were discarded from the Eppendorf tube to make the remaining volume 1 ml. The plate was placed in the plate reader and a reading was taken at OD_{595}. The results were saved.

The timer was started, then 20 µL of the Nile red stock solution were added to each Eppendorf tube quickly. Each tube was whirlimixed and then the contents were transferred to a row of the reagent reservoir. Stained samples were transferred to appropriate wells using a 200 µl multichannel pipette. The plate was placed in the plate reader, and the lid was removed. When the appropriate time elapsed for peak fluorescence to occur, the start button was clicked. When finished, the data were saved on a memory stick and the plate was removed from the reader.

The 15 ml Falcon tube from step 1 were retrieved, and centrifuged at full speed for 10 minutes**.** The supernatant was discarded, and re-suspended in 5 ml of distilled water (gently pipette to avoid breaking up the pellet), then the centrifugation step was repeated. The supernatant was discarded again, and 1 ml of distilled water was added and re-suspended as before. Each of the

oven dried crucibles was weighed and the contents of each tube were transferred into a pre-weighed oven dried crucible.

The drying oven was set at 50°C, the sample in the crucible was placed in the oven and left overnight. The weight was measured the next day and compared with the pre-weighed values to determine the net biomass with respect to cell number (via the OD measurement) (Chen *et al*., 2009; Alonzo and Mayzaud, 1999; Pick and Rachutin-Zalogin, 2012; Gardner *et al*., 2011; Cooksey *et al*., 1987; Elsey *et al*., 2007; Bertozzini *et al.,* 2011).

2.10 Lipid Determination by Direct Transesterification Method

3 x 20 ml of a well grown algal culture were centrifuged for 10 minutes at 3000 *g*. The supernatant was discarded and each pellet was re-suspended in 5 ml of distilled water and centrifuged again for 5 minutes at 3000 *g*. The supernatant was again discarded and the pellet was re-suspended in 1 ml of distilled water. Three Eppendorf tubes were labelled and each tube was weighed on the fine balance. Then 1 ml samples were transferred to the three pre-weighed Eppendorf tubes. Three lids of Eppendorf tubes were cut off and a hole was made in them using fine blade scissors. These lids were put on the three Eppendorf tubes containing the samples.

The samples were frozen at minus 80°C overnight and then freeze dried (lyophilized) for 24 hours. The Eppendorfs were re-weighed and 10 mg of the biomass were transferred to threaded glass tubes. 0.2 ml chloroform/methanol (2:1: v/v) and 0.3 ml HCl/ Methanol (5% v/v) were added to each tube. The tubes were sealed and placed in a hot plate set at

85°C within the fume cupboard for 1 hour. The tubes were opened and 1 ml of hexane was added to each tube. The tubes were re-sealed and left at room temperature for at least 1 hour. A 1:10 dilution of the extract in hexane was then quantified by gas chromatography (GC).

2.11 Nuclear Magnetic Resonance (NMR) Analysis of Compatible Solutes

2.11.1 Bacterial Isolates

A 5 ml sample of DP or WP cells was put into a 15 ml Falcon tube and centrifuged at 3000 *g* for 10 min and the supernatant was discarded. Then the cells were washed twice by re-suspension in 5 ml of M9 Minimal medium free of glucose and centrifuged as mentioned above. The pellet was kept in a freezer until the analysis was due.

The sample was thawed, resuspended in 1 ml M9 minimal medium free of carbon source and whirlimixed for 1 minute at room temperature. Then it was sonicated (3 x 20 seconds) and centrifuged at full speed in the microfuge for 10 minutes. The supernatants were transferred into two Eppendorf tubes and kept in -80^{o}C freezer overnight and then freeze dried for two days.

Dried samples were dissolved in 500 µl of deuterium oxide (D_2O) and 5 µl of trimethyl silyl propionate (TSP), then transferred to 5 mm NMR tubes and hand centrifuged. NMR spectra were measured on a Bruker AMX-500 spectrometer operating at 125.8 MHz and 500 MHz for the 13C and 1H nucleus (Leighton *et al*., 2001; Zhu *et al*., 2008).

2.11.2 Algal isolates

A 100 ml sample of algal cells was put into 2 x 50 ml Falcon tubes and centrifuged at 3000 g for 10 min, and the supernatant was discarded. The pellet was kept in a freezer until the analysis was due. The sample was thawed, resuspended in 1 ml distilled water and whirlimixed for 1 minute at room temperature. Then it was sonicated (3 x 20 seconds) and centrifuged at full speed in the microfuge for 10 minutes. The supernatants were transferred into two Eppendorf tubes and kept in -80^{o}C freezer overnight and then freeze dried for two days.

2.12 Molecular Identification Techniques

2.12.1 Extraction of Genomic DNA (gDNA)

gDNA was extracted from both bacterial and microalgae strains by using one of the following methods.

2.12.1.1 CTAB method

Extraction of gDNA using CTAB (2% cetyltrimethyl ammonium bromide, 2% β-mercaptoethanol, 0.1 M Tris-HCl pH8.0, 1.4 M NaCl, 20 mM EDTA) was achieved as described by Aguilera *et al*. (2006). Using freshly grown bacteria diluted with fresh M9 minimal medium (nutrient medium for WP2) to give an optical density (OD_{600}) of approximately 0.5, the samples were centrifuged at 3000 *g* for 10 min. The supernatant was discarded and the pellet resuspended in 500 µl of CTAB buffer in a 1.5 ml Eppendorf tube. The mixture was thoroughly vortexed and then incubated at 65^{o}C for 1 hour. DNA

was extracted with an equal volume of phenol-chloroform isoamylalcohol (25:24:1 v/v) and then centrifuged at 11,400 *g* for 5 min at room temperature. The aqueous layer was transferred into a clean 1.5 ml Eppendorf tube and 500 µl chloroform was added, vortexed and centrifuged at full speed in a microfuge for 5 min at room temperature. The top layer was transferred into a clean 1.5 ml Eppendorf tube. The DNA was precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volumes of 100% cold ethanol and then pelleted by centrifugation at 11,400 *g* for 15 min at 4^{o}C. The supernatant was discarded and then the DNA pellet washed with 1 ml of 70% cold ethanol and precipitated by centrifugation at 11,400 *g* for 5 min. The supernatant was discarded and the DNA pellet air dried. The resulting DNA pellets for each sample were re-suspended in 30-40 µl of MilliQ water and then left overnight on bench. In the morning the sample were incubated at 50^{o}C for 60-90 minutes to ensure solubilisation of the DNA. It was then analysed on an agarose gel as described in section 2.12.1.4.

2.12.1.2 QIAGEN kit

As an alternative to CTAB, the QIAGEN kit was used to extract gDNA following the protocol described in the QIAGEN Genomic DNA Handbook. The DNA was solubilised as described in section 2.12.1.1 and analysed on an agarose gel to check for the successful extraction of gDNA as described in section 2.12.1.4.

2.12.1.3 Key Prep kit

3 ml of algae were centrifuged and the KeyPrep manufacturer's instructions followed. The extracted DNA was solubilised as described above and visualised on a 1% agarose gel as described below.

2.12.1.4 Agarose gel electrophoresis

All DNA samples were analysed (separated) by gel electrophoresis using 0.8 – 1.3% gels. To prepare gels, 0.8 - 1.3 g low melting point agarose (Roche) were added to 250 ml flask containing 2 ml of 50 X TAE (242 g Tris, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA) and 98 ml distilled water. This mixture was heated in the microwave until the agarose had melted. The solution was allowed to cool before 5 µl ethidium bromide (BioRad # 161-0.433) was added and then the gel poured to set in the BioRad Subcell GT electrophoretic tank with 14, 20 or 30 well comb. Once the gel had set, it was covered with 1 X TAE buffer (20 ml of 50 X TAE made up to 1000 ml distilled water).

To load the gel, 10 µl of each DNA sample was mixed with 2 µl Orange G dye and carefully pipetted into a well. A 1 µl DNA ladder (10 kb) was added to permit the estimation of the size of DNA fragments in the sample to assure the presence of high molecular weight gDNA and the absence of RNA. The voltage was set at 80 V and left to run for 45 minutes. The gDNA was visualized and photographed under ultra violet light using the Uvitec "Uvidoc" mounted camera system. If the gel showed smearing, this indicates the presence of RNA. It was removed by adding 1 µl of RNAse to the genomic DNA preparation with incubation at 4°C overnight.

2.12.2 Polymerase Chain Reaction (PCR) Amplification and Purification

The amplification of 16S rRNA gene was carried out with universal bacterial forward primer (fD1) and universal bacterial reverse primer (rP2) (Table 2.13).

Primer	Stock Solution	Designed for
fD1	CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG	16S rRNA gene (forward)
rP2	CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT	16S rRNA gene (reverse)
NS1	GTAGTCATATGCTTGTCTC	18S rRNA gene (forward)
18L	CACCTACGGAAACCTTGTTACGACTT	18S rRNA gene (reverse)

Table 2.13: Primers used to amplify 16S and 18S rRNA genes. The primers were synthesised by Eurofins MWG.

50 µl of typical PCR mixture contained 38 µl of sterile distilled water, 5 µl of 10x buffer, 2.5 µl of 50 mM $MgCl_2$, 0.5 µl of forward primer, 0.5 µl of reverse primer, 1.0 µl dNTPs, 2.0 µl of gDNA and 0.5 µl of Bioline Taq. 30 cycles of denaturation at 94^{o}C for 3 minutes, annealing at 60^{o}C for 1 minute and elongation at 72^{o}C for 1 minute per cycle and then a final extension at 72^{o}C for 5 minutes were employed for bacterial 16S rRNA gene amplification. The products of PCR were analyzed on agarose gel to check for successful 16S rRNA gene amplification.

QIAquick PCR purification kit was used to purify the PCR products (QIAquick Spin Handbook, 2006).

2.12.3 TOPO Cloning Reaction

Fresh PCR product 4 µl, Salt Solution 1 µl and TOPO vector 1 µl were added together to create TOPO Cloning Reaction. The following steps were done:

- The reaction was mixed gently and incubated for 5 minutes at room temperature (22-23^{o}C).
- The cloning reaction was placed on ice.
- In preparation of the transformation of competent cells, 500 ml of LB medium and 500 ml of LB agar were prepared both containing filter sterilised ampicillin at a concentration of 50 µg ml^{-1}.
- 40 µl of 40 mg ml^{-1} X-gal were spread on LB medium plate which contained per half litre: (ampicillin 100 µg ml^{-1}, tryptone 5 g, yeast extract 2.5 g, NaCl 5 g and 7.5 g Bacteriological agar with pH value 7-7.2) and incubated at 37^{o}C until ready for use.
- 2 µl of TOPO Cloning Reaction were transformed in to One Shot Competent Cells; mixed gently and incubated on ice for 5-30 minutes.
- The cells were heated for 30 seconds at 42^{o}C and transferred immediately to ice to make heat-shock.
- 250 µl of room temperature S.O.C medium (tryptone 20 g, yeast extract 5 g NaCl 0.5 g and 1 M KCl 2.5 ml per one litre) were added
- The tubes were capped tightly and shaken horizontally (200rpm) at 37^{o}C for one hour.

- 10-50 µl of each transformation were spread on a pre-warmed selective plate and incubated overnight at 37^{o}C.
- Mix of white and blue colonies were visible on the plates from the transformation and individual white colonies were picked using 200 µl pipette tips and dropped directly into LB medium in Falcon tubes. These tubes were then shaken overnight at 37^{o}C.

2.12.4 Mini-Preps

QIAgen MiniPrep kit was used to get plasmid DNA, basic procedure was as follows:

- Overnight cultures were centrifuged in bench centrifuge at 3000 *g* for 5-10 minutes; supernatants were poured off.
- The pellets were re-suspended in 250 µl buffer P1 and transferred to micro-centrifuge tubes.
- 250 µl of buffer P2 were added; mixed by inverting 4-6 times and left up to 5 minutes.
- 350 µl of buffer N3 were added and mixed immediately by inverting 4-6 times. Then centrifuged at 13000 *g* for 10 minutes.
- The supernatant from the last step was applied to the QIAprep spin column by pipette and centrifuged for 60 seconds; the flow through was discarded.
- The QIAprep spin column was washed with 0.75 ml of buffer PE; centrifuged for 60 seconds; the flow through was discarded and centrifuged for 1 minute.

The QIAprep spin column was placed into a clean micro-centrifuge tube and 250 µl of buffer EB was added to the centre of the spin column; left for 30-60 seconds then centrifuged for 1 minute.

2.12.5 Restriction Digest

1 µl of *Eco*R1 enzyme, 1 µl of 10x buffer H and 8 µl of plasmid DNA were added to a PCR tube and incubated at 37^{o}C for two hours. Restriction digest samples were analyzed on agarose gel to check for the correct products.

2.12.6 Analysis of 16S and 18S rRNA Gene Sequences

10 µl of the products from the minipreps of samples along with 10 µl of primer were sent for nucleotide sequencing at the School of Medicine, University of Sheffield, Hallamshire Hospital, UK. The sequencing data were returned by email in the form of chromatograms and using the software FinchTV (Geospiza, Inc., Seattle, USA) the fluorescence data remaining unassigned to a nucleotide were manually assigned a nucleotide and the useable length of the read defined by deleting non-assigned data from either side of the sequence. The sequences were then exported as text files. The Basic Local Alignment Search Tool (BLAST) from the website of the National Centre for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/) was used to identify homologous sequenc

Chapter 3

ISOLATION AND CHARACTERISATION OF BACTERIAL ISOLATES

3.1 Introduction

The whole range of salt concentrations from fresh water to marine biotopes and hypersaline environments shows evidence of microbial life (Zahran, 1997). Evaporation of sea water caused by high temperatures, low rainfall, low humidity and high wind speed creates some of the hypersaline environments found on Earth (Schreiber, 1986). However, a number of hypersaline environments are athalassohaline (i.e. they are not just concentrated seawater, but contain different proportions of some ions e.g. the Dead Sea which is very high in Mg) (Oren, 2002).

A wide range of salt tolerance is demonstrated by microorganisms; some demonstrate a clear requirement for salt and are termed halophiles, whilst others can grow in high salinities, but do not require salt and are thus halotolerant (Gilmour, 1990; Joo and Kim, 2005; Oren, 2006; Bowers *et al*., 2009; Hezayen, 2010). The isolation of extremophiles can occur from environments in which they are not expected to be actively growing (Echigo *et al*., 2005). Therefore, the isolation of halotolerant or even halophilic bacteria from a wide range of environments including fresh water is possible (Schieper *et al*., 1997; Purdy *et al*., 2004; Oren, 2008). A good example of halotolerant bacteria is the *Staphylococcus* genus which can grow well in both freshwater and at NaCl concentrations as high as 1.7 – 2.6 M or even higher (Oren, 2006). Bacteria that can grow and survive in a range of saline environments and must maintain their cytoplasmic osmotic balance by using a physiological mechanism to allow them to adapt to life in saline habitats. Therefore, any microorganism living at high salt concentrations is expected to

keep its cytoplasm at least isotonic with the extracellular environment (Oren, 2008).

In the work described in this chapter, the isolation of salt tolerant bacteria capable of growth from 0 M to 0.75 M NaCl was demonstrated using water samples from ponds in the Derbyshire Peak District and in Sheffield. Growth and abundance of the isolates was measured using optical density, but this method must be used with caution and carefully calibrated with an alternative method such as direct cell counts (Tsuzuki *et al*., 1990; Becker, 1994). However, optical density measurement is a fast method for calculating the approximate growth curve of the microbes (Borowitzka and Moheimani, 2012).

3.2 Results and Discussion

3.2.1 Isolation of Bacteria

Minimal M9 medium was used to isolate bacteria from the samples of water collected from Weston Park pond in Sheffield (WP) and Derbyshire Peak District dew pond (DP) (section 2.1). The isolation protocol was to increase the NaCl concentration in the M9 minimal medium from 0.25 M up to 0.75 M NaCl to select for strains that were tolerant to increased salt concentration. One ml of each M9 minimal medium culture was inoculated in to 50 ml of minimal M9 medium at a range of salinities from 0.25 to 0.75 M NaCl. Flasks were incubated overnight at 25ºC with shaking (250 rpm). The best growth was found at low salinities, however some growth was found at 0.5 M NaCl and 0.75 M NaCl. Samples from the 0.5 and 0.75 M NaCl flasks were spread on minimal medium M9 agar plates of the same salinity and single colonies from these plates were used to purify two strains of bacteria designated WP and DP. Strain WP was able to grow well in minimal M9 medium at salinities up to 0.75 M NaCl and strain DP grew well in the same medium containing 0.5 M NaCl. Good growth was defined as reaching at least an OD_{600} of 1 after overnight growth at 25°C with shaking (250 rpm).

3.2.2 Initial Characterisation of Strains WP and DP

On minimal M9 agar medium the colony form of the two strains was observed to be regular, flat, shiny and opaque. The DP strain had white colonies on plates, about 2 mm in diameter (Figure 3.1), whereas WP strain had creamy colonies on plates, which were larger than the DP colonies (Figure 3.2).

Table 3.1 illustrates the basic phenotypic characteristics of WP and DP strains. Light microscopy examination of overnight cultures showed that cells of strain WP were motile, rod shaped cells that stained Gram-negative (Figure 3.3), whereas DP cells were non motile, Gram-negative and short rods (Figure 3.4). The WP strain was able to grow under anaerobic conditions, but the DP strain did not grow anaerobically.

Strain Characteristics	**WP**	**DP**
Pigmentation	cream	white
Gram stain	negative	negative
Morphology	rod	rod
Motile	negative	positive
Facultative Anaerobe	positive	negative

Table 3.1: The basic characteristics of WP and DP strains. Cells were grown in minimal M9 medium and were shaken at 250 rpm in a 25°C constant temperature room overnight.

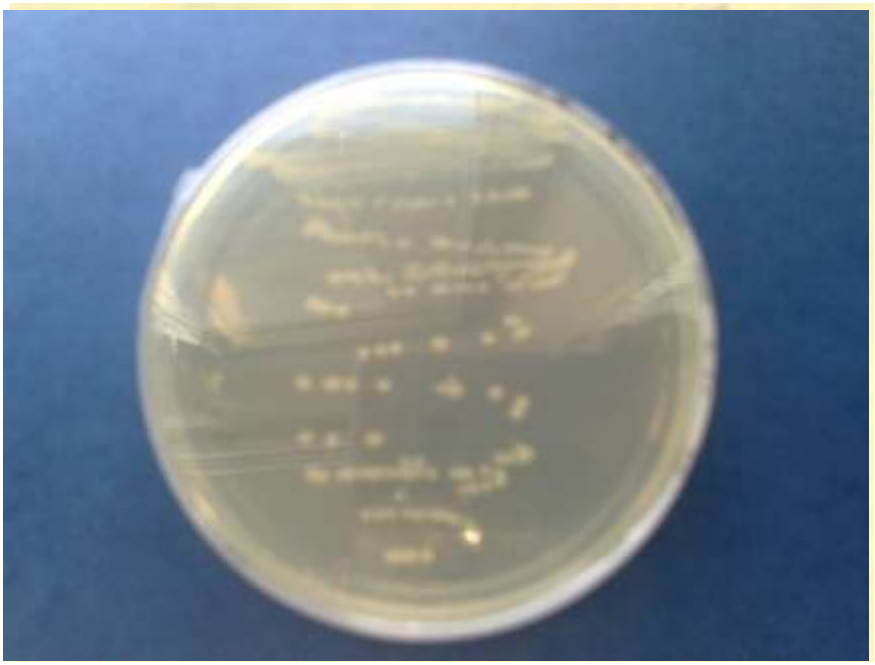

Figure 3.1: White colonies of DP strain grown on an M9 minimal medium agar plate.

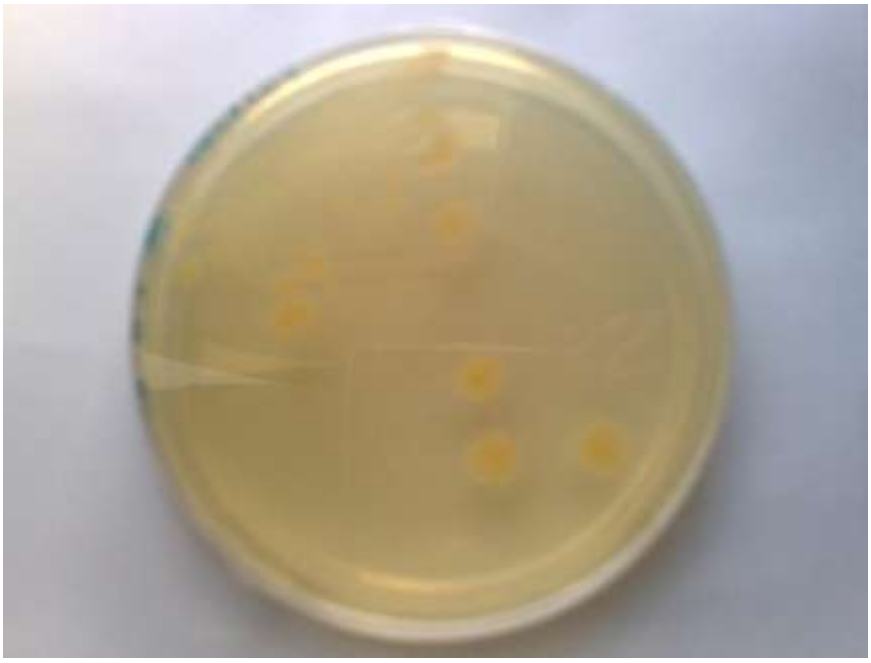

Figure 3.2: Cream colonies of WP strain grown on an M9 minimal medium agar plate.

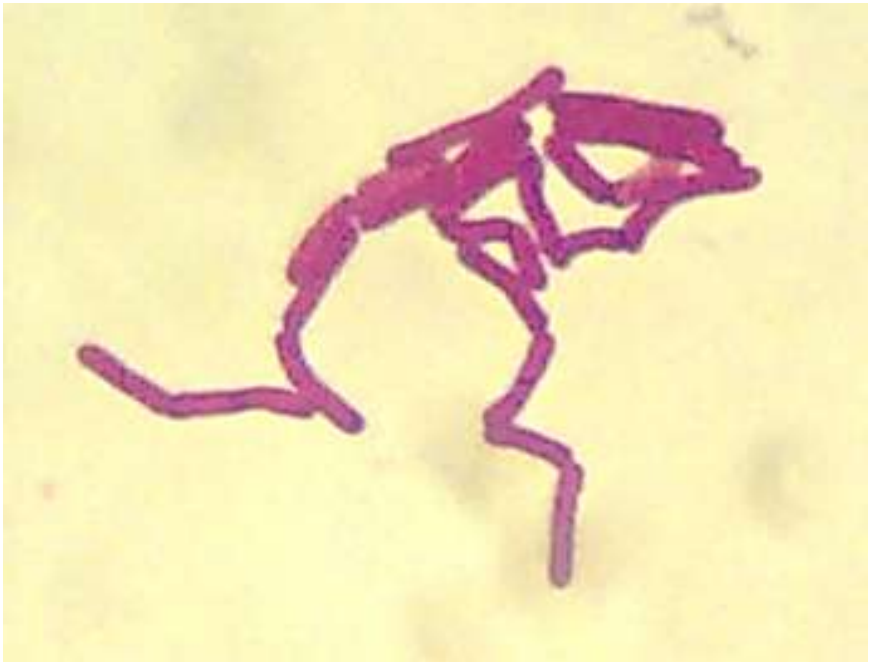

Figure 3.3: Rod-shaped Gramnegative cells of WP strain under light microscope, magnification x 1000.

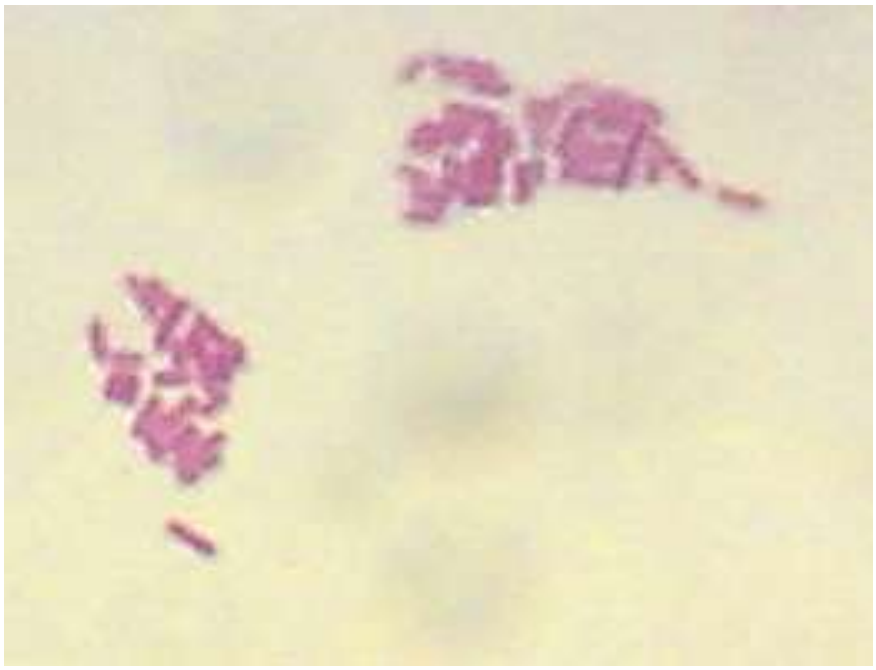

Figure 3.4: Rod-shaped Gramnegative cells of DP strain under light microscope, magnification x 1000**.**

3.2.3 Adaptation of Strains WP and DP to Different Salinities

In order to investigate the range of salinities that the two freshwater strains could tolerate cells of each strain were repeatedly sub-cultured in minimal M9 medium containing higher levels of NaCl on an incremental basis. Initially, WP was adapted to grow in 0.25 M NaCl, then 0.5 M NaCl and 0.75 M NaCl over a period of several weeks. The same process was repeated with the DP strain. Figures 3.5 and 3.6 show growth curves for the two strains. WP strain had the ability to grow across the full range of salt concentrations up to 0.75 M NaCl, but the maximum salinity tolerated by DP strain was 0.5 M NaCl. Table 3.3 shows the doubling times for each strain at each salinity tested and it is noteworthy that the DP strain grew faster than the WP strain, despite its more limited salt tolerance. It is also interesting to note that the fastest doubling time for the DP strain was at 0.25 M NaCl, possibly suggesting some requirement for NaCl. The growth rates of the WP and DP strains were fairly slow reflecting the minimal salts composition of M9 minimal medium. Figures 3.7 to 3.12 show the parts of the growth curves used to calculate the doubling times shown in Table 3.3.

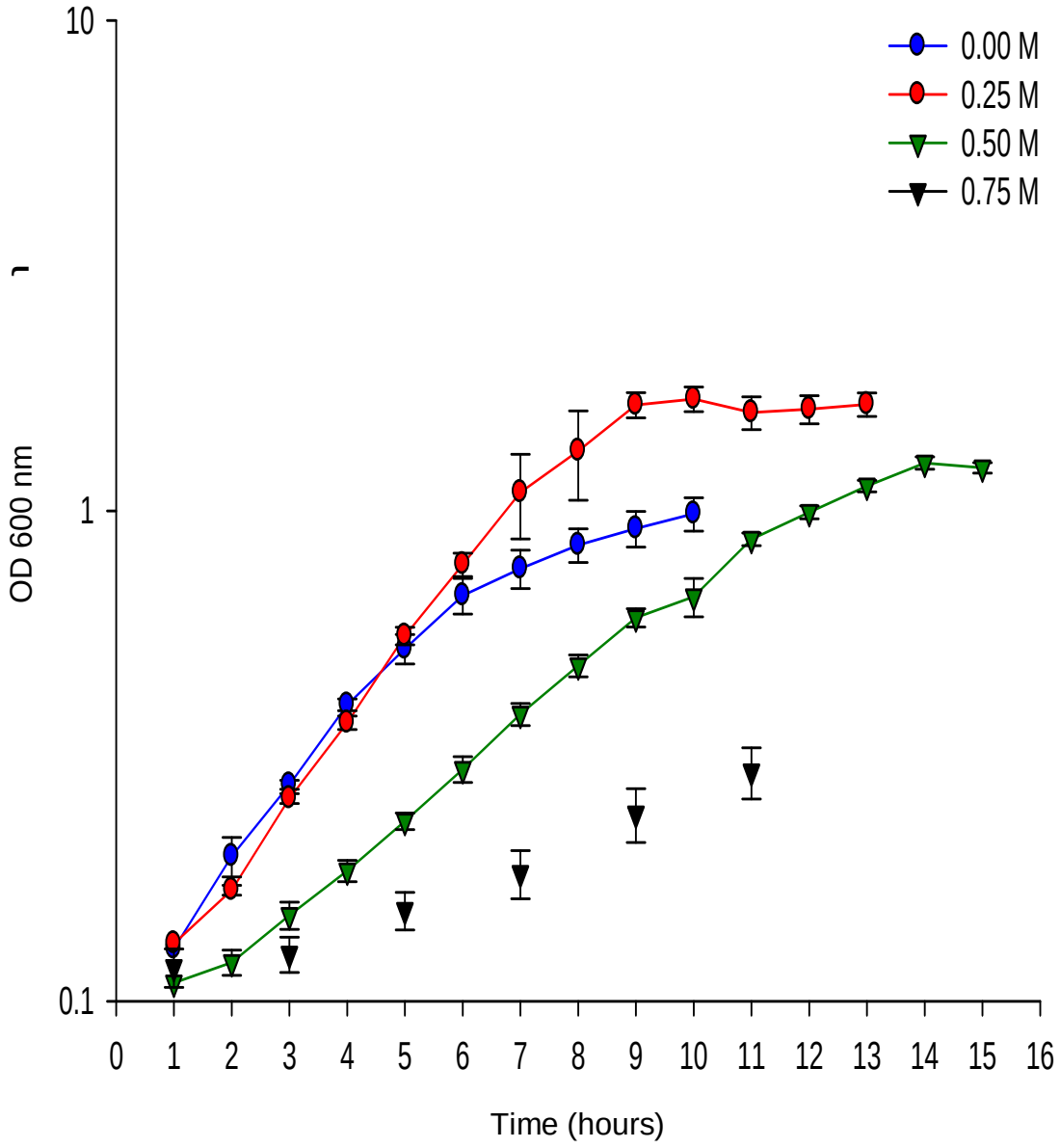

Figure 3.5: Growth curves of WP strain showing their ability to tolerate high salt concentrations. Cells were grown in minimal M9 medium from 0 - 0.75 M NaCl, incubated in a 25°C constant room temperature on an orbital shaker at 250 rpm. The OD was measured at 600 nm and data points are the means of four replicates plus or minus standard deviations.

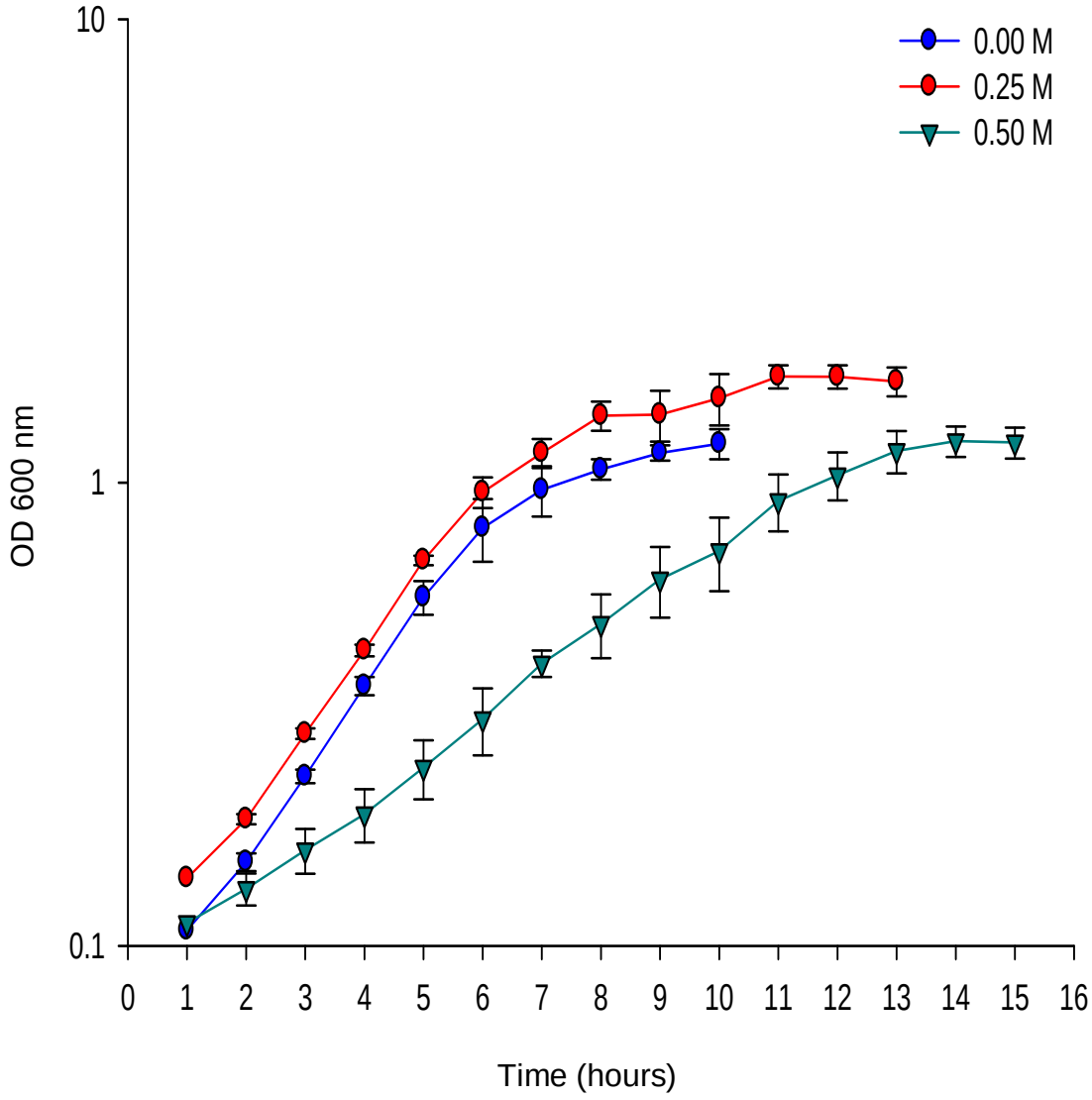

Figure 3.6: Growth curves of DP strain showing their ability to tolerate high salt concentrations. Cells were grown in minimal M9 medium from 0 - 0.5 M NaCl, incubated in a 25°C constant room temperature on an orbital shaker at 250 rpm. The OD was measured at 600 nm and data points are the means of four replicates plus or minus standard deviations.

	Doubling Time of Strains (minutes)	
NaCl (M)	**WP**	**DP**
0	135	108
0.25	112	70
0.5	180	130
0.75	258	-

Table 3.2: Effect of NaCl concentrations (0 to 0.75 M NaCl) on the doubling times of strains WP and DP. Each point represents the mean from four replicate samples.

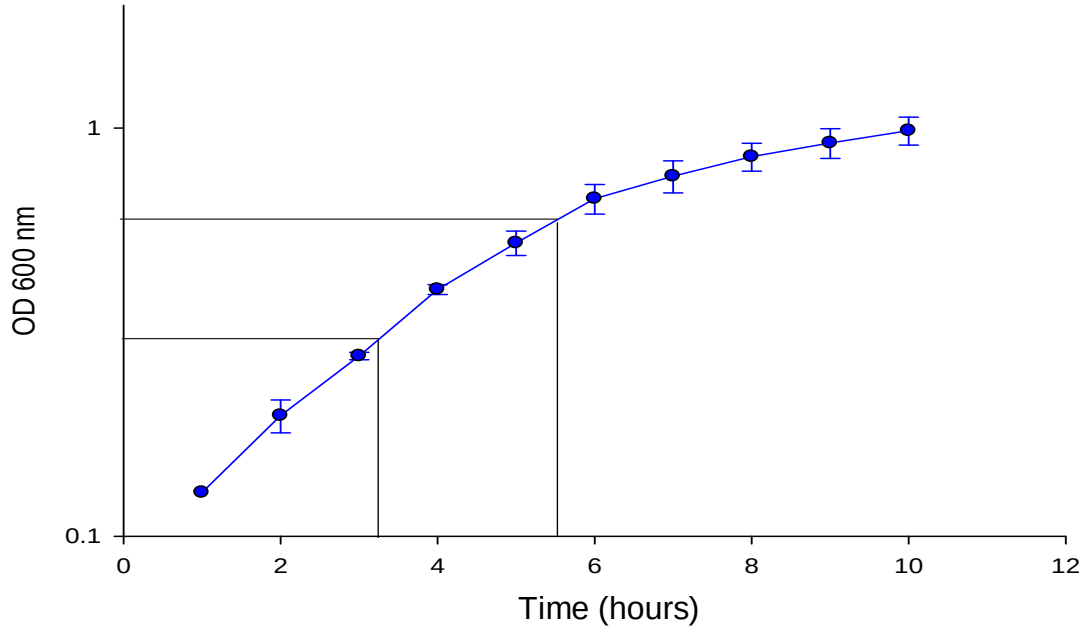

Figure 3.7: This figure shows the part of the growth curve used to calculate the doubling time for the WP strain in minimal M9 medium without added NaCl.

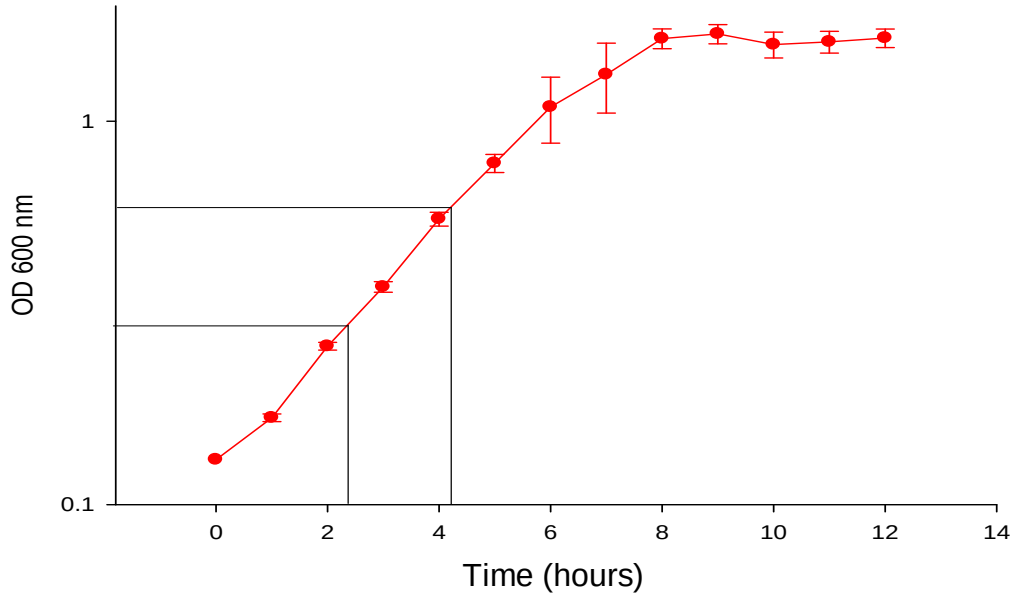

Figure 3.8: This figure shows the part of the growth curve used to calculate the doubling time for the WP strain in minimal M9 medium with 0.25 M NaCl.

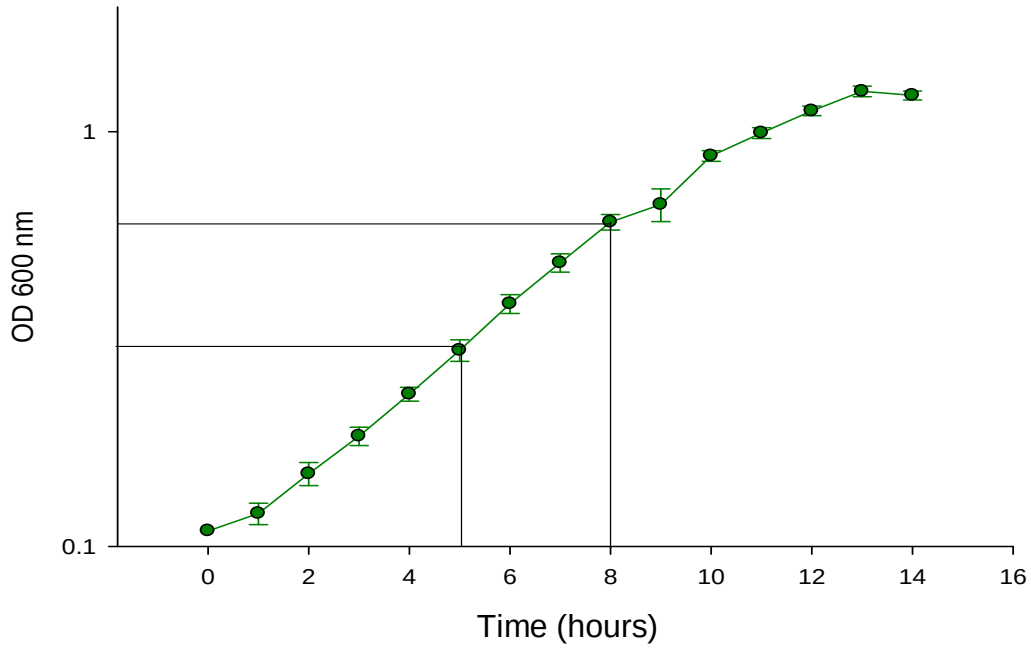

Figure 3.9: This figure shows the part of the growth curve used to calculate the doubling time for the WP strain in minimal M9 medium with 0.5 M NaCl.

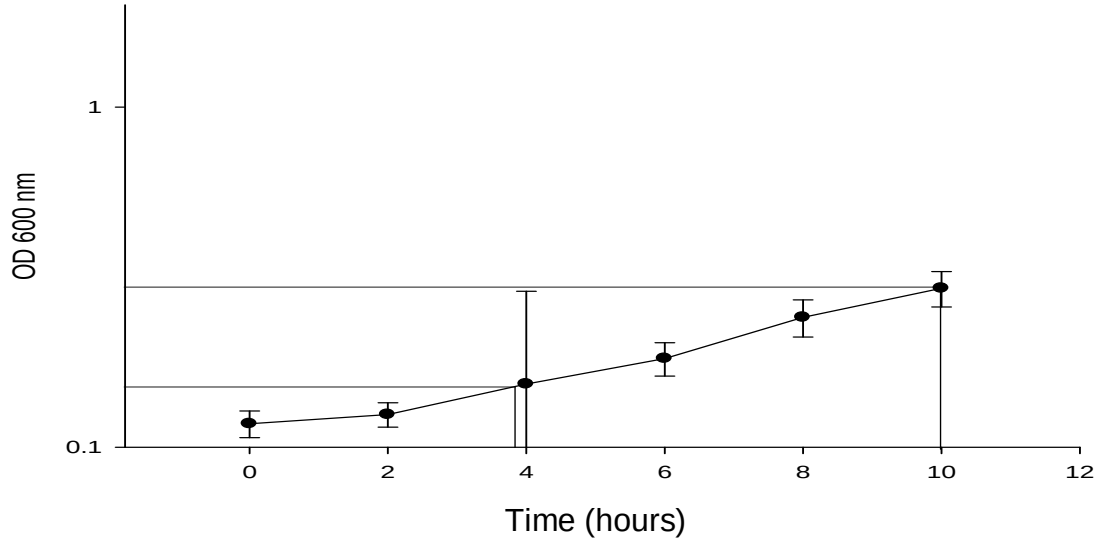

Figure 3.10: This figure shows the part of the growth curve used to calculate the doubling time for the WP strain in minimal M9 medium with 0.75 M NaCl**.**

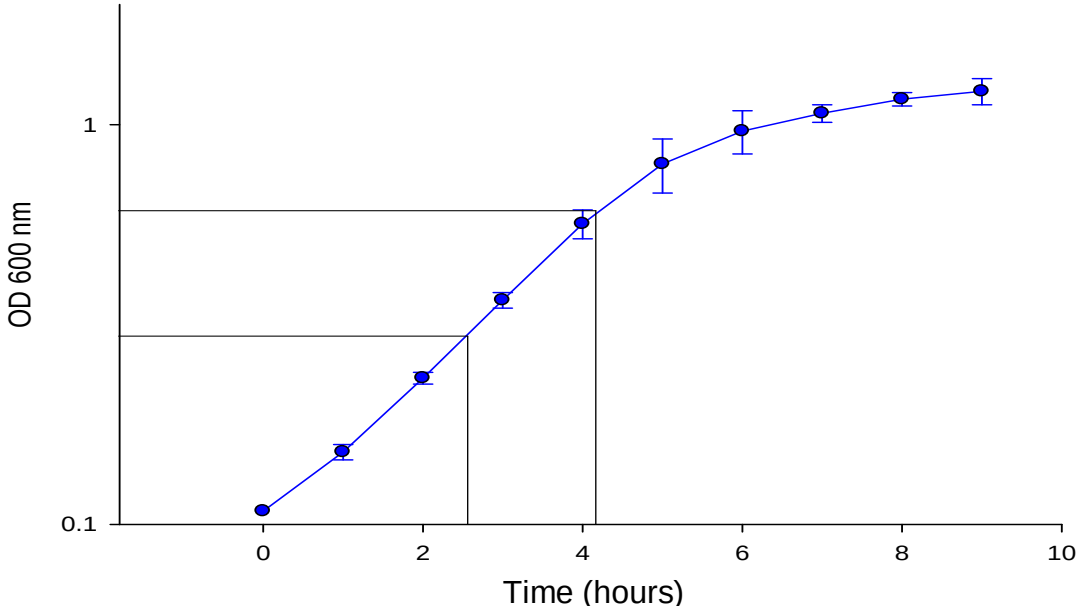

Figure 3.11: This figure shows the part of the growth curve used to calculate the doubling time for the DP strain in minimal M9 medium without added NaCl.

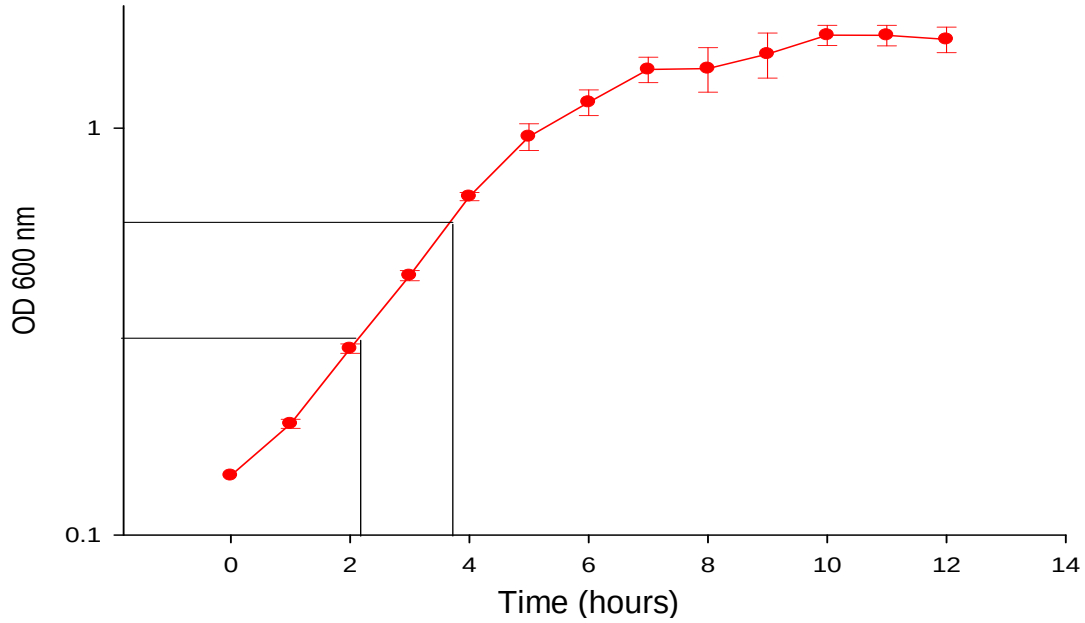

Figure 3.12: This figure shows the part of the growth curve used to calculate the doubling time for the DP strain in minimal M9 medium with 0.25 M NaCl**.**

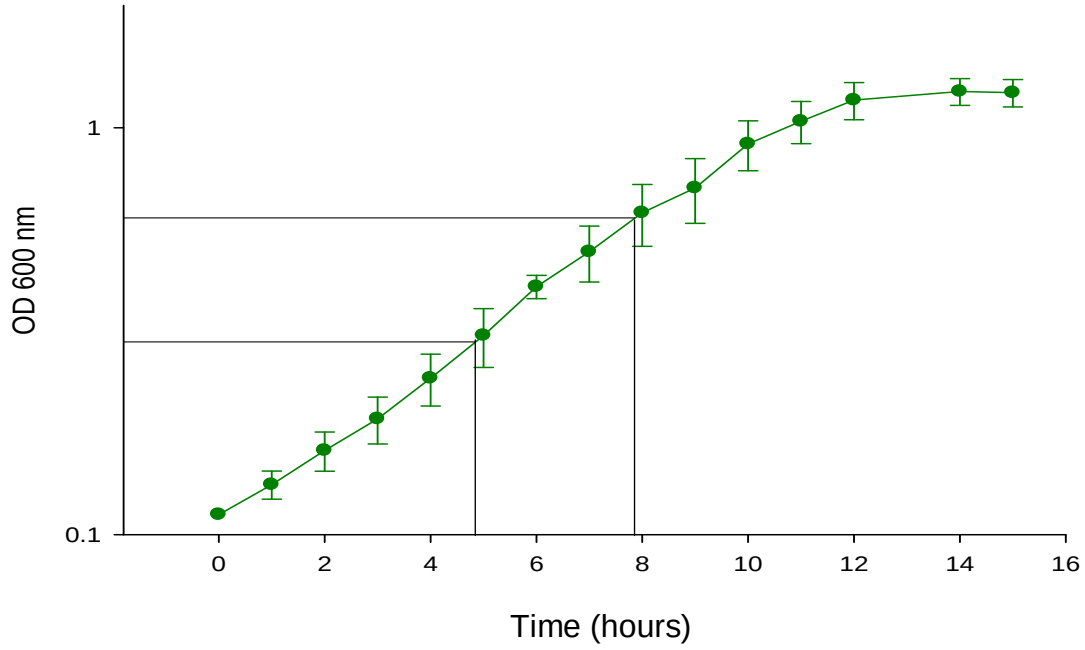

Figure 3.13: This figure shows the part of the growth curve used to calculate the doubling time for the DP strain in minimal M9 medium with 0.5 M NaCl**.**

3.3 Conclusion

Two organisms were successfully isolated from fresh water samples that could grow in salinities up to 0.5 M NaCl (strain DP) or 0.75 M NaCl (strain WP) (Figures 3.5 and 3.6). These salinities are not particularly high (0.5 M NaCl is approximately equivalent to the salinity of sea water), but the fact that minimal M9 medium was used makes it much harder for organisms to adapt to increased salinity. In rich medium (e.g. LB medium), the precursors of compatible solutes are present allowing easy accumulation of the compatible solutes (Oren, 2002). This method of accumulating metabolism-friendly (i.e. compatible solutes) is a very common way to allow growth in increased salinities. However, in M9 minimal medium, no such compatible solute precursors are present and this means that the compatible solutes must be synthesized *de novo*, which is a much more energetically demanding process.

In the next chapter, 16S rDNA sequencing will be used to identify the WP and DP strains and then the compatible solutes utilized by the two strains will be determined using NMR. Further characterization of their responses to salinity and temperature changes will be carried out as will a survey of the carbon sources suitable for growth

Chapter 4

IDENTIFICATION AND FURTHER CHARACTERISATION OF BACTERIAL ISOLATES

4.1 Introduction

The identification of microorganisms in most microbiology laboratories has usually been achieved by traditional identification techniques including Gram staining, cell morphology, culture requirements and biochemical tests. However, two major drawbacks have been reported for these methods. First, some microorganisms are non cultivable and therefore the conventional techniques cannot be used to identify such microorganisms. Second, some microorganisms have biochemical characteristics that do not fit into the pattern of a previously identified species (Woo *et al*., 2000). Therefore, nucleic acid sequence information (16S rRNA gene) has been selected and used to determine the similarity between groups of organisms because this gene is present in all prokaryotes (Amann *et al*., 1997).

In this chapter, the two bacterial isolates described in Chapter 3 were identified to the genus level by 16S rRNA gene sequencing. The 16S gene was amplified by the polymerase chain reaction (PCR) and sequenced. The sequence was compared with known 16S rRNA gene sequences in the BLAST database as described in Section 2.12.6. The presence of compatible solutes in the two strains was determined by NMR. Further physiological characterisation was undertaken to examine the respiration rates of the bacterial isolates under normal and saline conditions and the range of carbon sources utilised for growth was also determined.

4.2 Results and Discussion

4.2.1 Molecular Identification of Bacterial Isolates using 16S rDNA Sequencing

4.2.1.1 Extraction of genomic DNA (gDNA)

Two methods were utilized to extract genomic DNA from the WP and DP bacterial isolates: CTAB and the Qiagen kit. CTAB did not extract DNA. However, the Qiagen kit extracted the gDNA from both isolates as shown in Figure 4.1.

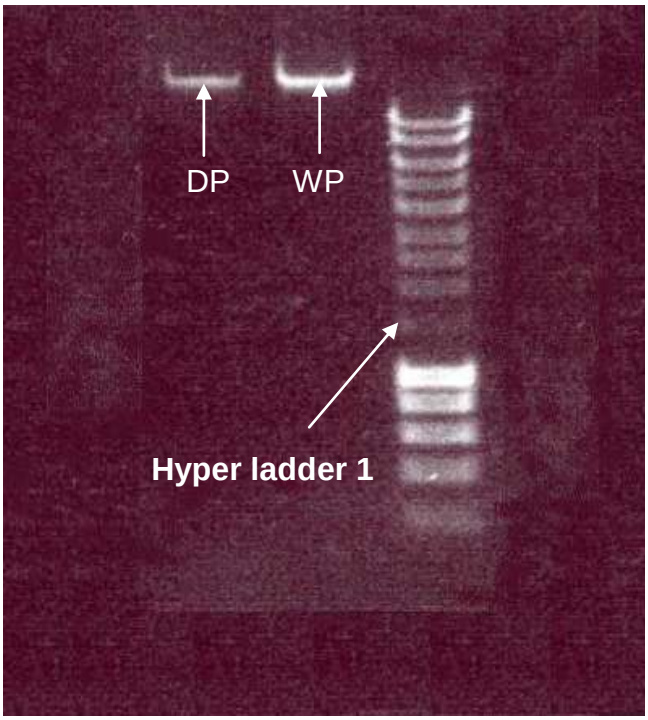

Figure 4.1: Genomic DNA extracted from both WP and DP strains using Qiagen kit.

4.2.1.2 Polymerase Chain Reaction (PCR) amplification

16S rRNA gene was amplified by PCR for WP and DP samples (Figure 4.2). It is clear that all PCR products were the correct size (1500 bp). The bright band on the ladder just below the bacterial DNA bands is 1000 bp and the band above is the 1500 bp band.

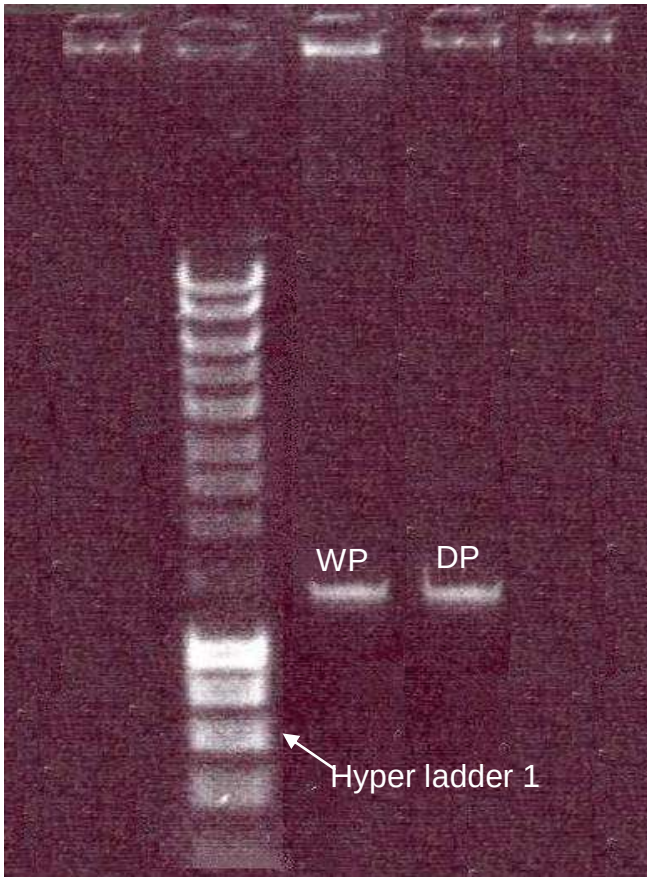

Figure 4.2: Amplification of the 1.5 kb product (16S rRNA gene) from the PCR involving the universal bacterial primers.

4.2.1.3 PCR purification

PCR products were purified by the QIAquick PCR purification kit for WP and DP samples (Figure.4.3), all purified PCR products were of the correct size (1500 bp).

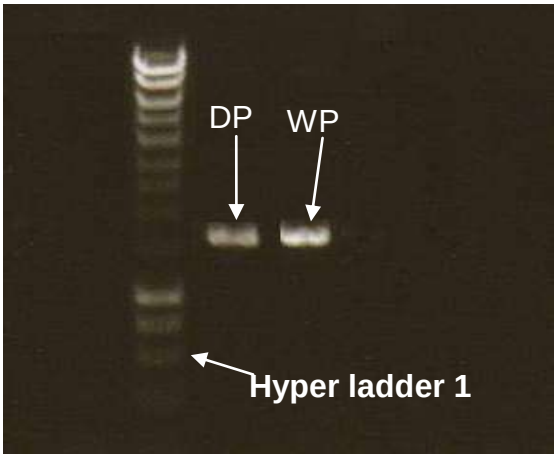

Figure 4.3: Purification of PCR products – very clear 1.5 kb bands remained after the purification process.

4.2.1.4 Sequencing of 16S rDNA gene of bacterial isolates.

The vectors containing the correct sized insert were sent to the Medical School for sequencing and produced good length sequences (Figures 4.4 and 4.6). The sequences were compared to other sequences using the NCBI BLAST database. The WP strain from Weston Park pond was shown to be a member of the *Enterobacter* genus with the closest species match as *E. amnigenus* (Table 4.1 and Figure 4.5). The DP strain from the Derbyshire Dew Pond near Tideswell was shown to be *Pseudomonas fluorescens* (Table 4.2 and Figure 4.7).

GGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCA
AAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGA
TTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCT
GGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCT
GATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTA
CTTTCAGCGAGGAGGAAGGCATTGTGGTTAATAACCACAGTGATTGACG
TTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTA
ATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCAC
GCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGG
AACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAA
TTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTG
GCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCG
TGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGA
TGTCGACTTGGAGGTTGTTCCCTTGAGGAGTG

Figure 4.4: 16S rRNA gene sequence of strain WP. The nucleotide sequence is 705 bp.

Match species/ strain	Percentage similarity
1- *Enterobacter amnigenus*	100%
2- *Enterobacter sp.*	100%
3- *Uncultured bacterium*	100%
4- *Enterobacter sp*	100%
5- *Enterobacter sp*	100%
6- *Klebsiella sp*	100%
7- *Bacterium*	100%
8-*. Enterobacter amnigenus*	100%
9- *Uncultured bacterium*	100%
10- *Buttiauxella gaviniae*	100%

Table 4.1: Similarity between 16S rRNA gene sequence of WP and other related species/strains based on MegaBlast.

Figure 4.5: Neighbour joining phylogenetic tree for WP strain shown in yellow. All the nearest matches are to uncultured bacteria or to bacteria belonging to the *Enterobacter* genus.

GGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCT
GCAGAATTCGCCCTTACGGGCGGTGTGTACAAGGCCCGGGAACGTATT
CACCGCGACATTCTGATTCGCGATTACTAGCGATTCCGACTTCACGCAG
TCGAGTTGCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGC
TCCACCTCGCGGCTTGGCAACCCTTTGTACCGACCATTGTAGCACGTGT
GTAGCCCAGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTT
CCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCATAACGTGC
TGGTAACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACAT
CTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAATGTTC
CCGAAGGCACCAATCTATCTCTAGAAAGTTCATTGGATGTCAAGGCCTG
GTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTG
TGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCC
CCAGGCGGTCAACTTAATGCGTTAGCTGCGCCACTAAGAGCTCAAGGC
TCCCAACGGCTAGTTGACATCGTTTACGGCGTGGACTACCAGGGTATCT
AATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCC
AGGTGGTCGCCTTCGCCACTGGTGTTCCTTCCTATATCTACGCATTTCA
CCGCTACACAGGAAATTCCACCACCCTCTACCATACTCTAGCTTGCCAG
TTTTGGATGCAGCTCCCGGTTTGAACCGGGGGCTTTCACATTCAACTTA
ACAAACCACCTACGCGCGCTTTACGCCCAGTAATT

Figure 4.6: 16S rRNA gene sequence of strain DP. The nucleotide sequence is 912 bp.

Match species/ strain	Percentage similarity
1- *Pseudomonas fluorescens*	100%
2- *Pseudomonas sp.*	100%
3- *Pseudomonas fluorescens*	100%
4- *Bacterium*	100%
5- *Uncultured bacterium*	100%
6- *Pseudomonas protegens*	100%
7- *Uncultured Pseudomonas*	100%
8-*. Uncultured Pseudomonas*	100%
9- *Pseudomonas sp*	100%
10- *Pseudomonas sp*	100%

Table 4.2: Similarity between 16S rRNA gene sequence of DP and other related species/strains based on MegaBlast.

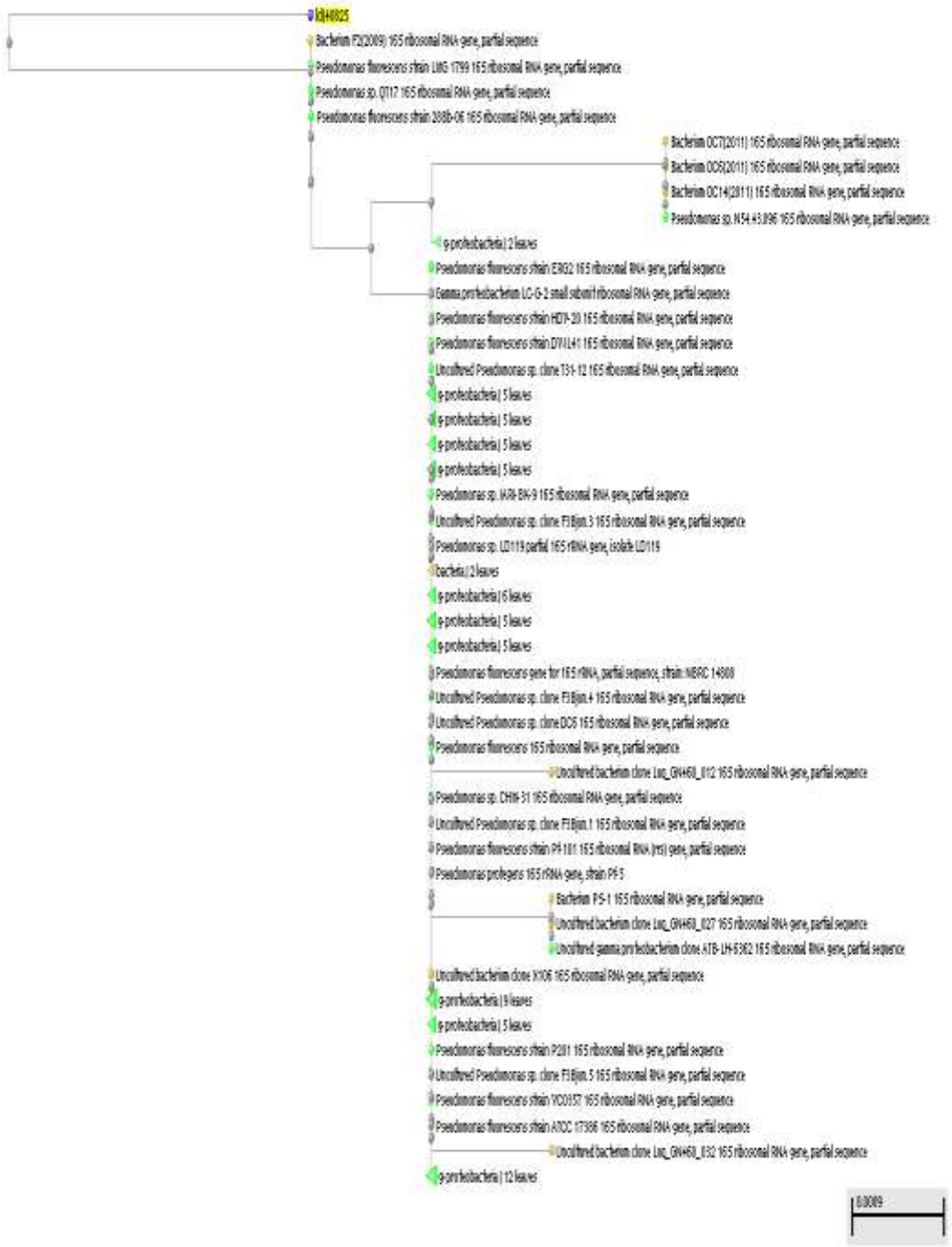

Figure 4.7: Neighbour joining phylogenetic tree for DP strain shown in yellow. The nearest matches are to *Pseudomonas fluorescens.*

4.2.2 Effect of Temperature and Salinity on the Respiration Rate of *Enterobacter amnigenus* and *Pseudomonas fluorescens*

Figures 4.8 to 4.11 show respiration rates for *E. amnigenus.* Each figure shows the respiration rate at three temperatures (25, 30 and 37°C) with the salinity increasing from 0 to 0.75 M NaCl. Increasing temperature appeared to increase the rate of respiration by showing a quicker and more complete use of oxygen indicated by the steeper slope and lower finishing value for oxygen content. The effect of increasing the salinity was much less clear. A lag time at the beginning of the oxygen traces was seen at 0.25 M NaCl (Figure 4.9), but was absent or much less pronounced at higher salinities of 0.5 and 0.75 M NaCl (Figures 4.9 and 4.10). At 25°C, it was possible to see a trend of decreasing respiration rate with increasing salinity (Figures 4.8 to 4.11).

Figures 4.12 to 4.14 show respiration rates for *P. fluorescens*. Each figure shows the respiration rate at three temperatures (25, 30 and 37°C) with the salinity increasing from 0 to 0.5 M NaCl. At 0.25 and 0.5 M NaCl (Figures 4.13 and 4.14) there was a clear trend of increasing respiration rate with increasing temperature, but this trend was much less clear at 0 M NaCl (Figure 4.12). The fastest respiration was at 37°C in 0.5 M NaCl medium (Figure 4.14).

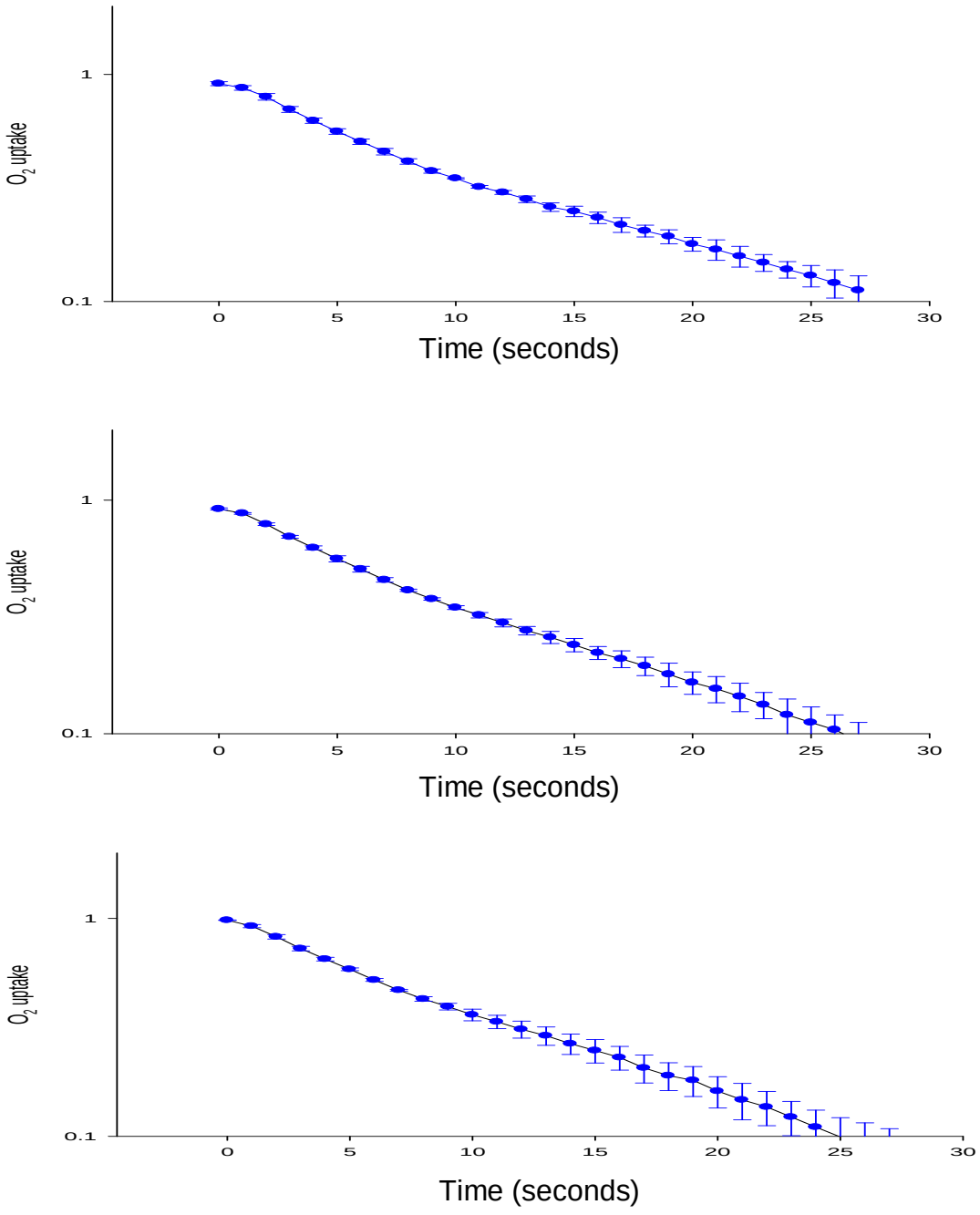

Figure 4.8: The effect of temperature (25, 30 and 37^{o}C) on O_2 uptake of *Enterobacter amnigenus* strain in minimal M9 medium. The units of O_2 content are dimensionless and are relative values based on the current flowing through the electrode**.**

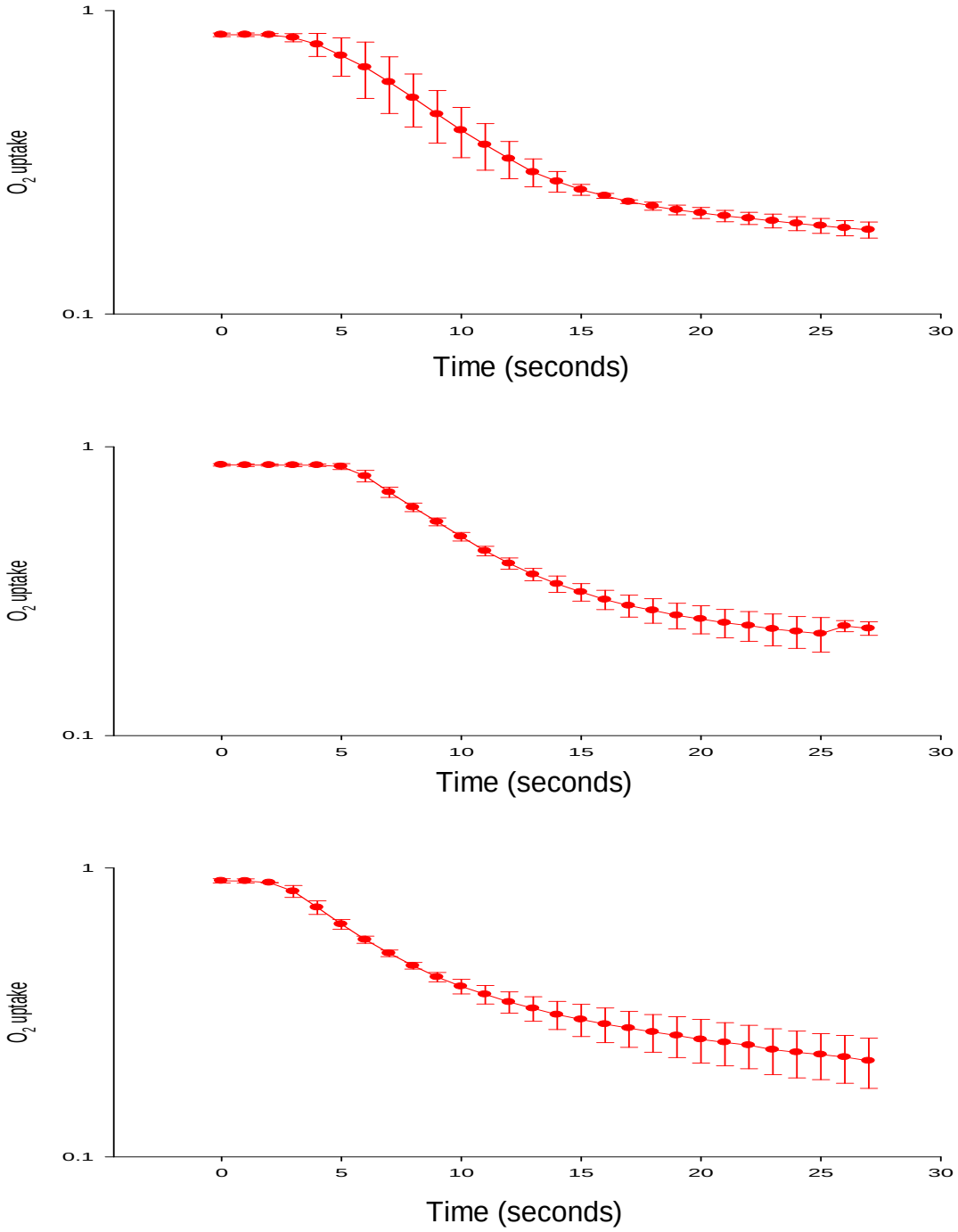

Figure 4.9: The effect of temperature (25, 30 and 37^{o}C) on O_2 uptake of *Enterobacter amnigenus* strain in minimal M9 medium containing 0.25 M NaCl. The units of O_2 content are dimensionless and are relative values based on the current flowing through the electrode.

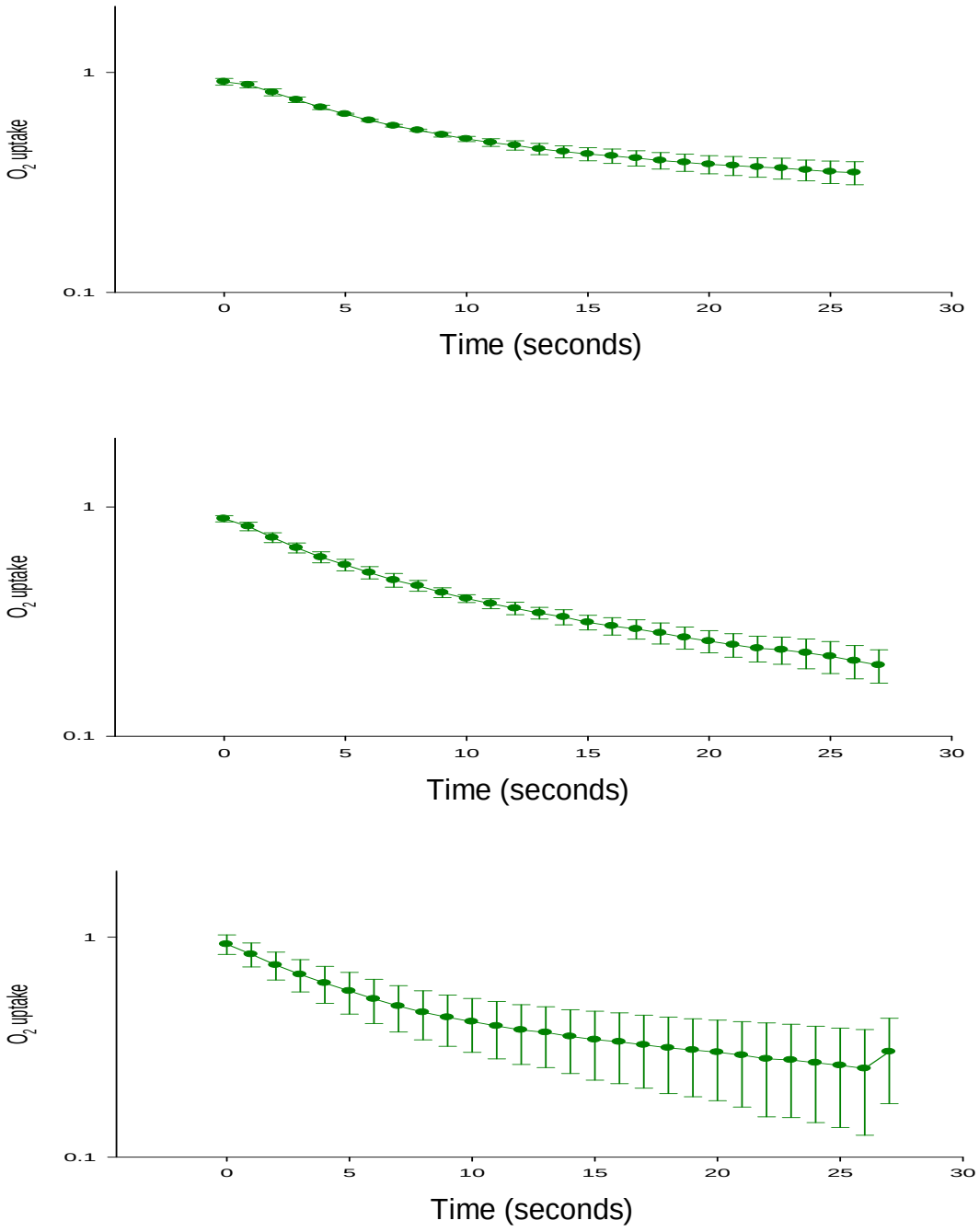

Figure 4.10: The effect of temperature (25, 30 and 37^{o}C) on O_2 uptake of *Enterobacter amnigenus* strain in minimal M9 medium containing 0.5 M NaCl. The units of O_2 content are dimensionless and are relative values based on the current flowing through the electrode.

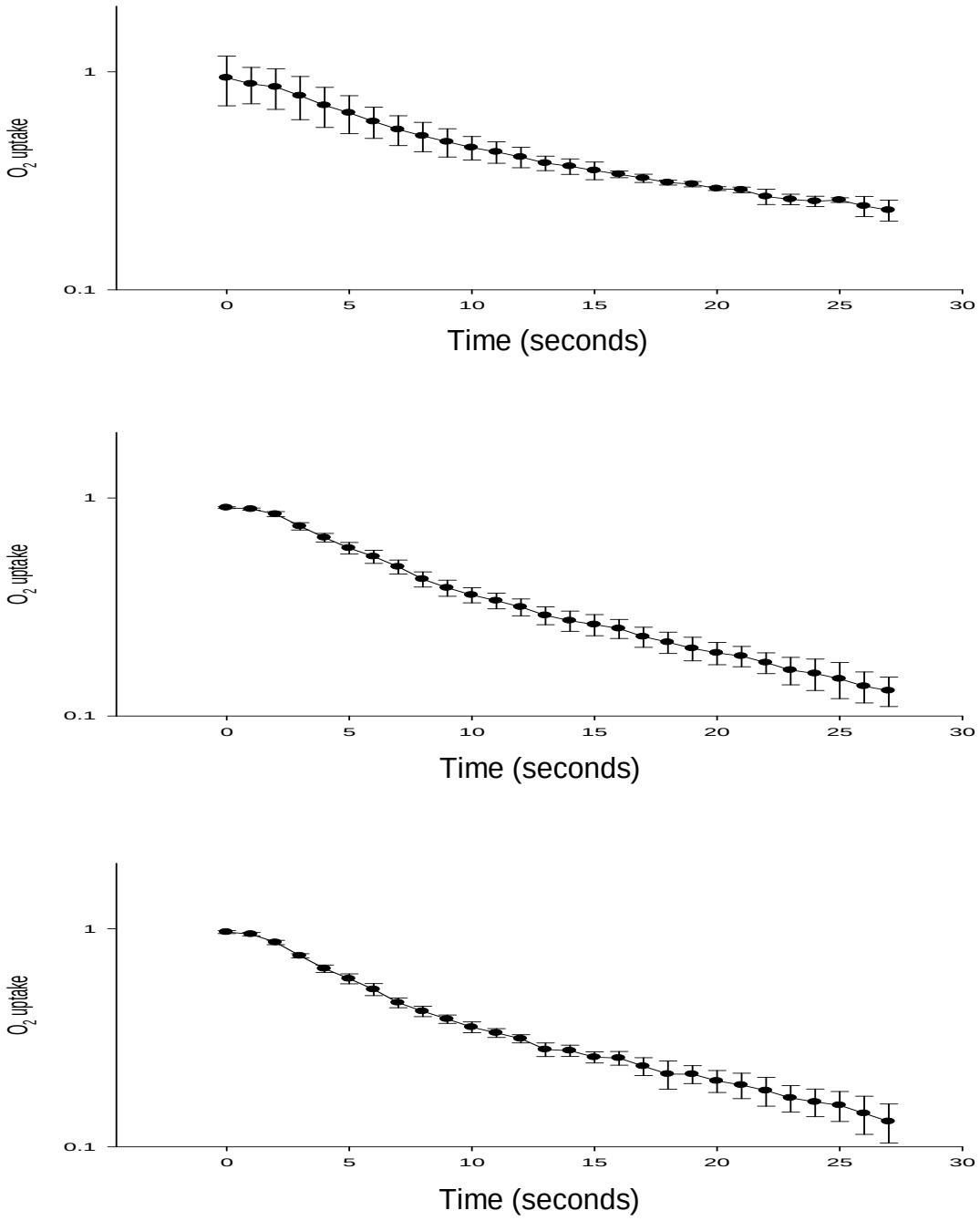

Figure 4.11: The effect of temperature (25, 30 and 37^{o}C) on O_2 uptake of *Enterobacter amnigenus* strain in minimal M9 medium containing 0.75 M NaCl. The units of O_2 content are dimensionless and are relative values based on the current flowing through the electrode.

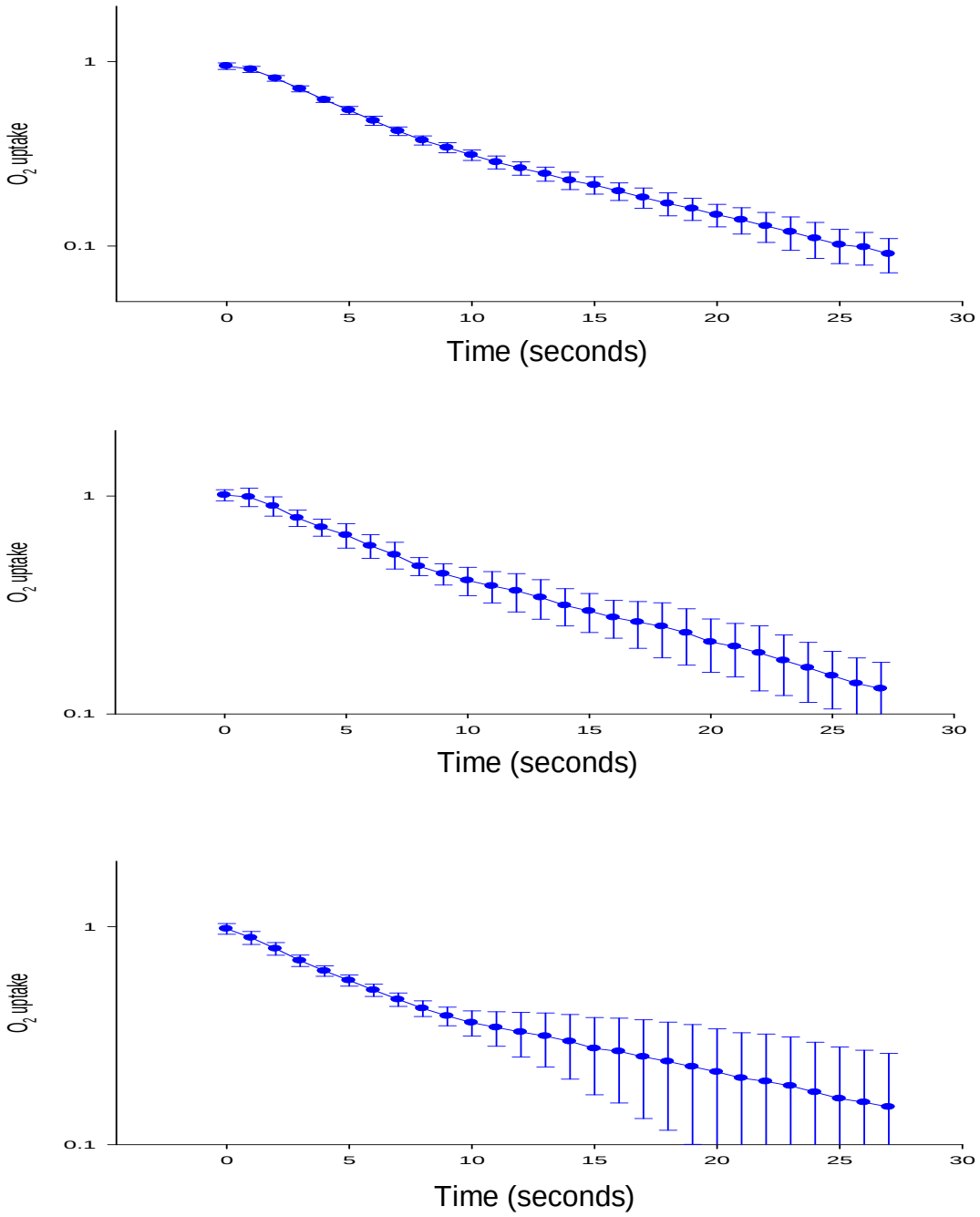

Figure 4.12: The effect of temperature (25, 30 and 37^{o}C) on O_2 uptake of *Pseudomonas fluorescens* strain in minimal M9 medium. The units of O_2 content are dimensionless and are relative values based on the current flowing through the electrode.

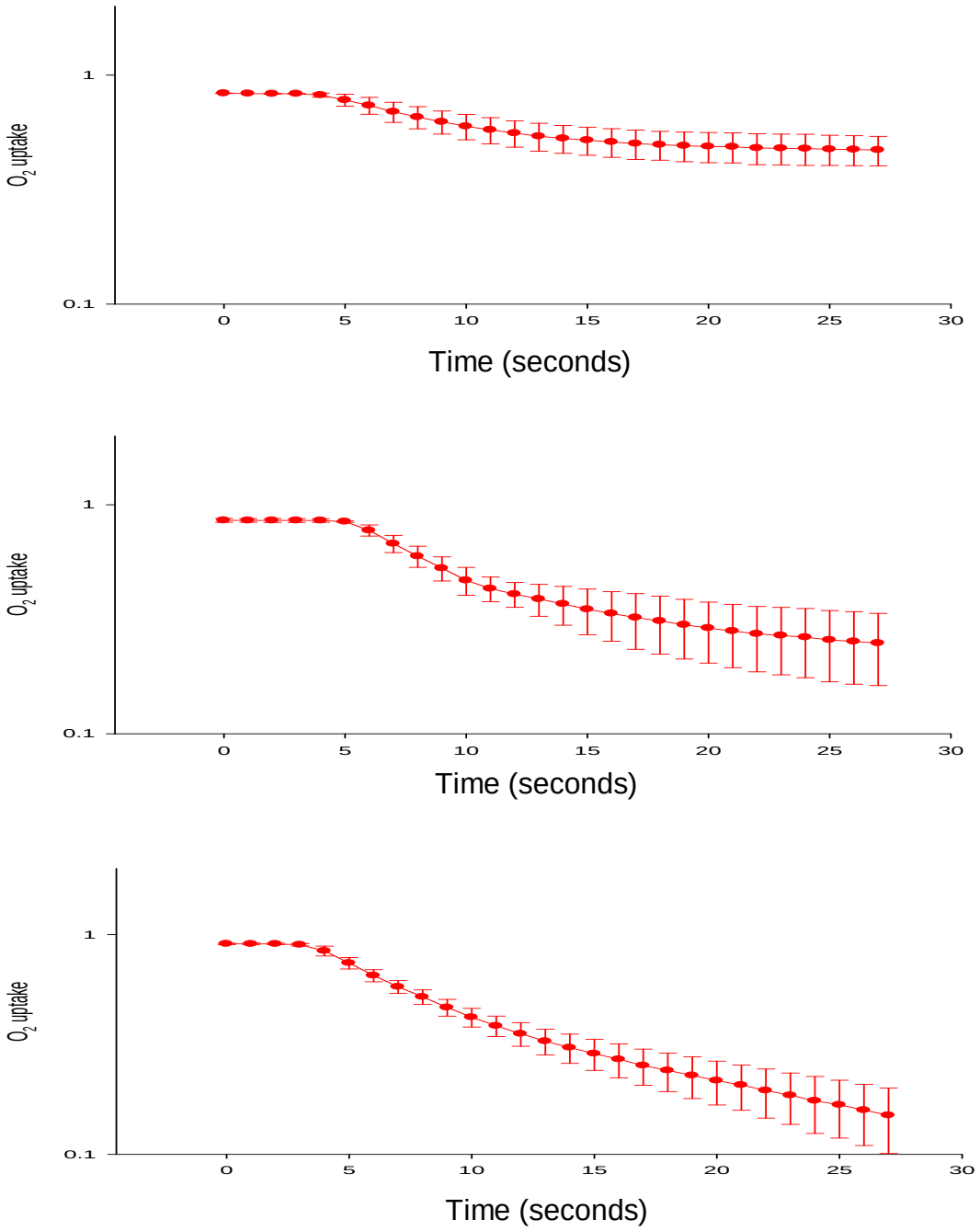

Figure 4.13: The effect of temperature (25, 30 and 37^{o}C) on O_2 uptake of *Pseudomonas fluorescens* strain in Minimal M9 medium containing 0.25 M NaCl. The units of O_2 content are dimensionless and are relative values based on the current flowing through the electrode.

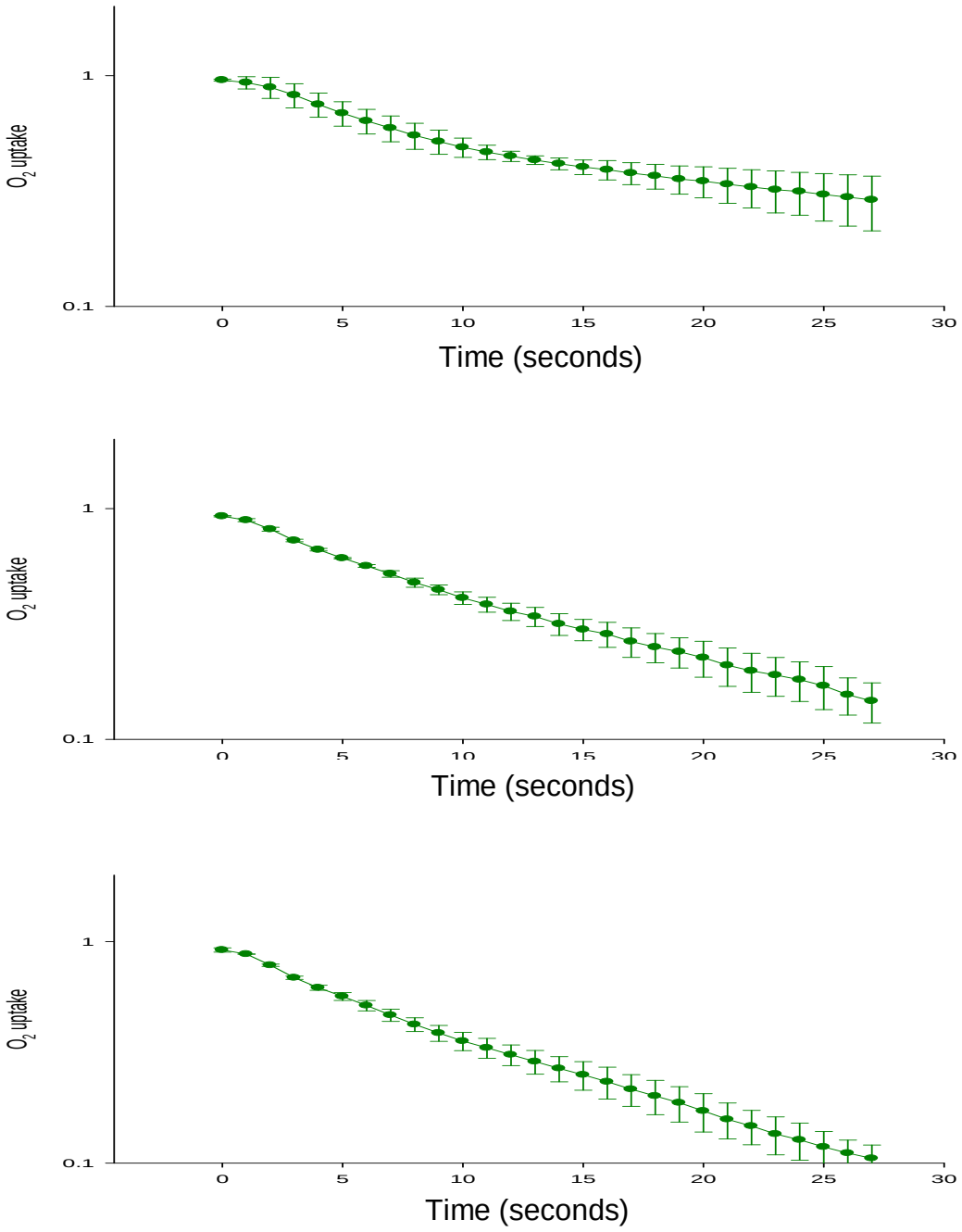

Figure 4.14: The effect of temperature (25, 30 and 37^{o}C) on O_2 uptake of *Pseudomonas fluorescens* strain in Minimal M9 medium containing 0.5 M NaCl concentration. The units of O_2 content are dimensionless and are relative values based on the current flowing through.

4.2.3 Determination of Compatible Solutes (Osmolytes) by Nuclear Magnetic Resonance (NMR) spectroscopy

The aim of this investigation was to identify the compatible solutes accumulated by *Enterobacter amnigenus* and *Pseudomonas fluorescens* strains when exposed to a range of external salinities from 0.25 M to 0.75 M NaCl (for *E. amnigenus*) and 0.25 to 0.5 M NaCl (for *P. fluorescens*) in M9 minimal medium (Figures 4.15 and 4.16).

Few obvious solutes were noted in the NMR spectra (i.e. not many compounds were apparently accumulated at the increased salinities). For *E. amnigenus*, lysine, lactate and glucose were detected at higher salinities (Figure 4.15) and for *P. fluorsecens* only glucose was detected (Figure 4.16).

Therefore, no common compatible solutes were detected for either organism, except for lysine for *E. amnigenus* and this helps to explain the relatively low salinities tolerated by *E. amnigenus* and *P. fluorescens.*

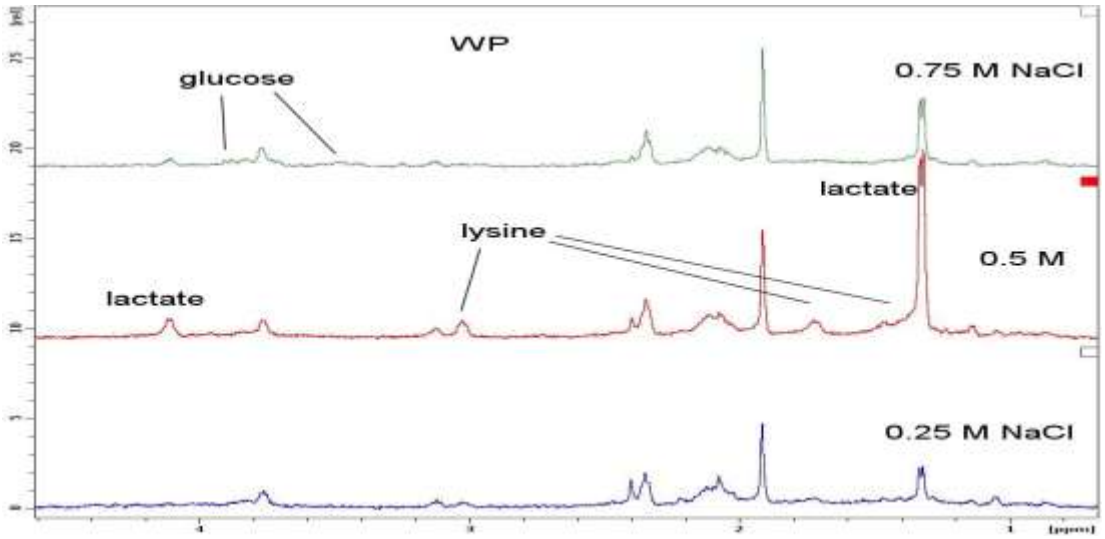

Figure 4.15: One-dimensional 1H-NMR spectra of cell extracts derived from *Enterobacter amnigenus* cells grown in M9 minimal medium supplemented with 0.25 to 0.75 M NaCl.

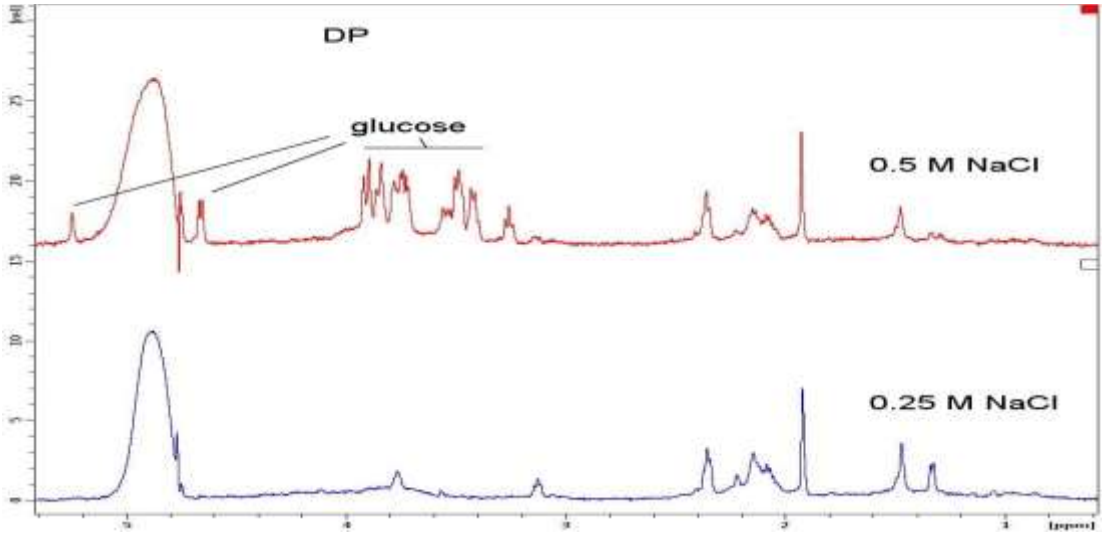

Figure 4.16: One-dimensional 1H-NMR spectra of cell extracts derived from *Pseudomonas fluorescens* cells grown in M9 minimal medium supplemented with 0.25 to 0.5 M NaCl.

4.2.4 Determination of Carbon Sources by BIOLOG test

BIOLOG GN2 plates were used to determine the carbon sources utilized *P. fluorescens* and *E. amnigenus* in M9 minimal medium. Table 4.3 shows the carbon utilization pattern for cells grown in normal M9 medium (i.e. without added salt).

Carbon Sources	*Pseudomonas fluorescens*	*Enterobacter amnigenus*
1. Water	-	-
2. a-Cyclodextrin	-	-
3. Dextrin	++	+
4. Glycogen	+	-
5. Tween 40	+	-
6. Tween 80	+	+
7. N-Acetyl-Dgalactosamine	++	++
8. N-Acetyl-Dglucosamine	+++	++
9. Adonitol	+++	-
10. L-Arabinose	+++	+++
11. D-Arabitol	+++	-
12. D-Cellobiose	+++	+++

Table 4.3: Carbon sources utilized by *Pseudomonas fluorescens* and *Enterobacter amnigenus* in M9 minimal medium as measured by the Biolog test.

Carbon Sources	*Pseudomonas fluorescens*	*Enterobacter amnigenus*
13. i-Erythritol	-	-
14. D-Fructose	+++	++
15. L-Fucose	++	-
16. D-Galactose	+++	+++
17. Gentiobiose	+++	++
18. Gentiobiose	+++	++
19. m-Inositol	+	-
20. α-D-Lactose	+++	+
21. Lactulose	+	-
22. Maltose	+++	++
23. D-Mannitol	+++	+++
24. D-Mannose	+++	+++

Table 4.3 (continued): Carbon sources utilized by *Pseudomonas fluorescens* and *Enterobacter amnigenus* in M9 minimal medium as measured by Biolog test.

Carbon Sources	*Pseudomonas fluorescens*	*Enterobacter amnigenus*
25. D-Melibiose	+++	+++
26. β-Methyl - D-Glucoside	+++	+++
27. D-Psicose	+	-
28. L-Rhamnose	+++	+++
29. L-Rhamnose	+++	++
30. D-Sorbitol	+++	-
31. Sucrose	++	+++
32. D-Trehalose	+++	+++
33. Turanose	++	++
34. Xylitol	-	-
35. Pyruvate Acid Methyl Ester	+++	++
36. Succinic Acid Mono-Methyl-Ester	-	-

Table 4.3 (continued): Carbon sources utilized by *Pseudomonas fluorescens* and *Enterobacter amnigenus* in M9 minimal medium as measured by Biolog test.

Carbon Sources	*Pseudomonas fluorescens*	*Enterobacter amnigenus*
37. Acetic Acid	-	-
38. Cis-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. -Hydroxy Butyric Acid	-	-
47. β-Hydroxy Butyric Acid	+	-
48. Y-Hydroxy Butyric Acid	-	-

Table 4.3 (continued): Carbon sources utilized by *Pseudomonas fluorescens* and *Enterobacter amnigenus* in M9 minimal medium as measured by Biolog test.

Carbon Sources	*Pseudomonas fluorescens*	*Enterobacter amnigenus*
49. p-Hydroxy Phenylacetic Acid	++	-
50. taconic 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	+	-

Table 4.3 (continued): Carbon sources utilized by *Pseudomonas fluorescens* and *Enterobacter amnigenus* in M9 minimal medium as measured by Biolog test.

Carbon Sources	*Pseudomonas fluorescens*	*Enterobacter amnigenus*
61. Bromosuccinic Acid	+	++
62. Succinamic Acid	-	-
63. Glucuronamide	+	-
64. -Alaninamide	+	-
65. D-Alanine	++	++
66. L-Alanine	++	++
67. L-Alanylglycine	+	-
68. L-Asparagine	+	-
69. L-Aspartic Acid	-	-
70. L-Glutamic Acid	-	-
71. Glycyl-LAspartic Acid	-	++
72. Glycyl-LGlutamic Acid	-	+

Table 4.3 (continued): Carbon sources utilized by *Pseudomonas fluorescens* and *Enterobacter amnigenus* in M9 minimal medium as measured by Biolog test.

Carbon Sources	*Pseudomonas fluorescens*	*Enterobacter amnigenus*
73. L-Histidine	++	-
74. Hydroxy-LProline	-	-
75. L-Leucine	-	-
76. -Ornithine	-	-
77. LPhenylalanine	-	-
78. -Proline	-	++
79. L-Pyroglutamic Acid	-	-
80. D-Serine	++	-
81. L-Serine	++	++
82. L-Threonine	-	-
83. D,L-Carnitine	-	-
84. $_{Y}$-Amino Butyric Acid	-	-

Table 4.3 (continued): Carbon sources utilized by *Pseudomonas fluorescens* and *Enterobacter amnigenus* in M9 minimal medium as measured by Biolog test.

Carbon Sources	*Pseudomonas fluorescens*	*Enterobacter amnigenus*
85. Urocanic Acid	-	-
86. Inosine	++	++
87. Uridine	+	-
88. Thymidine	++	++
89. Phenyethylamine	-	-
90. Putrescine	+	-
91. 2-Aminoethanol	-	-
92. 2,3-Butanediol	-	-
93. Glycerol	+++	+++
94. D,L-α-Glycerol Phosphate	+	++
95. Glucose-1- Phosphate	+++	++
96. Glucose-6- Phosphate	+++	+++

Table 4.3 (continued): Carbon sources utilized by *Pseudomonas fluorescens* and *Enterobacter amnigenus* in M9 minimal medium as measured by Biolog test.

Table 4.4 shows the carbon utilization pattern for cells of *Pseudomonas fluorescens* and *Enterobacter amnigenus* grown in M9 minimal medium with 0.5 M NaCl.

Carbon Sources	*Pseudomonas fluorescens*	*Enterobacter amnigenus*
1. Water	-	-
2. a-Cyclodextrin	-	-
3. Dextrin	+	-
4. *Glycogen*	-	-
5. Tween 40	-	-
6. Tween 80	-	-
7. N-Acetyl-Dgalactosamine	+	-
8. N-Acetyl-Dglucosamine	+++	++
9. Adonitol	-	-
10. L-Arabinose	+/-	+/-
11. D-Arabitol	++	-
12. D-Cellobiose	+	-

Table 4.4: Carbon sources utilised by *Pseudomonas fluorescens* and *Enterobacter amnigenus* in M9 minimal medium with 0.5 M NaCl as measured by Biolog test.

Carbon Sources	*Pseudomonas fluorescens*	*Enterobacter amnigenus*
13. i-Erythritol	-	-
14. D-Fructose	++	-
15. L-Fucose	++	-
16. D-Galactose	++	++
17. Gentiobiose	+	-
18. Gentiobiose	++	++
19. m-Inositol	++	-
20. α-D-Lactose	-	-
21. Lactulose	-	-
22. Maltose	-	++
23. D-Mannitol	++	-
24. D-Mannose	++	-

Table 4.4 (continued): Carbon sources utilised by *Pseudomonas fluorescens* and *Enterobacter amnigenus* in M9 minimal medium with 0.5 M NaCl as measured by Biolog test.

Carbon Sources	*Pseudomonas fluorescens*	*Enterobacter amnigenus*
25. D-Melibiose	-	-
26. β-Methyl - D-Glucoside	++	-
27. D-Psicose	-	-
28. L-Rhamnose	+	-
29. L-Rhamnose	++	-
30. D-Sorbitol	++	-
31. Sucrose	-	-
32. D-Trehalose	++	-
33. Turanose	-	-
34. Xylitol	-	-
35. Pyruvate Acid Methyl Ester	++	++
36. Succinic Acid Mono-Methyl-Ester	-	-

Table 4.4 (continued): Carbon sources utilised by *Pseudomonas fluorescens* and *Enterobacter amnigenus* in M9 minimal medium with 0.5 M NaCl as measured by Biolog test.

Carbon Sources	*Pseudomonas fluorescens*	*Enterobacter amnigenus*
37. Acetic Acid	-	-
38. Cis-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. -Hydroxy Butyric Acid	-	-
47. β-Hydroxy Butyric Acid	-	-
48. Y-Hydroxy Butyric Acid	-	-

Table 4.4 (continued): Carbon sources utilised by *Pseudomonas fluorescens* and *Enterobacter amnigenus* in M9 minimal medium with 0.5 M NaCl as measured by Biolog test.

Carbon Sources	*Pseudomonas fluorescens*	*Enterobacter amnigenus*
49. p-Hydroxy Phenylacetic Acid	-	-
50. taconic 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	-	-

Table 4.4 (continued): Carbon sources utilised by *Pseudomonas fluorescens* and *Enterobacter amnigenus* in M9 minimal medium with 0.5 M NaCl as measured by Biolog test.

Carbon Sources	*Pseudomonas fluorescens*	*Enterobacter amnigenus*
61. Bromosuccinic Acid	-	-
62. Succinamic Acid	-	-
63. Glucuronamide	-	-
64. -Alaninamide	-	-
65. D-Alanine	-	-
66. L-Alanine	-	++
67. L-Alanylglycine	-	-
68. L-Asparagine	-	-
69. L-Aspartic Acid	-	-
70. L-Glutamic Acid	-	-
71. Glycyl-LAspartic Acid	-	-
72. Glycyl-LGlutamic Acid	-	-

Table 4.4 (continued): Carbon sources utilised by *Pseudomonas fluorescens* and *Enterobacter amnigenus* in M9 minimal medium with 0.5 M NaCl as measured by Biolog test.

Carbon Sources	*Pseudomonas fluorescens*	*Enterobacter amnigenus*
73. Bromosuccinic Acid	-	-
74. Succinamic Acid	-	-
75. Glucuronamide	-	-
76. -Alaninamide	-	-
77. D-Alanine	-	-
78. L-Alanine	-	-
79. L-Alanylglycine	-	-
80. L-Asparagine	++	-
81. L-Aspartic Acid	-	-
82. L-Glutamic Acid	-	-
83. Glycyl-LAspartic Acid	-	-
84. Glycyl-LGlutamic Acid	-	-

Table 4.4 (continued): Carbon sources utilised by *Pseudomonas fluorescens* and *Enterobacter amnigenus* in M9 minimal medium with 0.5 M NaCl as measured by Biolog test.

Carbon Sources	*Pseudomonas fluorescens*	*Enterobacter amnigenus*
85. Urocanic Acid	-	-
86. Inosine	-	-
87. Uridine	-	-
88. Thymidine	-	-
89. Phenyethylamine	-	-
90. Putrescine	-	-
91. 2-Aminoethanol	-	-
92. 2,3-Butanediol	-	-
93. Glycerol	++	+++
94. D,L-α-Glycerol Phosphate	-	++
95. Glucose-1- Phosphate	++	++
96. Glucose-6- Phosphate	++	++

Table 4.4 (continued): Carbon sources utilised by *Pseudomonas fluorescens* and *Enterobacter amnigenus* in M9 minimal medium with 0.5 M NaCl as measured by Biolog test.

4.3 Conclusion

The two strains isolated and partly characterised in Chapter 3 were identified in this chapter as *Enterobacter amnigenus* (WP) and *Pseudomonas fluorescens* (DP). The identifications seem secure – certainly to the genus level, but the species identification also seems fairly reliable (Tables 4.1 and 4.2). The respiration data shown in Figures 4.8 to 4.14 confirm that both strains can metabolise at temperatures between 25 and 37°C and at salinities of at least 0.5 M NaCl. Both organisms can utilize a wide range of carbon sources in the absence of NaCl and at 0.5 M NaCl (Tables 4.3 and 4.4.)

E. amnigenus has been studied previously, but not extensively, with some 26 publications (Web of Knowledge, accessed July 2013) with this organism in the title. It has been recognized as a potential pathogen and Bollet *et al.* (1991) isolated *E. amnigenus* from an intravenous catheter inserted in a heart transplant patient. However, *E. amnigenus* has also been shown to be of industrial importance (e.g. Hungund and Gupta (2010) produced bacterial cellulose from a strain (GH-1) of *E. amnigenus*). Bacterial cellulose differs from plant cellulose in being easily biodegradable and has good water-holding capacity. Very recently, new taxonomic analysis, using multilocus sequence analysis, has suggested that *E. amnigenus* should be removed from the *Enterococcus* genus and placed in the new genus *Lelliottia* as *L. amnigena* (Brady *et al.,* 2013).

In contrast to *E. amnigenus*, the other species identified (*P. fluorescens*) is very well characterized with over 10000 papers published with this organism in the title (Web of Knowledge, accessed July 2013). *P. fluorescens* is

widespread in nature being a common soil organism, but also being found in marine environments. One of the reasons for the large amount of published information on *P. fluorescens* is its ability to form biofilms (Mastropaolo *et al*., 2012). It is also known as a rhizobacterium and *P. fluorescens* has been associated with the economically important black truffle found in southern Europe (Dominguez *et al*., 2012). However, like many species of pseudomonads, *P. fluorescens* is an opportunistic nosocomial pathogen. For example, Benito *et al*. (2012) reported an outbreak of *P. fluorescens* in a coronary care unit.

Chapter 5

ISOLATION AND IDENTIFICATION OF ALGAL ISOLATES

5.1 Introduction

In Chapters 3 and 4, halotolerant bacterial species were successfully isolated from fresh water samples. In the second phase of the work described in this thesis, it was decided to use fresh water samples from the Weston Park pond to look for halotolerant microalgal species with potential for producing biofuels. The isolation and screening of new microalgae strains with potential for biofuels production is not new. For example, in 1983 the Solar Energy Research Laboratory (SERI, now the National Renewable Energy Laboratory, NREL) in Boulder, Colorado, USA started a widespread isolation and screening program of microalgae from saline habitats and compiled extensive and detailed information on many of the strains isolated (Barclay *et al*., 1985; Sheehan *et al*. 1998). This programme ended in the mid 1990s and the close out report suggested that the main barriers to successful exploitation of microalgae for biofuel production were at the biological end of the multi-disciplinary techniques required (Sheehan *et al.*, 1998) (i.e. microalgae species that produced more neutral lipid under saline conditions were necessary for successful commercial exploitation).

As part of the isolation process, it is very important to accurately identify the microalgae. Although, microalgae cells are considerably bigger than bacteria, they still have relatively few phenotypic characters, however, pigment composition (cell colour) can be very helpful in putting the algae into one of the major groups of green algae, diatoms etc (van den Hoek *et al.,* 1995). Nevertheless, molecular techniques involving rRNA sequencing are now commonly used to confirm the identity of microalgal species. It was shown that 18S rRNA gene is highly conserved within an algal species and

among species of the same genus. Therefore, this gene can be used for identifying eukaryotes (Woo *et al*., 2000). Employing these genes in the identification of microorganisms has many advantages over using the conventional techniques. First, 16S and 18S rRNA genes are present in prokaryotes and eukaryotes respectively and perform the same function. Second, their sequences are highly conserved, but contain regions of conserved, variable and hypervariable sequences. Third, their sizes (around 1500 bases for 16S rRNA gene and 1800 bases for 18S rRNA gene) are relatively easy to sequence but large enough to contain sufficient information for identification and phylogenetic analyses of organisms (Spratt, 2004).

In this chapter, two microalgal strains were isolated and identified to the genus level by 18S rRNA gene sequencing. The 18S algal rRNA gene was amplified by the polymerase chain reaction (PCR) and sequenced. The sequence of the PCR product was compared with known 18S rRNA gene sequences in the GenBank database as described in Section 2.12 and the taxonomic placement of the alga was tested. Phylogenetic trees were constructed for both microorganisms to predict their genetic relatedness.

5.2 Results and Discussion

5.2.1 Isolation of Algae

Bold's Basal Medium (BBM – a classic fresh water algal medium) and f/2 Medium (a commonly used artificial sea water medium) were used to isolate algae from the samples of water collected from Weston Park pond in Sheffield (section 2.1). The isolation protocol was to increase the NaCl concentration in the BBM medium from 0.4 M up to 0.8 M NaCl to select for strains that were tolerant to increased salt concentration. Five ml of each BBM medium culture was inoculated in to 50 ml of BBM medium at a range of salinities from 0.4 to 0.8 M NaCl and f/2 medium (which contains approximately 0.5 M NaCl). Flasks were incubated overnight at 25ºC under constant illumination. The best growth was found at low salinities, however some growth was found at 0.6 M NaCl and 0.8 M NaCl.

5.2.2 Initial characterization of strains B1.4BBM and B1F2

On BBM medium, the colour of B1.4BBM strain was observed to green (Figure 5.1), whereas B1F2 strain which was grown in f/2 medium has a yellow colour (Figure 5.2). The colour difference between the two strains was less evident on agar plates (Figures 5.3 to 5.4), but there was still a yellow/green colour for the B1F2 strain.

Examination of the two strains under the light microscope (Figures 5.5 and 5.6) show that their cell morphology is completely different. Strain B1.4BBM has large round green individual cells, whereas strain B1F2 has smaller cells grouped together as packets of four cells.

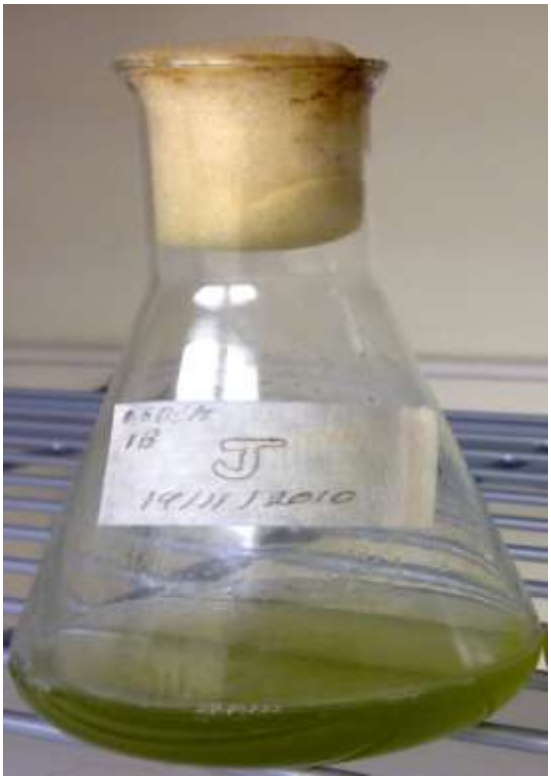

Figure 5.1: Green culture of B1.4BBM Isolate in 0.4 M NaCl BBM Medium.

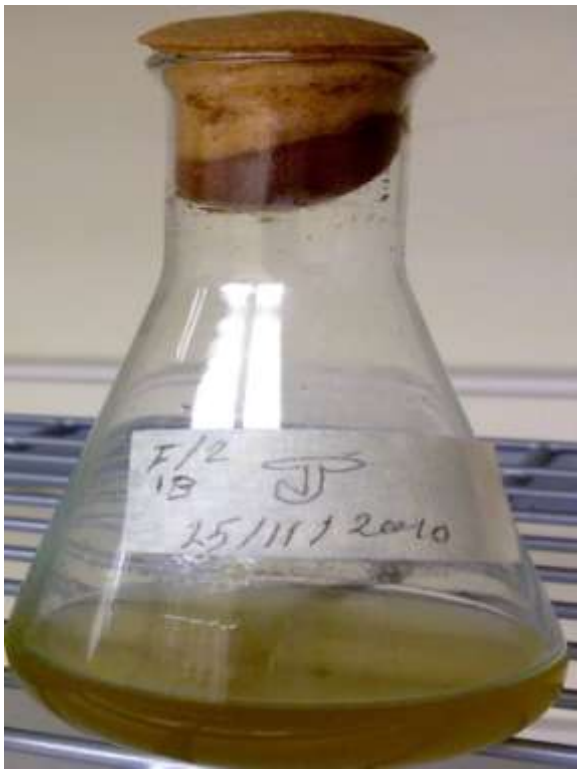

Figure 5.2: Yellow culture of B1F2 Isolate in f/2 Medium.

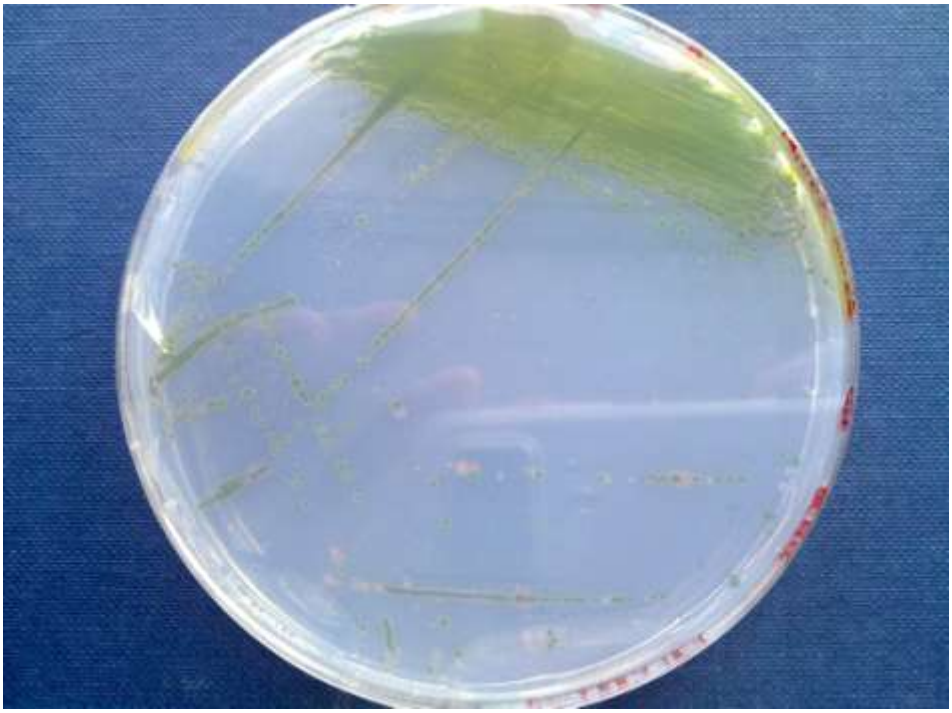

Figure 5.3::Green colonies of B1,4BBM strain grown on BBM medium agar plate.

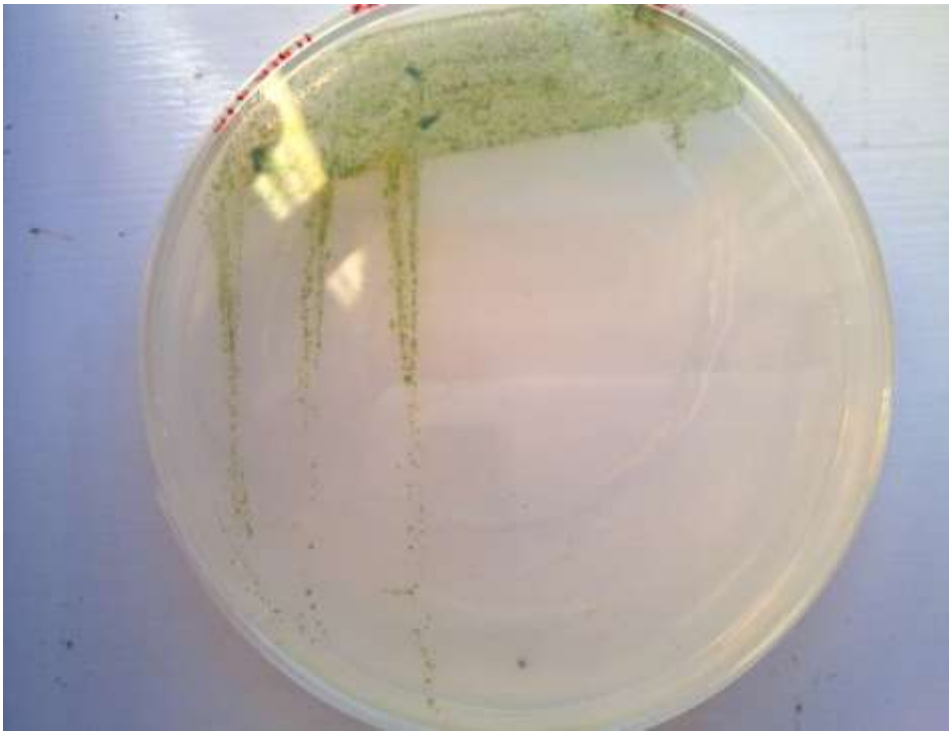

Figure 5.4: Yellow/green colonies of B1F2 strain grown on BBM medium agar plate.

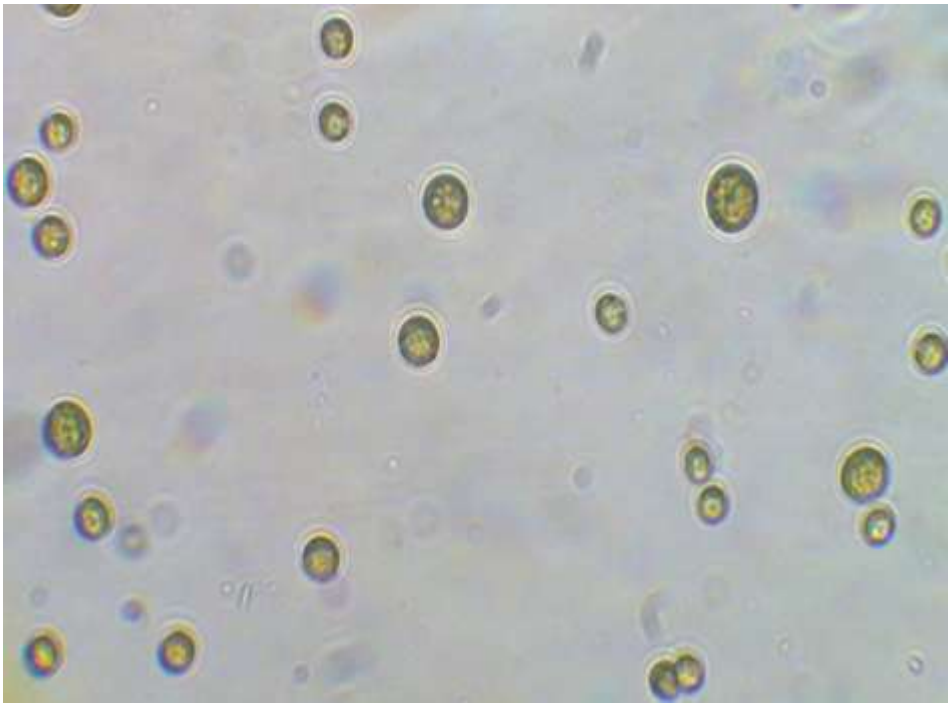

Figure 5.5: Rounded-shaped cells of B1.4BBM strain under light microscope, magnification x 400.

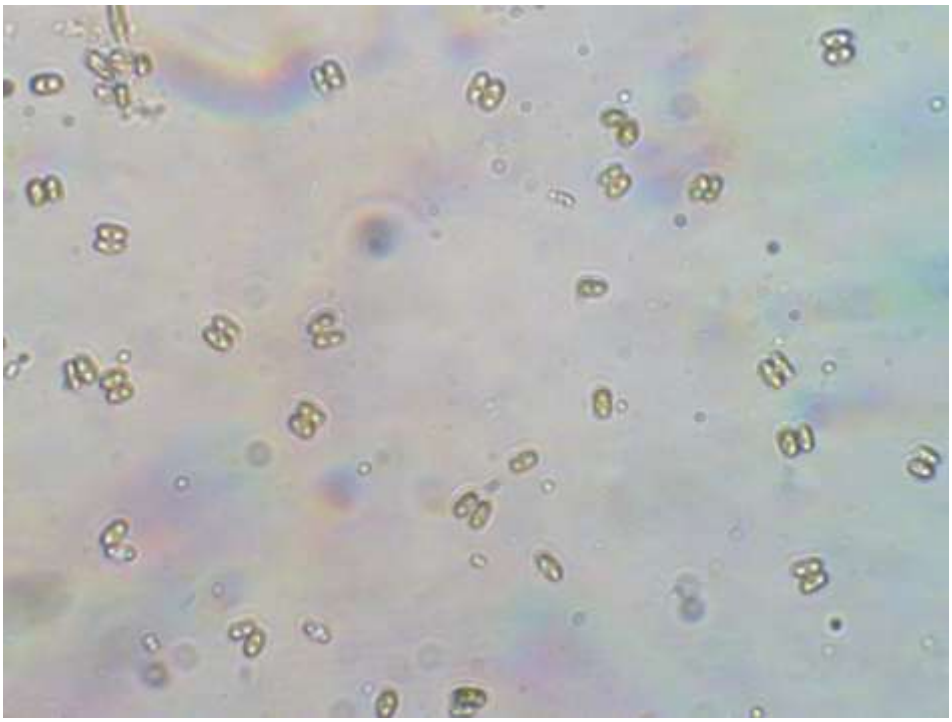

Figure 5.6: Cells of B1F2 strain under light microscope show a four-cell packet type of morphology, magnification x 400.

5.2.3 Molecular Identification of Algal Isolates using 18S rDNA Sequencing

In addition to the two strains described in section 5.2.2, two other strains (2AF2 and AAB) were also identified using 18S rDNA sequencing.

5.2.3.1 Extraction of genomic DNA (gDNA)

Two methods were utilized to extract genomic DNA from the 1BF2, 2AF2, 2AB and 1B.4BBM algal isolates: CTAB and the Qiagen kit. The Qiagen kit extracted the gDNA from all isolates as shown in (Figure 5.7).

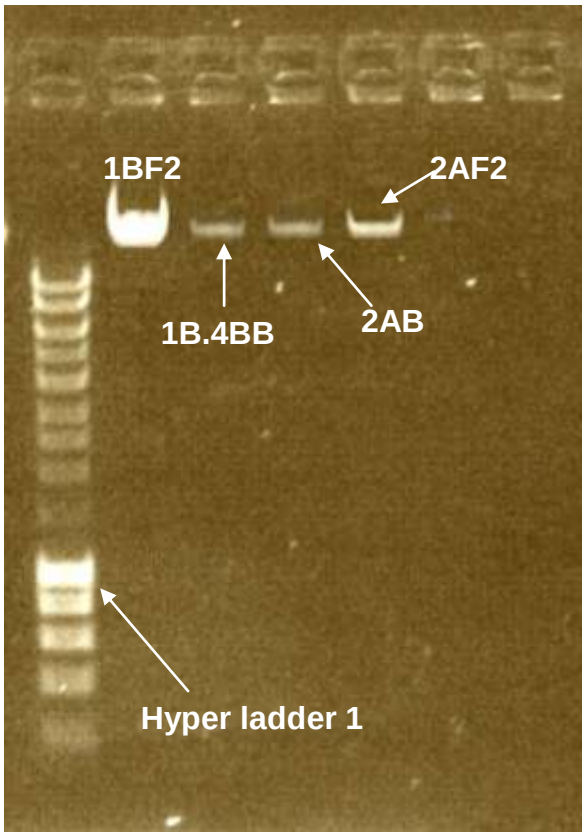

Figure 5.7: Genomic DNA extracted from 1BF2, 1B.4BBM, 2AB and 2AF2 strains using the Qiagen kit.

5.2.3.2 Polymerase Chain Reaction (PCR) amplification.

18S rRNA gene was amplified by PCR for all four algal strains (Figure 5.8). It is clear that all PCR products were approximately the correct size (1500 bp).

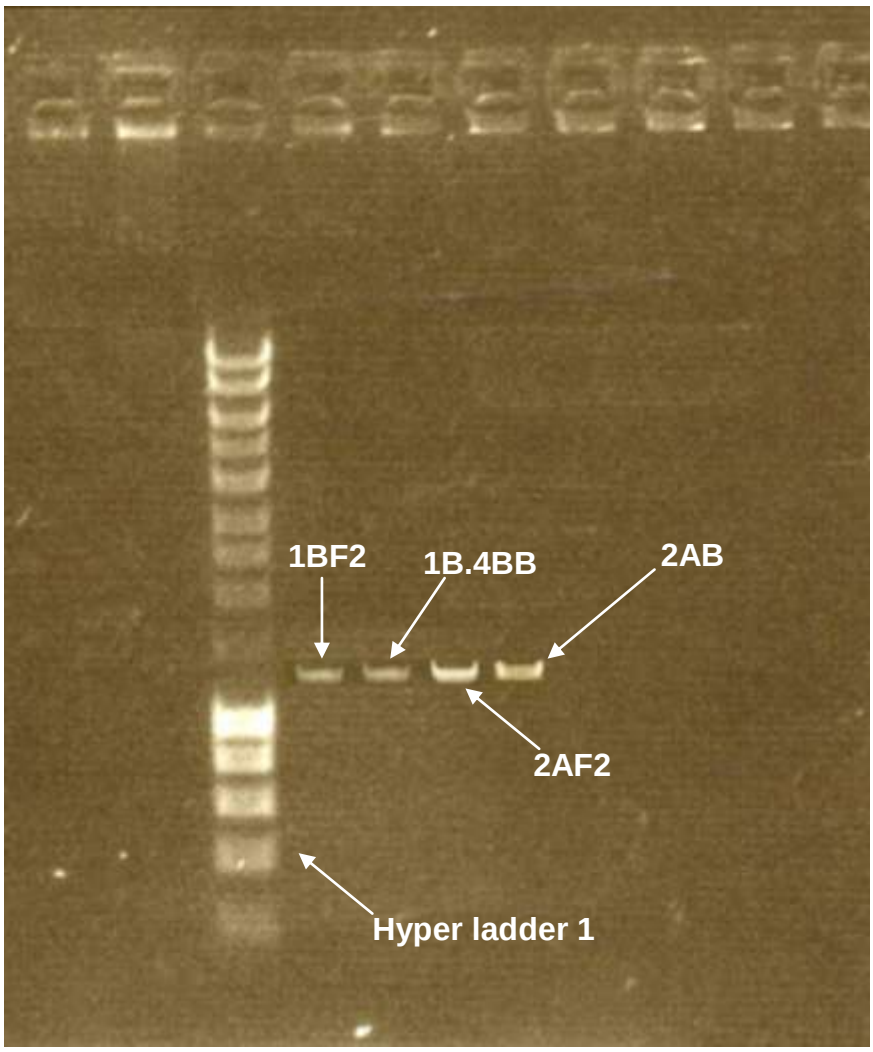

Figure 5.8: Amplification of the 18S rRNA gene product from the PCR involving the universal eukaryote primers.

5.2.3.3 Ligation Using TOPO Cloning

Ligation was carried out using the TOPO Cloning Reaction for all four algal rRNA samples (Figure 5.9). Successful ligation of 18S rRNA into the vector was confirmed by white colonies being produced on the plates. Blue colonies confirmed the presence of the plasmid, but that there was no insertion and the lacZ gene was transcribed and the X-gal substrate was used by *E. coli* cells**.**

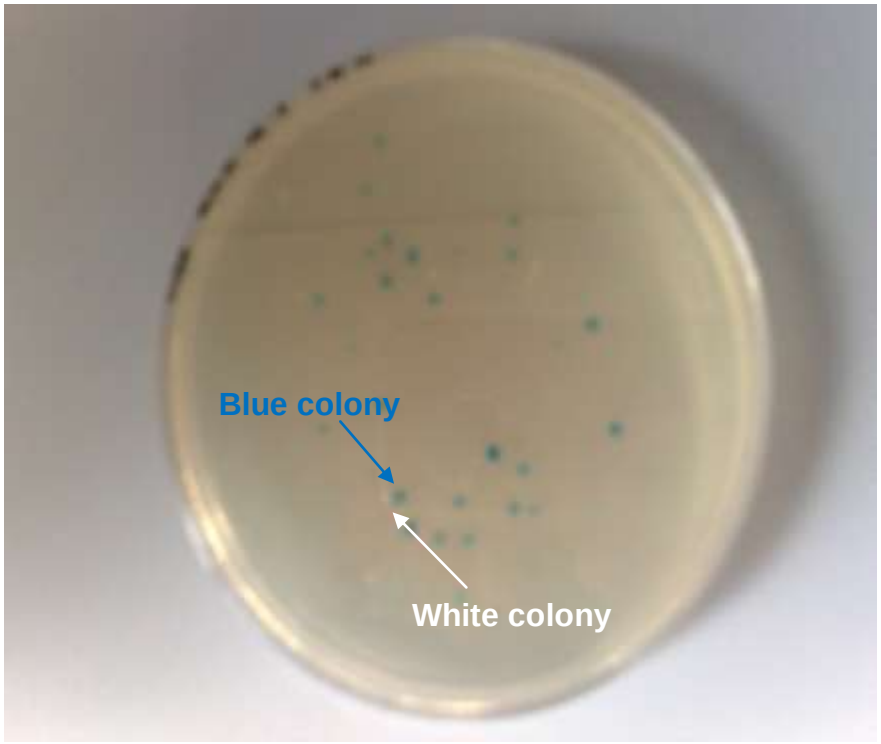

Figure 5.9: Blue-white screen for the detection of successful ligation. Selective LB agar plate contained 50 µl ml^{-1} ampicillin and 40 µl 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

5.2.3.4 Mini-preps and Digestion Restriction

Plasmid was isolated by QIAgen Mini-prep kit from all four algal samples. Figure 5.10 shows successful cutting of plasmid DNA by using *Eco*R1 for both samples.

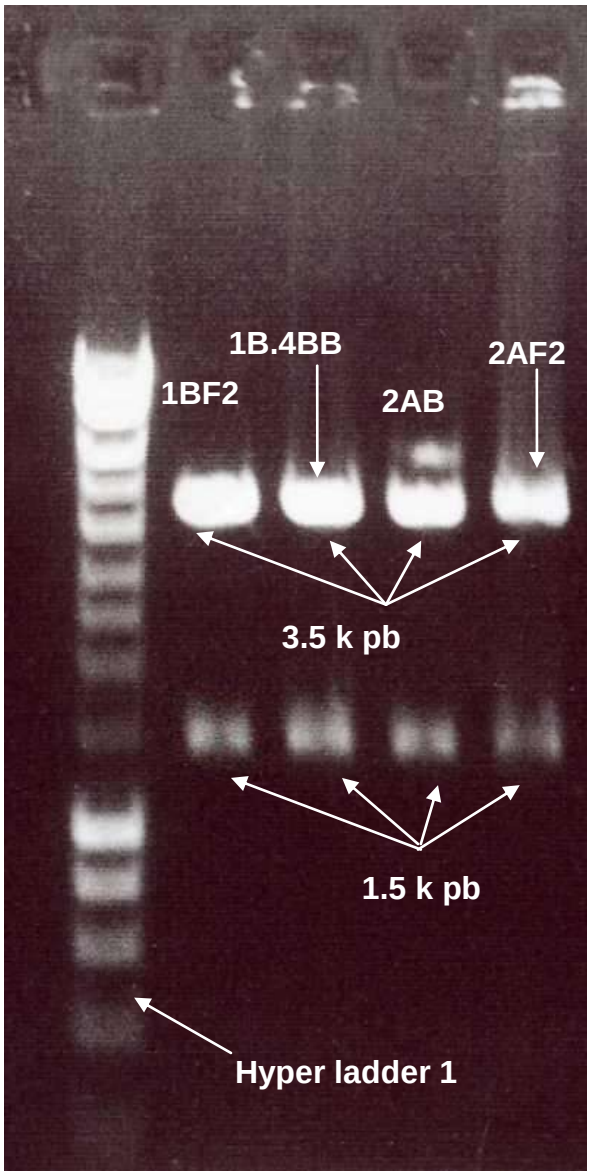

Figure 5.10: Mini-prep digest showing the expected two bands after *Eco*R1 treatment

5.2.3.5 Sequencing of 18S rDNA gene of algal isolates

The vector containing the correct sized insert was sent to the Medical School for sequencing and produced good length sequences (Figures 5.11, 5.13, 5.15 and 5.17). The sequences were compared to other sequences using the NCBI Genbank (Figures 5.12, 5.14, 5.16 and 5.18). The closest matches for each rDNA sequence are shown in Tables 5.1 to 5.4.

Strain 1BF2, which has the yellow/green colour and the morphology of four cells joined together was identified as a diatom of the genus *Navicula*, with *N. pelliculosa* as the most likely species (Table 5.1).

The other strains were all identified as *Chlorella* or closely related genera to *Chlorella* such as *Micractinium* and *Hindakia* (Tables 5.2 to 5.4).

AGCCCCTTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGTGC

GTGATGACTCTTCGGAGTTGTATTTATTAGATGGAAACCAACTGCCTCG

GCATGATGTGGTGATTCATAATAAGCTTGCGGATCGCATGCTTCGGCGG

CGATGGATCATTCAAGTTTCTGCCCTATCAGCTTTGACGGTACTGTATTG

GAGTACCGTGGCGGTAACGGGTAACGGGAAATTAGGGTTTGACACCGG

AGAGGGAGCCTGAGAGACGGCTACCACATCCAAGGAAGGCAGCAGGC

GCGTAAATTACCCAATCTTGACACAAGGAGGTAGTGACAATAAATAACA

ATGCCGGGCCTTTTTGGGTCTGGCAATTGGAATGAGAACAATTTAAACC

CCTTATCGAGGATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGG

TAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTC

GTAGTTGGATTTGTGGCGCGTGTTGCGGCGTCCATTCGTTTGGTTCTGC

CGTGTCCGCGCCATCCTTGGGTGGAATCTGTGTGGCATTAGGTTGTCG

CGCAGGGGATGCCCATCGTTTACTGTGAAAAAATTAGAGTGTTCAAAGC

AGGCTTATGCCGGTGAATATATTAGCATGGAATAATAAGATAGGTCTAG

GGTCCTATTTTGTTGGTTTGCGGTCCTTAGAATGATTTAACAAGGACAGT

TGGGGGTATTCGTATTCCATTGTCAGAGTGAAATTCTGGATTTCTGG

Figure 5.11: 18S rRNA nucleotide gene sequence of 1BF2 strain (780 letters).

Match species/ strain	**Percentage similarity**
Navicula pelliculosa	100%
Navicula saprophila	100%
Craticula importuna	100%
Prestauroneis integra	100%
Stauroneis anceps	100%
Stauroneis kriegeri	100%
Craticula molestiformis	100%
Craticula molestiformis	100%
Bacillariophyta sp	100%
Stauroneis phoenicenteron	100%

Table 5.1: Similarity between 18S rRNA gene sequence of 1BF2 and other related species/strains based on Blast.

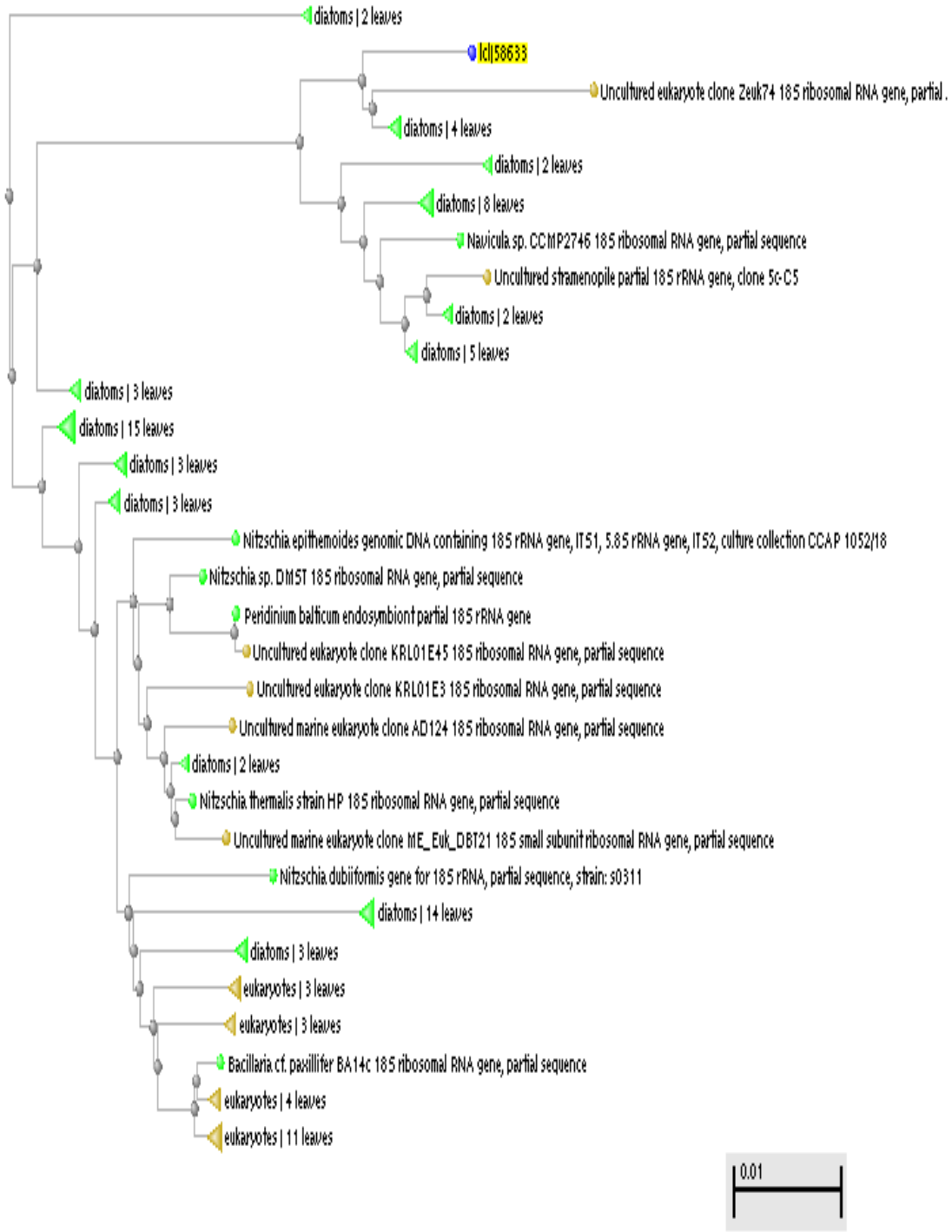

Figure 5.12: Neighbour joining phylogenetic tree for 1BF2 strain (shown in yellow).

TGGTTCCTACTACTCGGATACCCGTACTAAATCTAGAGCTAATACGTGC
GTAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATAAAAGGCCGAC
CGGGCTCTGCCCGACTCGCGGTGAATCATGATAACTTCACGAATCGCAT
GGCCTTGTGCCGGCGATGTTTCATTCAAATTTCTGCCCTATCAACTTTCG
ATGGTAGGATAGAGGCCTACCATGGTGGTAACGGGTGACGGAG,GATTA
GGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAG
GAAGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGT
GACAATAAATAACAATACTGGGCCTTTTCAGGTCTGGTAATTGGAATGA
GTACAATCTAAACCCCTTAACGAGGATCAATTGGAGGGCAAGTCTGGTG
CCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTTAAGTTGCTG
CAGTTAAAAAGCTCGTAGTTGGATTTCGGGTGGGGCCTGCCGGTCCGC
CGTTTTCGGTGTGCACTGGCAGGGCCCACCTTGTTGTCGGGGACGGGC
TCCTGGGCTTTCACTGTCCGGGACTCGGAGTCGACGCTGTTACTTTGAG
TAAATTAGAGTGTTCAAAGCAGGCCTACGCTCTGAATACATTAGCATGG
AATAACACGATAGGACTCTGGCCTATCCTGTTGGTCTGTAGGACCGGAG
TAATGATTAAGAGGGACAGTCGGGGGCATTCGTATTTCATTGTCAGAGG
TGAAATTTCTTGGATTT

Figure 5.13: 18S rRNA nucleotide gene sequence of 1B.4BBM strain (797 letters).

Match species/ strain	**Percentage similarity**
Chlorella sp.	100%
Heynigia riparia	100%
Hindakia fallax	100%
Hindakia fallax	100%
Actinastrum hantzschii	100%
Micractinium sp.	100%
Micractinium sp.	100%
Chlorella sp.	100%
Chlorella sp.	100%
Chlorella vulgaris	100%

Table 5.2: Similarity between 18S rRNA gene sequence of 1B.4BBM and other related species/strains based on Blast.

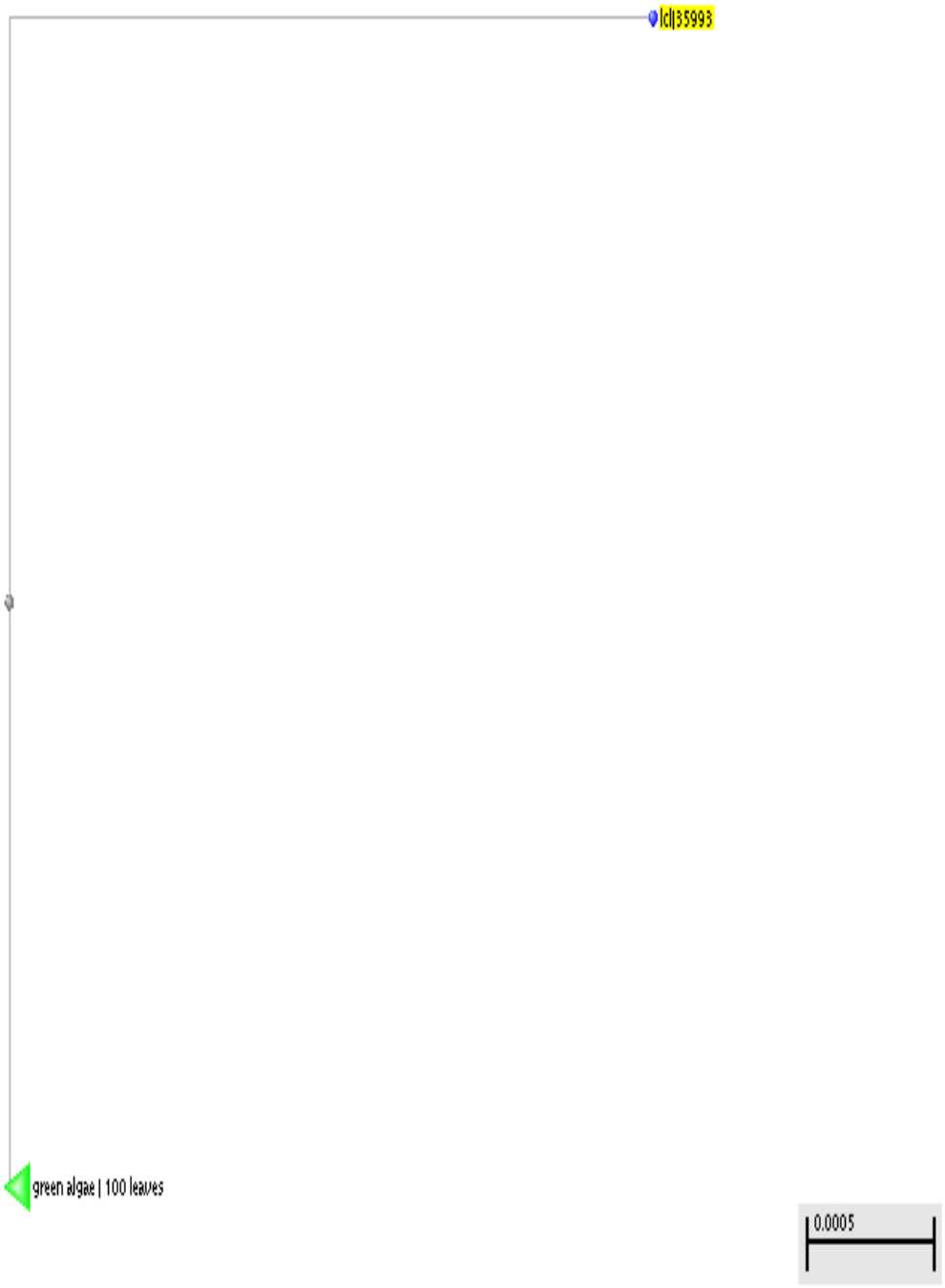

Figure 5.14: Neighbour joining phylogenetic tree for 1B.4BBM strain (shown in yellow). This tree looks strange because a large number of *Chlorella*-like organisms are all equally related to strain 1B.4BBM.

ATGACTTTTCGGCGGCTGAGAGCGGAGACCGCCCCCAGTCGCCAATCC
GAACACTTCACCAGCACACCCAATCGGTAGGAGCGACGGGCGGTGTGT
ACAAAGGGCAGGGACGTAATCAACGCAAGCTGATGACTTGCGCTTACTA
GGCATTCCTCGTTGAAGATTAATAATTGCAATAATCTATCCCCATCACGA
TGCAGTTTCAAAGATTACCCGGGCCTCTCGGCCAAGGCTAAGCTCGTTG
AATGCATCAGTGTAGCGCGCGTGCGGCCCAGAACATCTAAGGGCATCA
CAGACCTGTTATTGCCTCATGCTTCCATTGGCTAGTCGCCAATAGTCCC
TCTAAGAAGTCCGCCGACTGGCGAGCCAATCGTGACTATTTAGCAGGCT
GAGGTCTCGTTCGTTACCGGAATCAACCTGACAAGGCAACCCACCAACT
AAGAACGGCCATGCACCACCACCCATAGAATCAAGAAAGAGCTCTCAAT
CTGTCAATCCTCACTATGTCTGGACCTGGTAAGTTTTCCCGTGTTGAGT
CAAATTAAGCCGCAGGCTCCACGCCTGGTGGTGCCCTTCCGTCAATTC
CTTTAAGTTTCAGCCTTGCGACCATACTCCCCCCGGAACCCAAAAACTT
TGATTTCTCATATGGTGCCGGCGGAGTCATCGAAGAAACATCCGCCGAT
CCCTAGTCGGCATCGTTTATGGTTGAGACTAGGACGGTATCTAATCGTC
TTCGAGCCCCCAACTTTCGTTCTTGATTAATGAAAACATCCTTGGCAAAT
GCTTTCGCAGTAGTTCGTCTTTCATAAATCCAAGAATTTCACCTCTGACA
ATGAAATACGAATGCCCCCGACTGTCCCTCTTAATCATTACTCCGGTCC
TACAGACCAACAGGATAGGCCAGAGTCCTATCGTGTTATTCCATGCTAA
TGTATTCAGAGC

Figure 5.15: 18S rRNA nucleotide gene sequence of 2AF2 strain (942 letters).

Match species/ strain	**Percentage similarity**
Micractinium sp.	100%
Chlorella vulgari	100%
Chlorella sp.	100%
Chlorella sp.	100%
Chlorella sp.	100%
Chlorella sp.	100%
Chlorella sp.	100%
Chlorella sp.	100%
Chlorella sp.	100%
Chlorella sp.	100%

Table 5.3: Similarity between 18S rRNA gene sequence of 2AF2 and other related species/strains based on Blast.

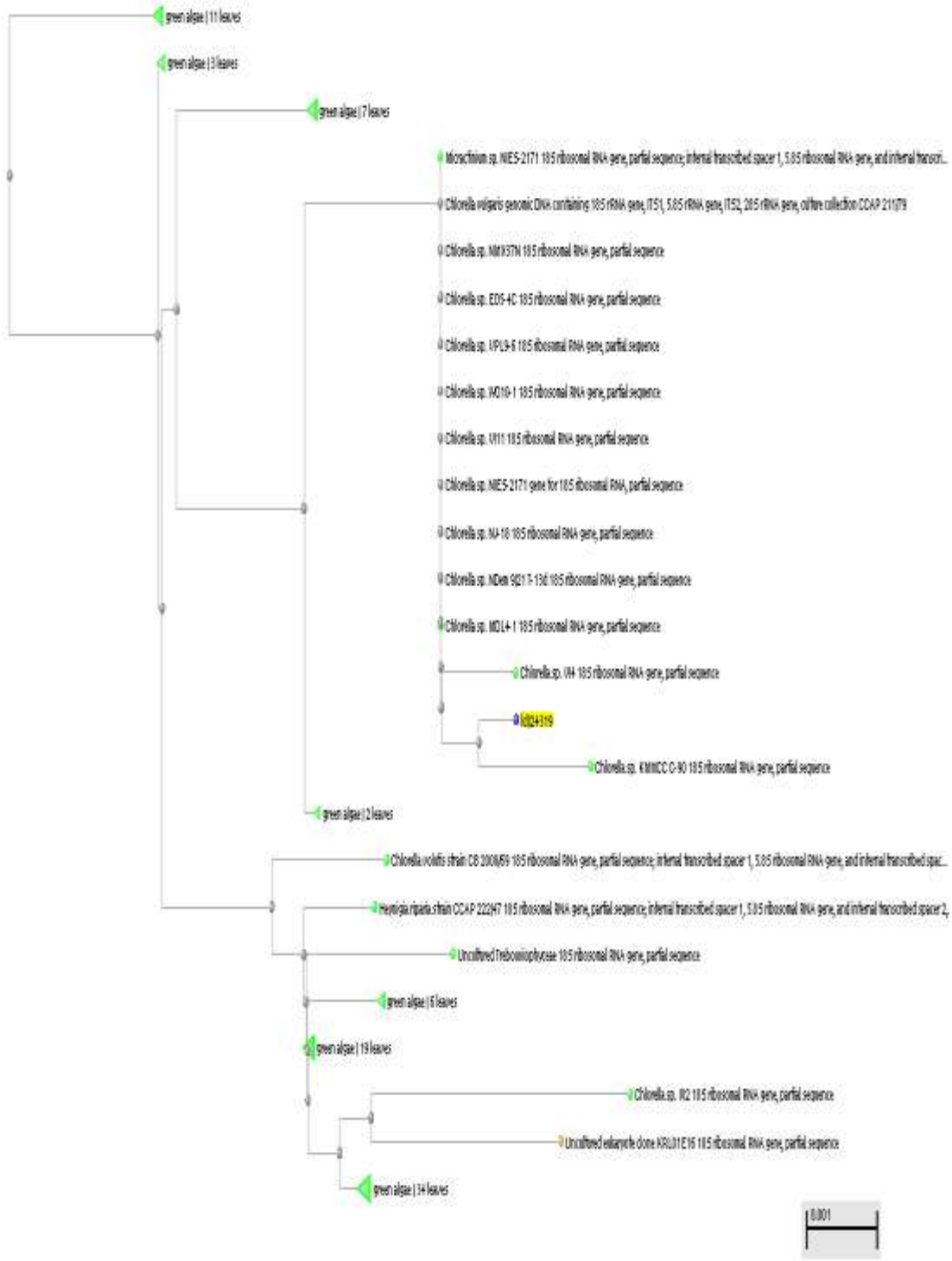

Figure 5.16: Neighbour joining phylogenetic tree for 2AF2 strain (shown in yellow).

ATGACTTTCGGCGGCTGAGAGCGGAGACCGCCCCCAGTCGCCAATCCG
AACACTTCACCAGCACACCCAATCGGTAGGAGCGACGGGCGGTGTGTA
CAAAGGGCAGGGACGTAATCAACGCAAGCTGATGACTTGCGCTTACTA
GGCATTCCTCGTTGAAGATTAATAATTGCAATAATCTATCCCCATCACGA
TGCAGTTTCAAAGATTACCCGGGCCTCTCGGCCAAGGCTAAGCTCGTTG
AATGCATCAGTGTAGCGCGCGTGCGGCCCAGAACATCTAAGGGCATCA
CAGACCTGTTATTGCCTCATGCTTCCATTGGCTAGTCGCCAATAGTCCC
TCTAAGAAGTCCGCCGACTGGCGAGCCAATCGTGACTATTTAGCAGGCT
GAGGTCTCGTTCGTTACCGGAATCAACCTGACAAGGCAACCCACCAACT
AAGAACGGCCATGCACCACCACCCATAGAATCAAGAAAGAGCTCTCAAT
CTGTCAATCCTCACTATGTCTGGACCTGGTAAGTTTTCCCGTGTTGAGT
CAAATTAAGCCGCAGGCTCCACGCCTGGTGGTGCCCTTCCGTCAATTC
CTTTAAGTTTCAGCCTTGCGACCATACTCCCCCCGGAACCCAAAAACTT
TGATTTCTCATATGGTGCCGGCGGAGTCATCGAAGAAACATCCGCCGAT
CCCTAGTCGGCATCGTTTATGGTTGAGACTAGGACGGTATCTAATCGTC
TTCGAGCCCCCAACTTTCGTTCTTGATTAATGAAAACATCCTTGGCAAAT
GCTTTCGCAGTAGTTCGTCTTTCATAAATCCAAGAATTTCACCTCTGACA
ATGAAATACGAATGCCCCCGACTGTCCCTCTTAATCATTTACTCCGGTC
CTACAGACCAACAG

Figure 5.17: 18S rRNA nucleotide gene sequence of 2AB strain (942 letters).

Match species/ strain	**Percentage similarity**
Micractinium sp.	100%
Chlorella vulgaris	100%
Chlorella sp.	100%
Chlorella sp.	100%
Chlorella sp.	100%
Chlorella sp.	100%
Chlorella sp.	100%
Chlorella sp.	100%
Chlorella sp.	100%
Chlorella sp.	100%

Table 5.4: Similarity between 18S rRNA gene sequence of 2AB and other related species/strains based on Blast.

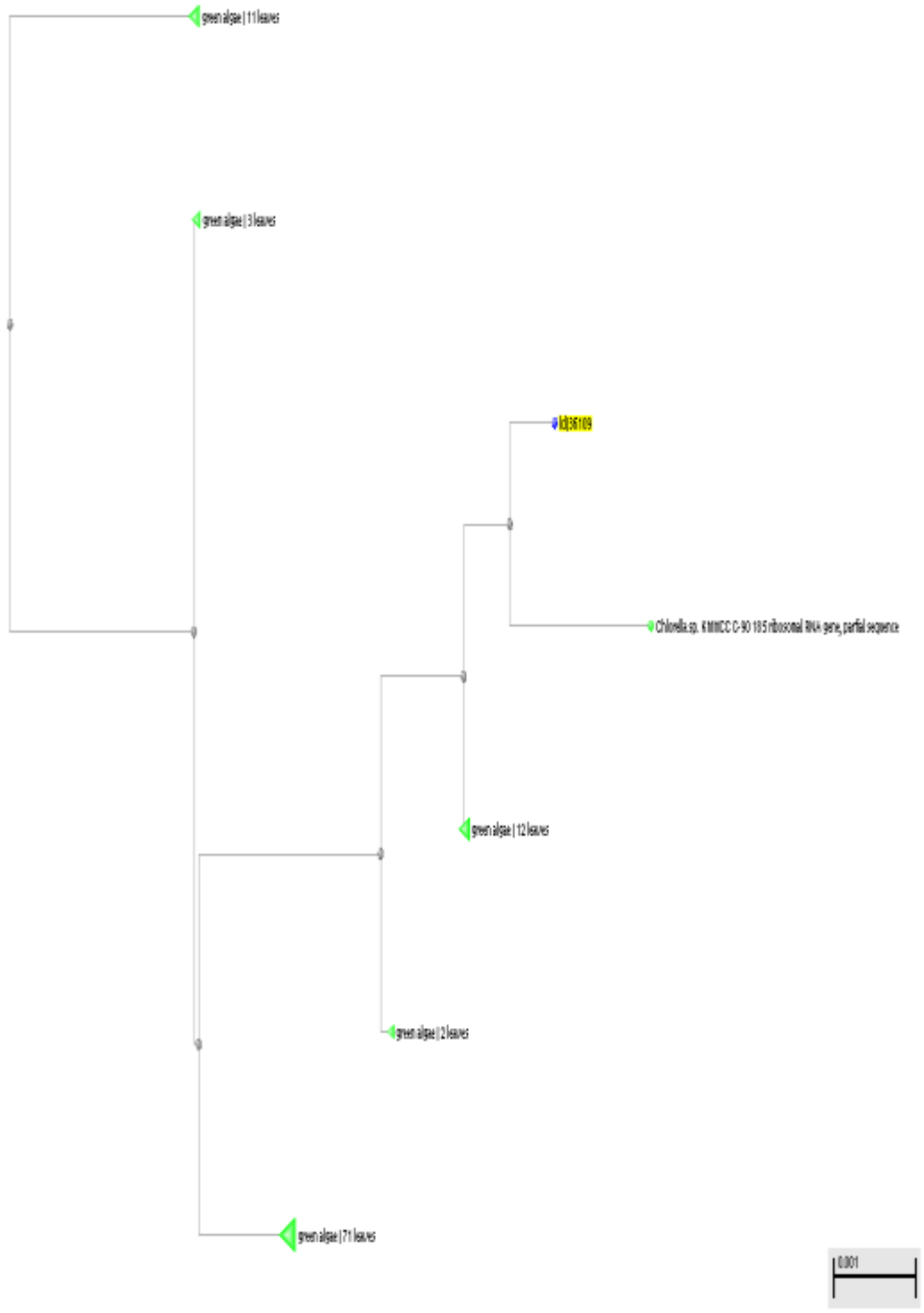

Figure 5.18: Neighbour joining phylogenetic tree for 2AB strain (shown in yellow).

5.3 Conclusion

Strain 1BF2 was clearly identified as a diatom within the genus *Navicula* and the closest species match is *N. pelliculosa.* Diatoms are widespread in nature and play an important part in the microbial biodiversity in rivers and streams (Patrick, 1961), so it is not surprising to find *N. pelliculosa* in the Weston Park pond. One of the key characteristics of diatoms is that their cell walls or frustules are composed of amorphous hydrated silica, also their cell size is relatively small being about (2 – 20 µm). They are golden-brown in colour due to the presence of chlorophyll c and fucoxanthin (Berzano *et. al*., 2012).

The other three strains (2AB, 2AF2 and 1B.4BBM) all fall within the *Chlorella* group of green algae. The taxonomy of *Chlorella*-like algae is complex (Huss *et al.,* 1999) and new genera such as *Micractinium* are being split away from the main *Chlorella* genus (Hoshina and Fujiwara, 2013).

Due to the fact that *Chlorella* strains have been well studied in general and their potential to produce biofuel has also been investigated (Phukan *et al.,* 2011), it was decided to further characterize *Navicula pelliculosa* and investigate its potential use in biofuel production.

Chapter 6

FURTHER CHARACTERISATION AND POTENTIAL BIOFUEL PRODUCTION OF *NAVICULA PELLICULOSA*

6.1 Introduction

Microalgal species used for biofuel production are likely to be grown in outdoor raceway ponds, which are open to the elements and are easily contaminated (Chisti, 2008). To avoid contamination issues, the use of extremophilic algae is recommended and halotolerant and/or alkalitolerant algae are likely to be suitable (Gilmour and Zimmerman, 2012; Gardner *et al*., 2011). Therefore, the diatom species *Navicula pelliculosa* isolated and identified in Chapter 5 will be further characterised in this chapter to see how well it grows in high salinity and high pH. Diatoms, with a very few exceptions such as *Phaeodactylum tricornutum*, require silica to grow and form their cell walls (van den Hoek*et al.,* 1995), therefore the requirement for silica will be examined in the *Navicula* isolate.

Determination of microalgae growth can be achieved by counting the number of algal cells under the microscope using a graduated counting chamber or by measuring the optical density (absorbance). Any indirect method such as OD measurement must be used with caution (Tsuzuki *et al*., 1990; Becker, 1994) and in the work described in this chapter both methods were used. Borowitzka and Moheimani (2012) stated that OD measurement is an exceedingly fast method for calculating an approximate growth curve. If the OD is used, it is important to find the correlation between cell count and the OD and how trustworthy this relationship is under different culture conditions. (Griffiths *et al*., 2011). It is also worth keeping in mind that alteration of the cell pigment content can increase the error associated with growth measurements made using absorbance (Anderson, 2005; Guillard and Sieracki, 2005).

Biodiesel consists of fatty acid methyl esters (FAMEs), which can be produced by the transesterification of triacylglycerol (TAG) (Chisti, 2007). TAG is neutral storage lipid with three fatty acid chains attached to each molecule of glycerol, and it is synthesized by microorganisms, plants and animals. FAME provides an increased energy yield in comparison to ethanol that is also derived biologically; it can be injected immediately into diesel or jet engines (Cunningham, 2007; Hill *et al*., 2006). However, by using TAG from crop plants, the availability of fuel will be limited because of other factors involved in crop production such as the food versus fuel debate (Chisti, 2008). Due to their large physiological diversity and fast growthrate (Hu *et al*., 2008), microalgae have been suggested to be the best suited group of organisms to overcome the limitations of variety of climates and the food versus fuel debate. The TAG content of plants such as soybean and oil palm is around 5% while it can be as high as 80% of microalgae dry weight, although 50% of dry weight is more likely to be achieved (Banerjee *et al*., 2002; Chisti, 2007, 2008; Benemann and Oswald, 1996.; Sheehan *et al*., 1998).

In this chapter, the strain of *Navicula* which was isolated and identified in Chapter 5 will be stressed using high pH and high salinity in both BBM and f/2 medium. The *Navicula* strain of the isolated microalgae was further studied based on its ability to produce lipid under different pH and saline conditions.

6.2 Results and Discussion

6.2.1 Adaptation of Navicula pelliculosa strain to different salinities

In order to investigate the range of salinities that the freshwater *Navicula* strain could tolerate, cells of the strain were repeatedly sub-cultured in BBM medium containing higher levels of NaCl on an incremental basis. Initially, *Navicula* was adapted to grow in 0.4 M NaCl, then 0.6 M NaCl and 0.8 M NaCl over a period of several weeks. The same process was repeated in f/2 medium. Figures 6.1 and 6.2 show growth curves for *Navicula* in BBM media. *Navicula* had the ability to grow across the full range of salt concentrations up to 0.8 M NaCl in BBM medium. It is noteworthy that cell counts showed significantly lower growth at 0.8 M NaCl, whereas this growth decreased at 0.8 M NaCl is not evident when OD_{600} is measured.

Figures 6.3 and 6.4 show that growth of the *Navicula* strain was decreased at both 0.6 and 0.8 M NaCl in f/2 medium, which demonstrates that *Navicula* was less salt tolerant in f/2 medium than in BBM medium (Figures 6.1 to 6.4).

6.2.2 Adaptation of Navicula pelliculosa strain to different pH values

In order to investigate the range of pH that the *Navicula* strain could tolerate, cells were repeatedly sub-cultured in BBM medium containing higher levels of pH on an incremental basis. Initially, *Navicula* was adapted to grow in pH 7, then pH 9 and pH 11 over a period of several weeks. The same process was repeated in f/2 medium with different levels of pH, it was adapted to grow in pH 7.6, pH 8.5, pH 9.25 and pH 10. Figures 6.5 and 6.6 show growth

curves for *Navicula* in BBM medium at the different pH values and Figures 6.7 and 6.8 show the growth in f/2 medium at different pH values. *Navicula* had the ability to grow across the full range of pH in both media, with good growth still evident at pH 11 in BBM medium (Figure 6.6). Again, the cell counts provided more discrimination than the OD measurements.

6.2.3 Growth curves of Navicula pelliculosa strain in different concentrations of silica

In all of the experiments described above, *Navicula* was grown in BBM and f/2 medium containing silica 0.1 mM. To check for any effects of silica on growth of *Navicula*, growth curves were measured in BBM and f/2 medium with different concentrations of silica. In BBM medium (Figures 6.9 and 6.10), there was no effect of lowering the silica concentration to 25% of normal value over the first 5 days of growth. Longer term effects were not measured in this experiment.

In f/2 medium, there was little or no effect on growth of *Navicula* when the silica concentration was varied between 25 and 200% (Figures 6.11 and 6.12).

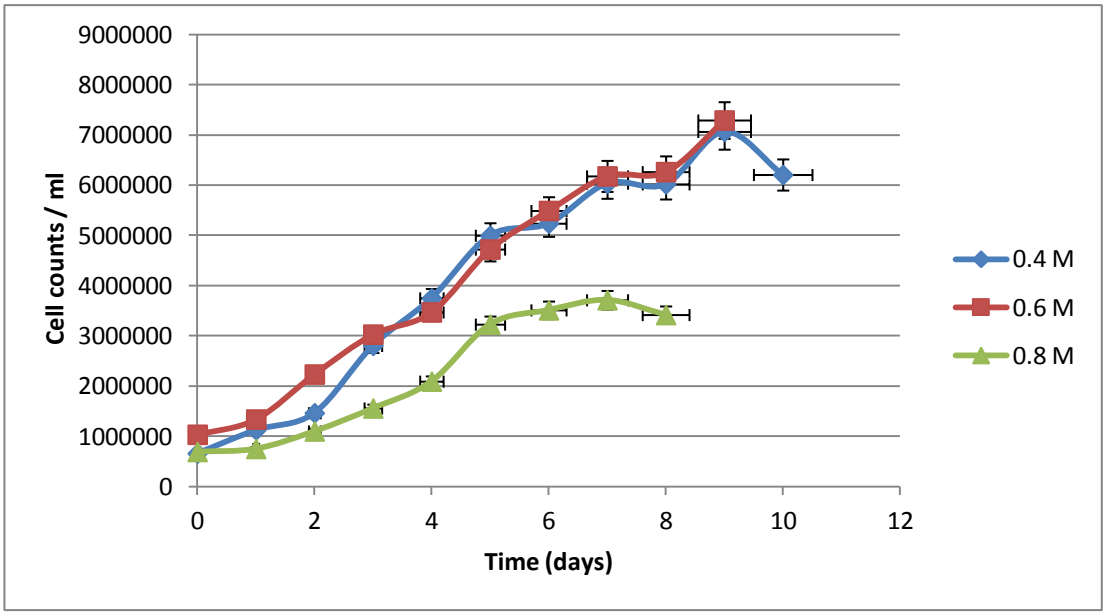

Figure 6.1: Cell counts for Navicula strain grown at a range of salinities. Cells were grown in BBM medium with silica containing 0.4, 0.6 or 0.8 M NaCl with error bars.

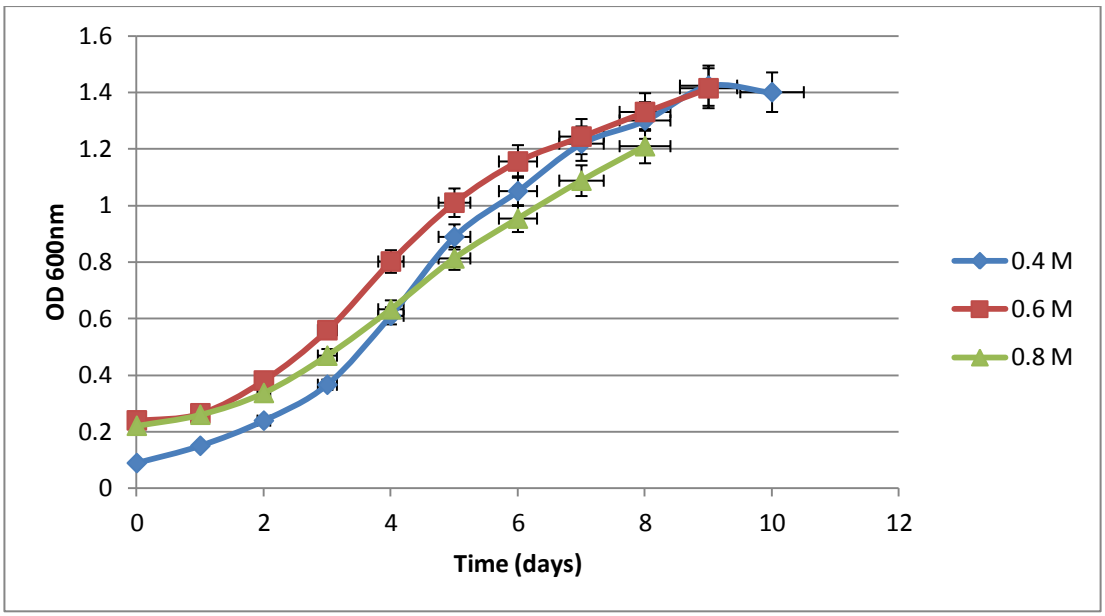

Figure 6.2: Growth curves for Navicula strain grown at a range of salinities. Cells were grown in BBM medium with silica containing 0.4, 0.6 or 0.8 M NaCl. The OD was measured at 600 nm against an appropriate medium blank with error bars.

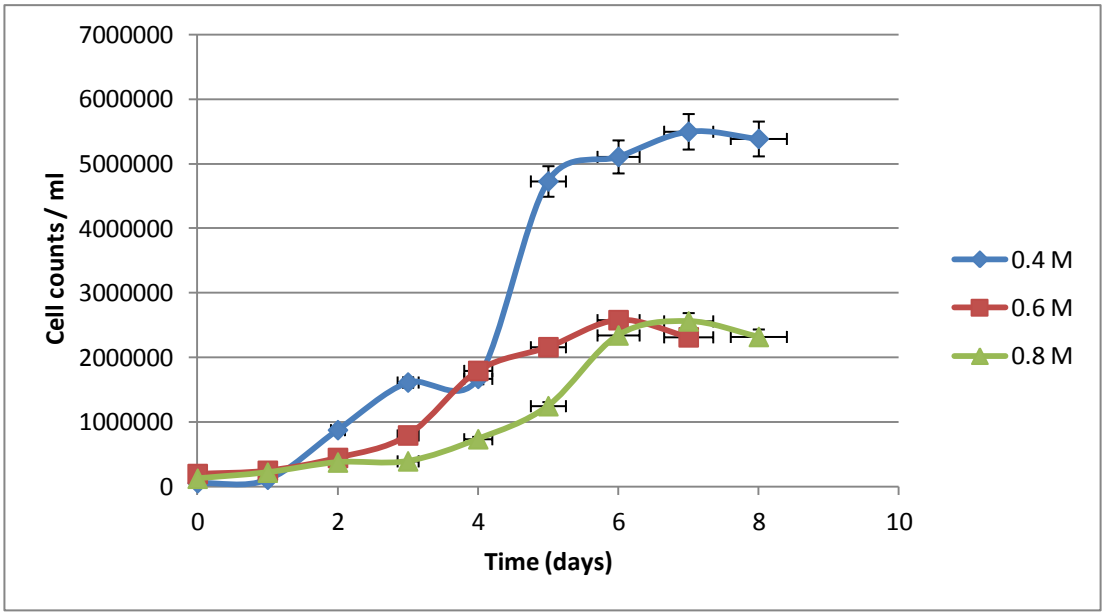

Figure 6.3: Cell counts for Navicula strain grown at a range of salinities. Cells were grown in f/2 medium with silica from 0.4 M to 0.8 M NaCl with error bars.

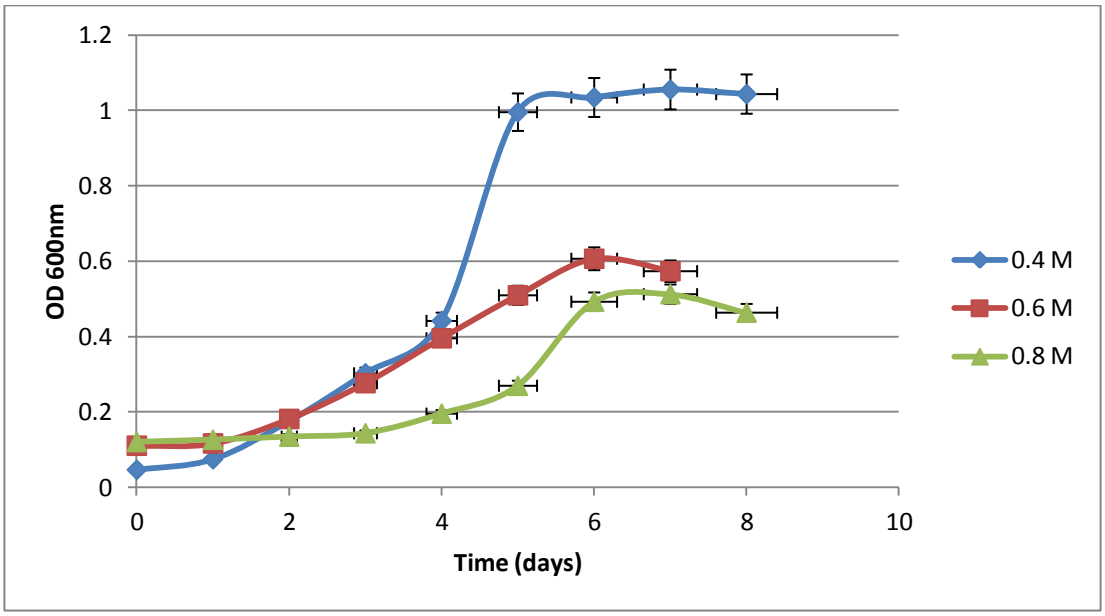

Figure 6.4: Growth curves for Navicula strain at a range of salinities. Cells were grown in f/2 medium with silica from 0.4 M to 0.8 M NaCl. The OD was measured at 600 nm against an appropriate medium blank with error bars.

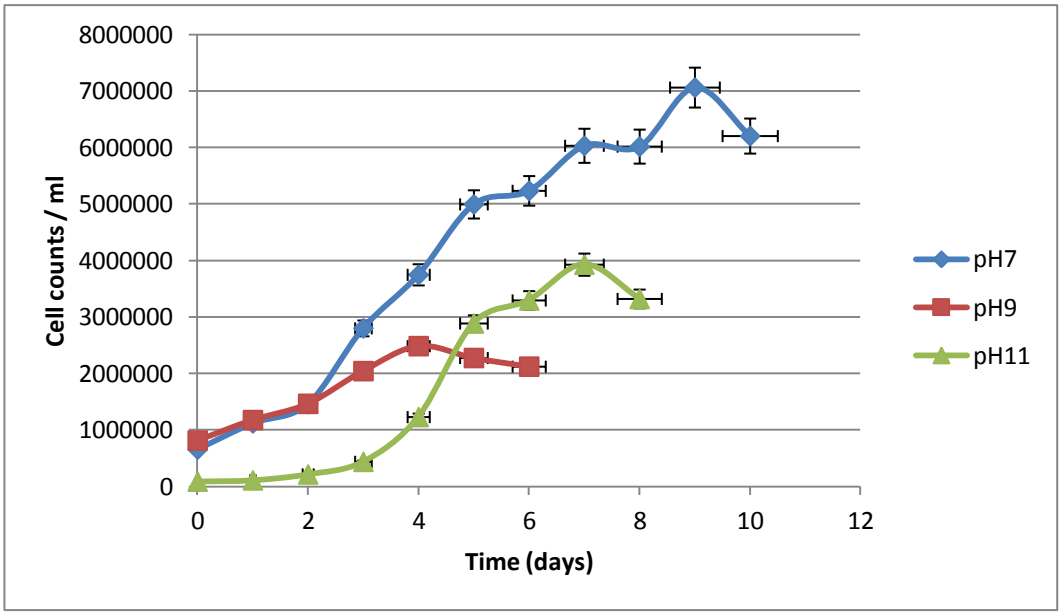

Figure 6.5: Cell counts for Navicula strain grown at a range of pH values. Cells were grown in 0.4 M NaCl BBM medium with silica at pH 7, pH 9 and pH 11 with error bars.

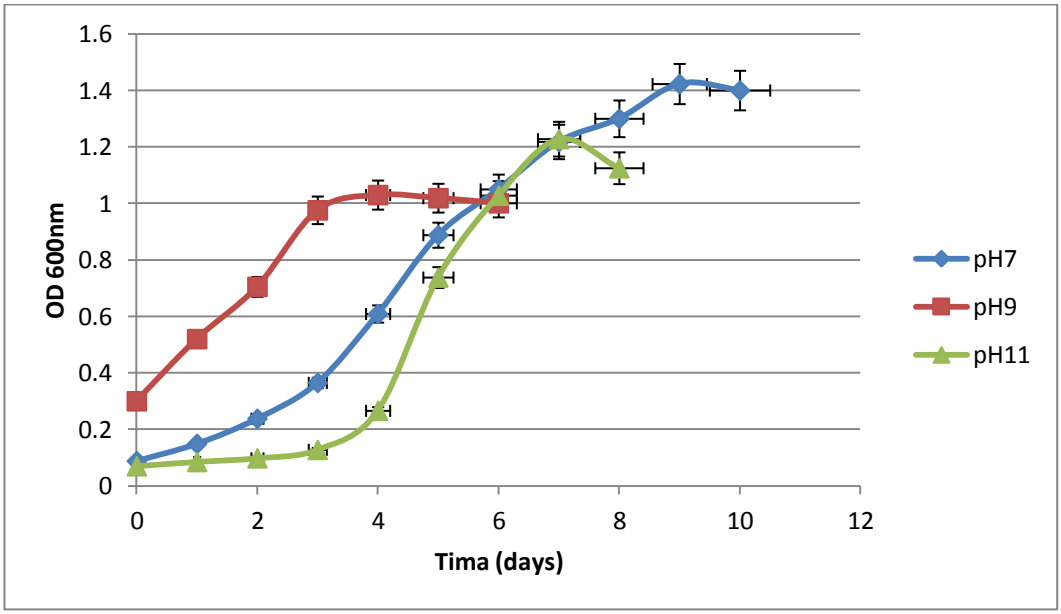

Figure 6.6: Growth curves for Navicula strain at a range of pH values. Cells were grown in 0.4 M NaCl BBM medium with silica at pH 7, pH 9 and pH 11. The OD was measured at 600nm against an appropriate medium blank with error bars.

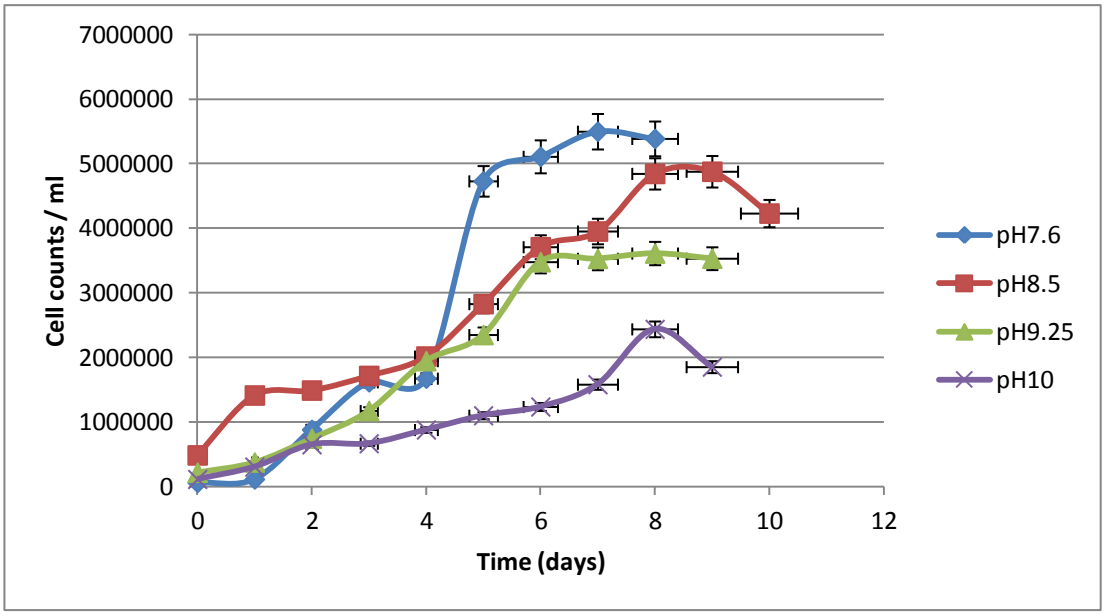

Figure 6.7: Cell counts for Navicula strain at a range of pH values. Cells were grown in f/2 medium with silica and the pH was adjusted to vary from 7.6 to 10 with error bars.

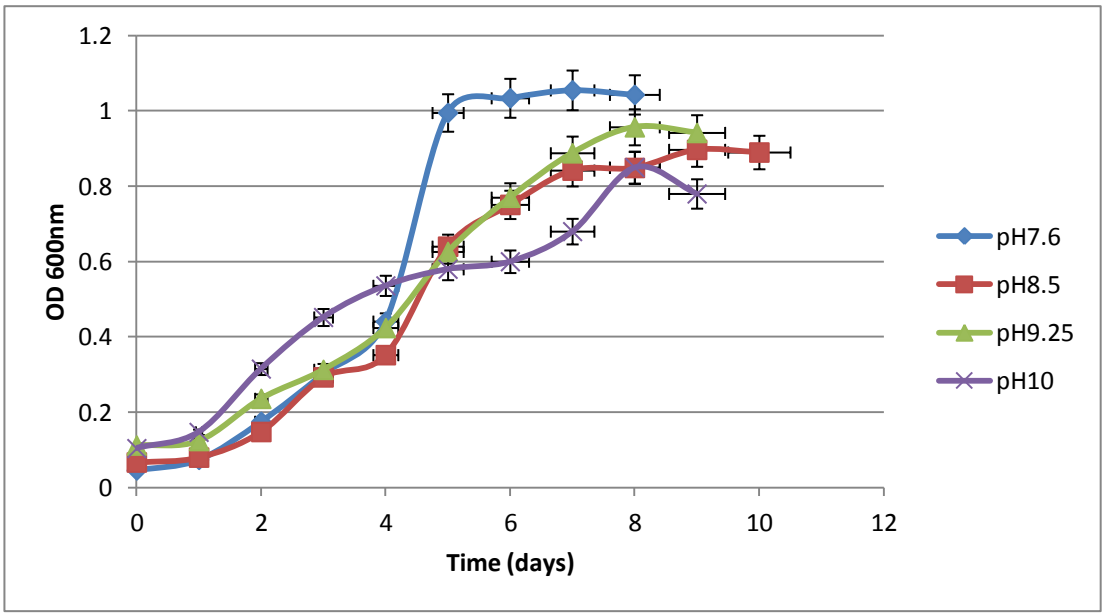

Figure 6.8: Growth curves for the Navicula strain at a range of pH values. Cells were grown in f/2 medium with silica and the pH was adjusted to vary from 7.6 to 10. The OD was measured at 600nm against the appropriate medium blank with error bars**.**

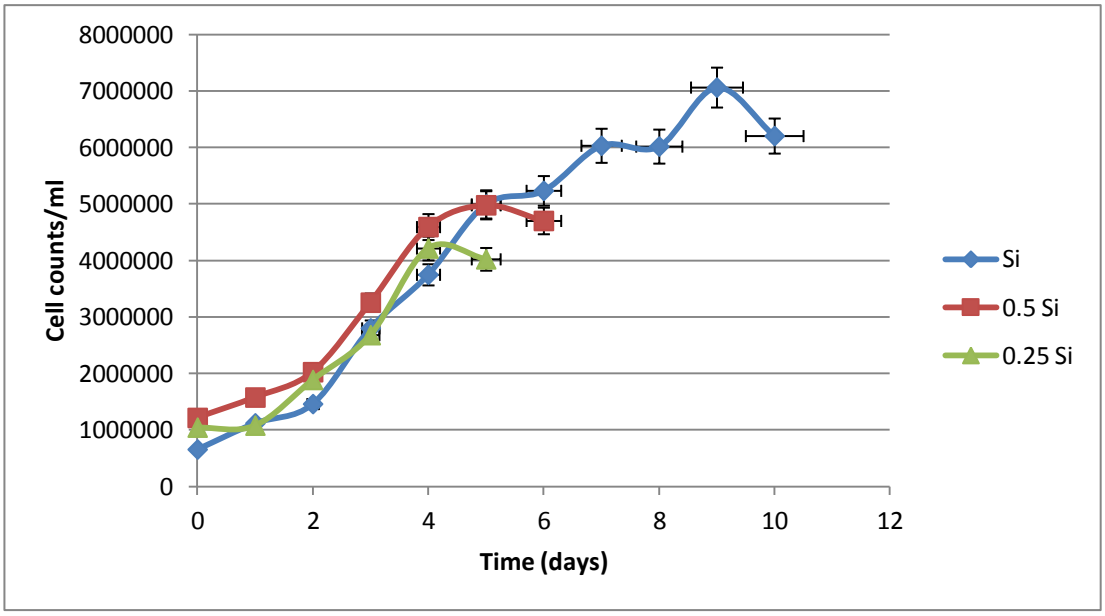

Figure 6.9: Cell counts for Navicula strain grown in different concentrations of silica. Cells were grown in BBM medium plus silica at 25%, 50% and 100% of normal value which is 0.1 mM with error bars.

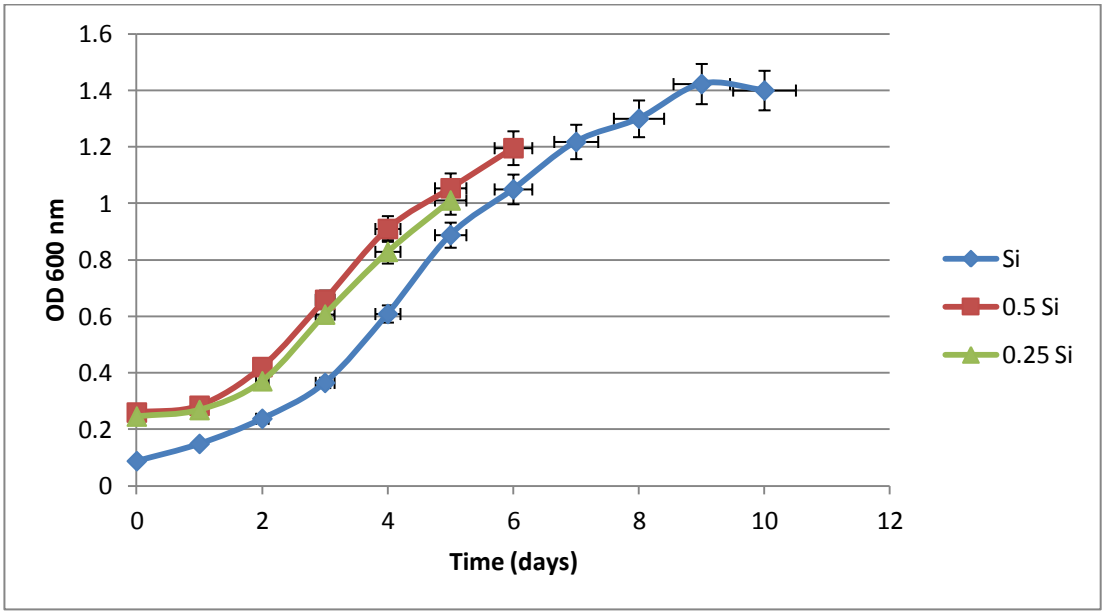

Figure 6.10: Growth curves for Navicula strain in different concentrations of silica. Cells were grown in BBM medium plus silica at 25%, 50% and 100% of normal value which is 0.1 mM. The OD was measured at 600 nm against an appropriate medium blank with error bars.

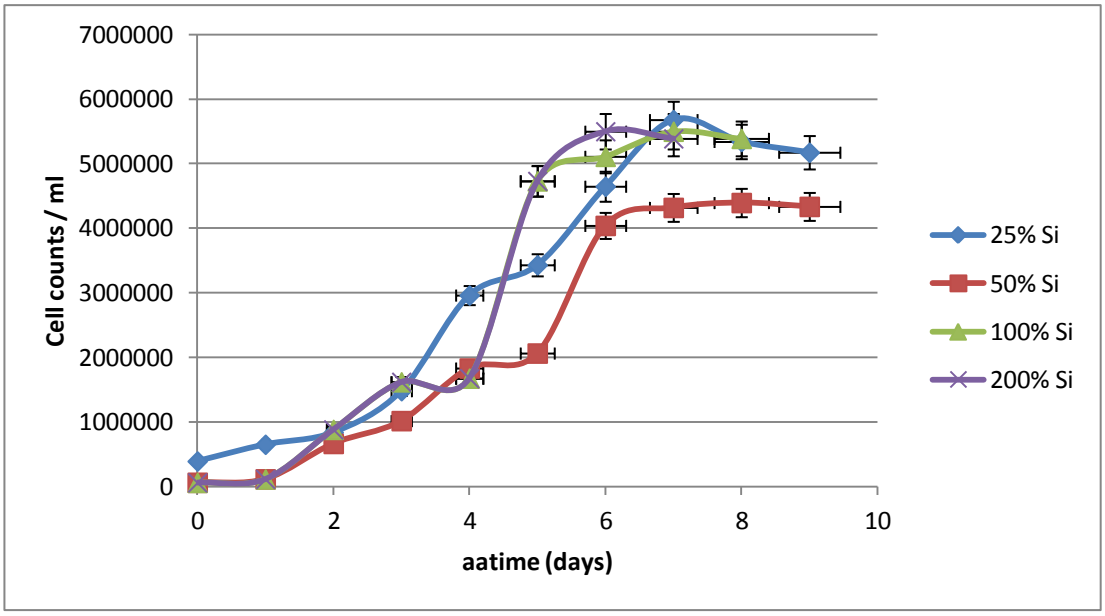

Figure 6.11: Cell counts for Navicula strain grown in different concentrations of silica. Cells were grown in f/2 medium plus silica at 25%, 50%, 100% and 200% of normal value which is 0.1 mM with error bars.

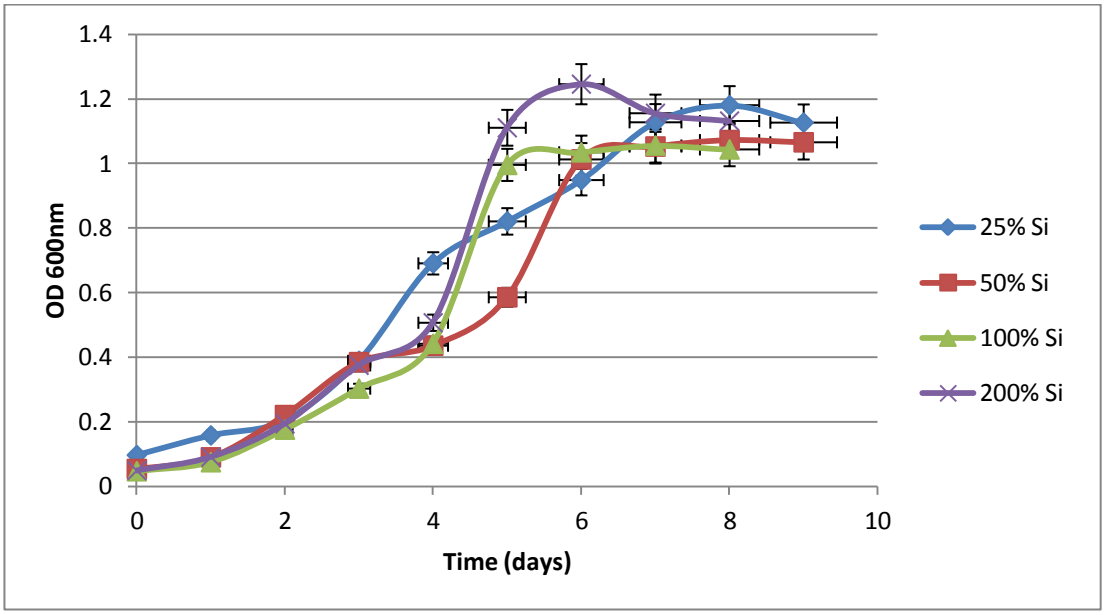

Figure 6.12: Growth curves for Navicula strain grown in different concentrations of silica. Cells were grown in f/2 medium with silica at 25%, 50%, 100% and 200% silica of normal value which is 0.1 mM. The OD was measured at 600nm against an appropriate with error bars.

6.2.4 Lipid Determination by Colorimetric Methods

The initial determination of lipid content involved all four strains isolated from the Weston Park pond. The sulphovanillin (SPV) method clearly showed that *Navicula* had the highest levels of total lipid with values up to nearly 20% (Figure 6.13).

Total lipid as a percentage of biomass can also be measured gravimetrically after a Bligh and Dyer lipid extraction using chloroform/methanol. The errors associated with the gravimetric method can be large and fairly substantial amounts of biomass are required to get consistent measurements. Figure 6.14 shows that many of the gravimetric measurements exceed 100% of biomass, which are clearly impossible. However, the values do correlate with the values measured by the SPV method and the greater than 100% values are probably due to underestimation of the total dry weight after sonication and freeze-drying (Figure 6.14).

Despite the substantial errors, the results in Figures 6.13 and 6.14 show *Navicula* as the most promising strain for biodiesel production, and all further lipid analysis was carried out on the *Navicula* strain only.

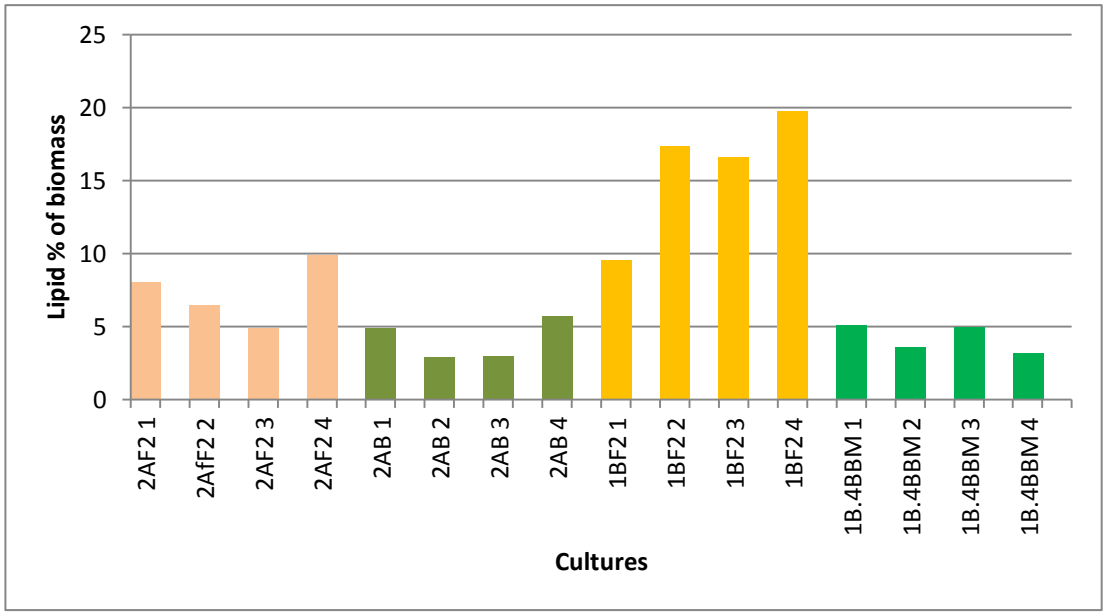

Figure 6.13: Total lipid content of Navicula (1BF2, yellow) and Chlorella-like strains (2AF2, 2AB1 and 1B.4BBM) sp. using the sulphovanillin (SPV) method.

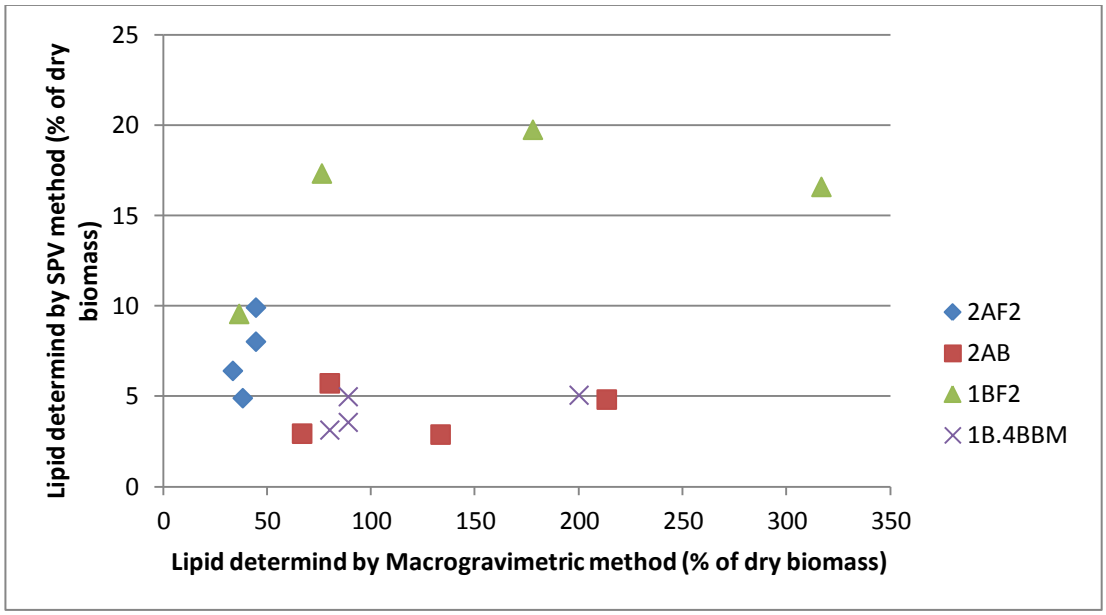

Figure 6.14: Correlation between total lipid (percentage of dry biomass) for the four algae strains as determined using the colorimetric (SPV) method and macro-gravimetric method. Note that the gravimetric method often gives values exceeding 100%**.**

6.2.5 Determination of Neutral Lipid (TAG) by Nile Red Fluorescence

Prior to measuring neutral lipid concentration in *Navicula* cells, a number of preliminary experiments must be carried out. Firstly, the optimum concentration of Nile Red (NR) dye must be established (Figure 6.15). 2 mM NR (dissolved in acetone) gave a good fluorescence signal, which was not increased by an increase in NR concentration to 3 mM.

The time taken for the highest (peak) NR fluorescence was measured in Figure 6.16. Peak fluorescence was reached about 15 minutes after adding NR.

A concentration curve was produced that related NR fluorescence to the concentration of the neutral lipid standard compound triolein (Figure 6.17). This allows the conversion of the arbitrary fluorescence units to triolein equivalents.

Further standardization involved relating the cell number of *Navicula* samples to OD_{595}. This was done for both the plate reader using 96 well plates (Figure 6.18) and using 1 ml cuvettes in the spectrophotometer (Figure 6.19). A comparison between the two methods is shown in Figure 6.20. As expected the results are very different for the two methods because of the different volumes and different machines used.

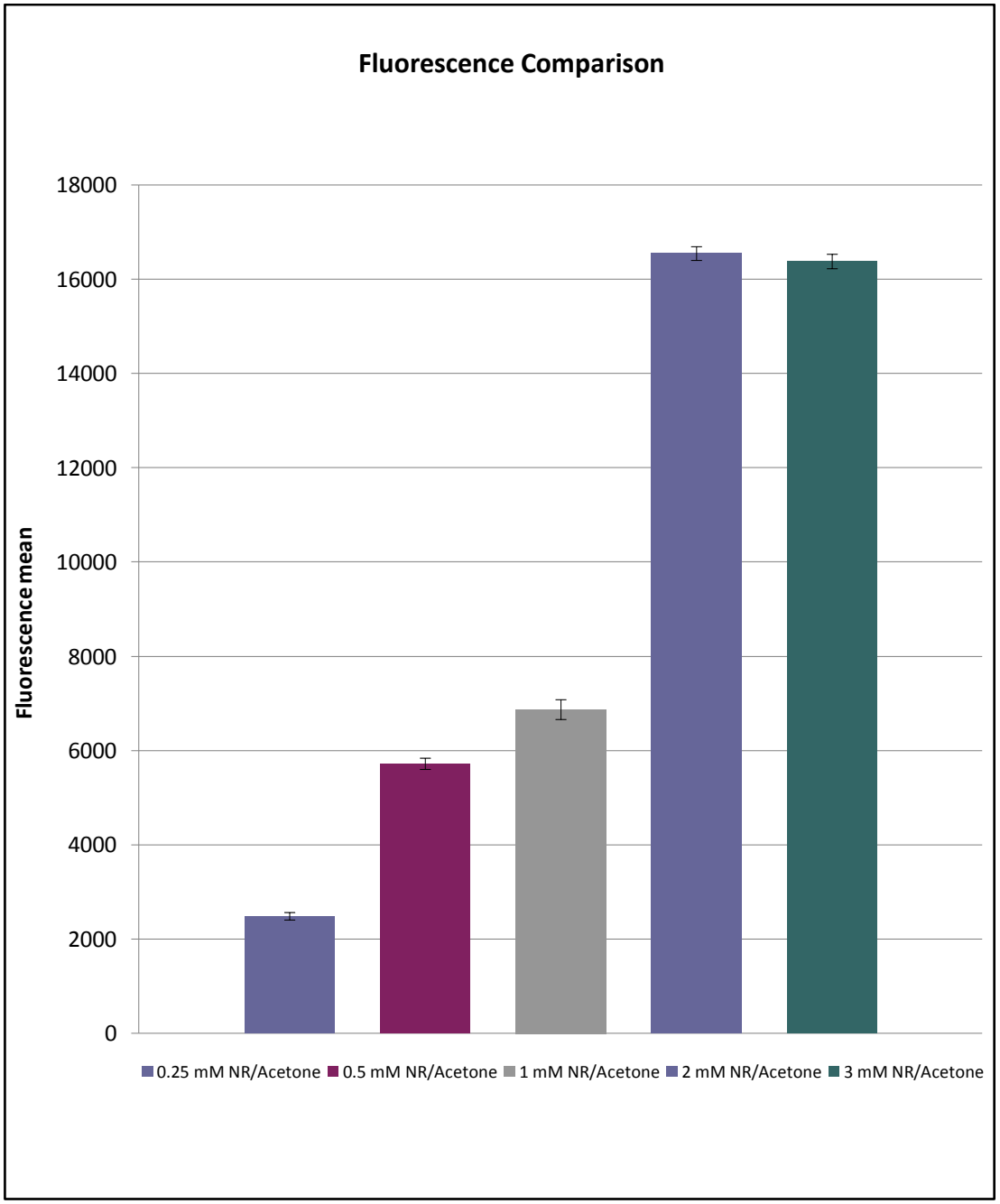

Figure 6.15: Nile Red concentration for measurement of lipid content in thesample

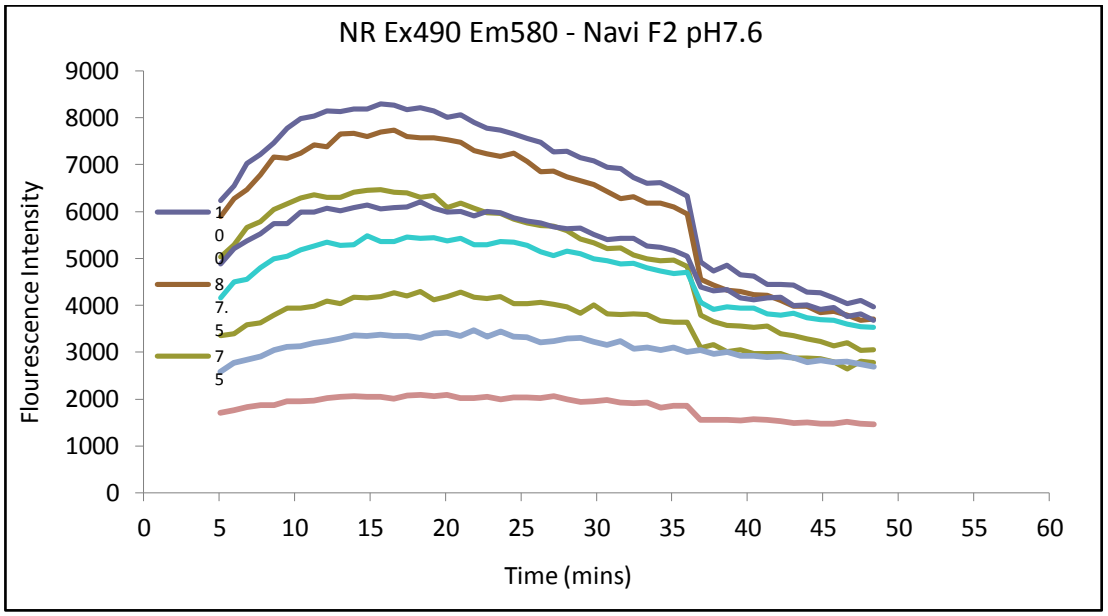

Figure 6.16: Nile Red Peak for time needed to measure lipid content in the sample.

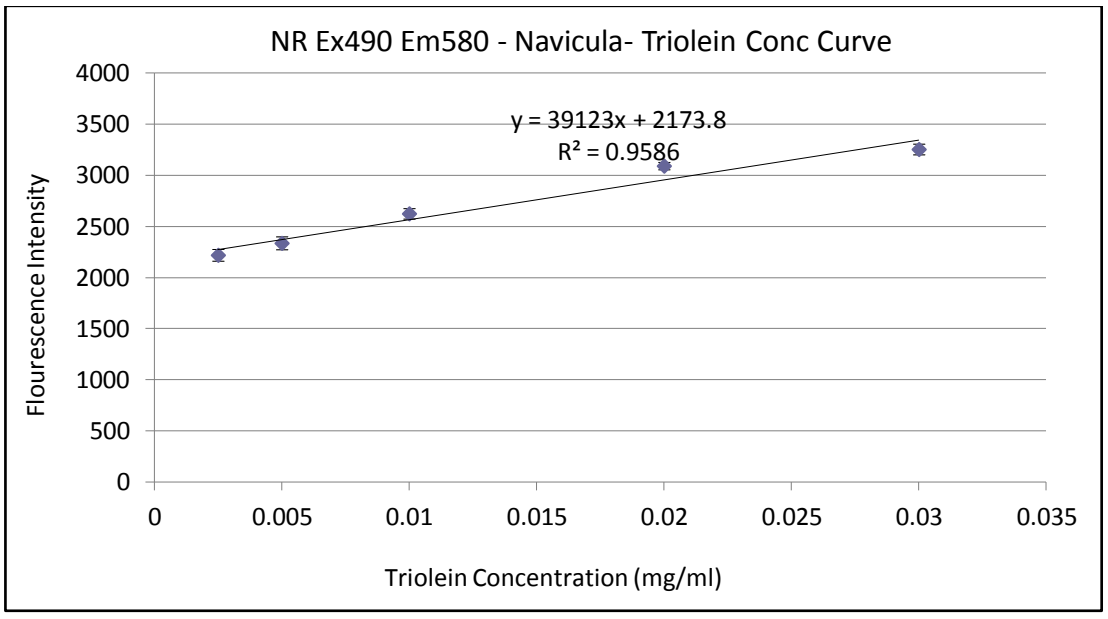

Figure 6.17: Concentration curve of NR fluorescence intensity versus triolein concentration

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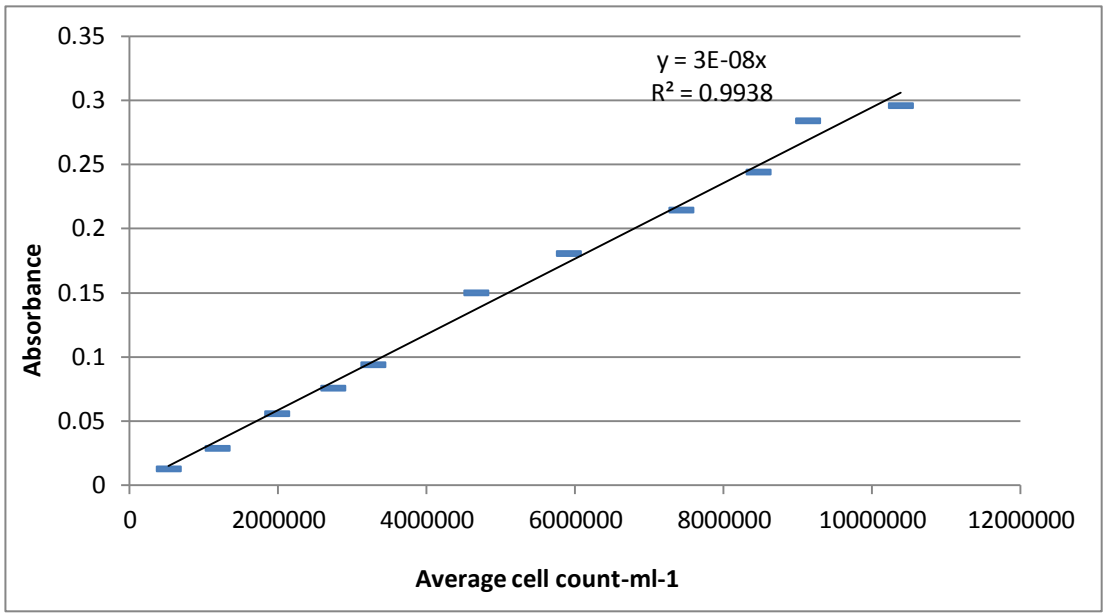


Figure 6.18: Correlation of mean cell count of *Navicula* samples with OD595using a 96 well plate read in the Plate Reader. The cells were grown in f/2 medium with silica**.**

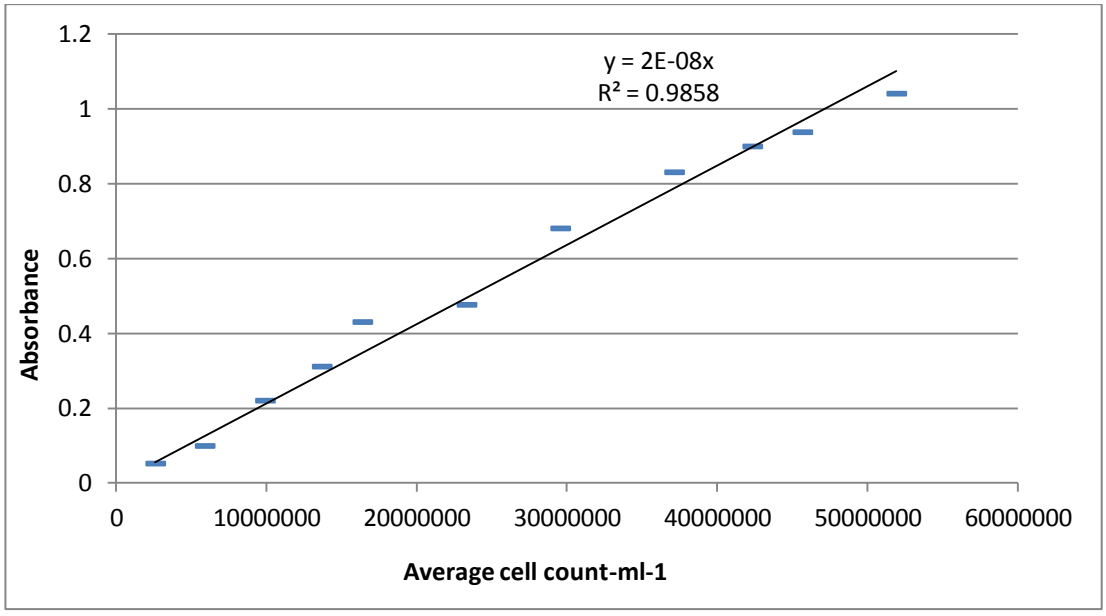


Figure 6.19: Correlation of mean cell count of *Navicula* samples with OD595 measured in the spectrophotometer. The cells were grown in of f/2 medium with silica**.**

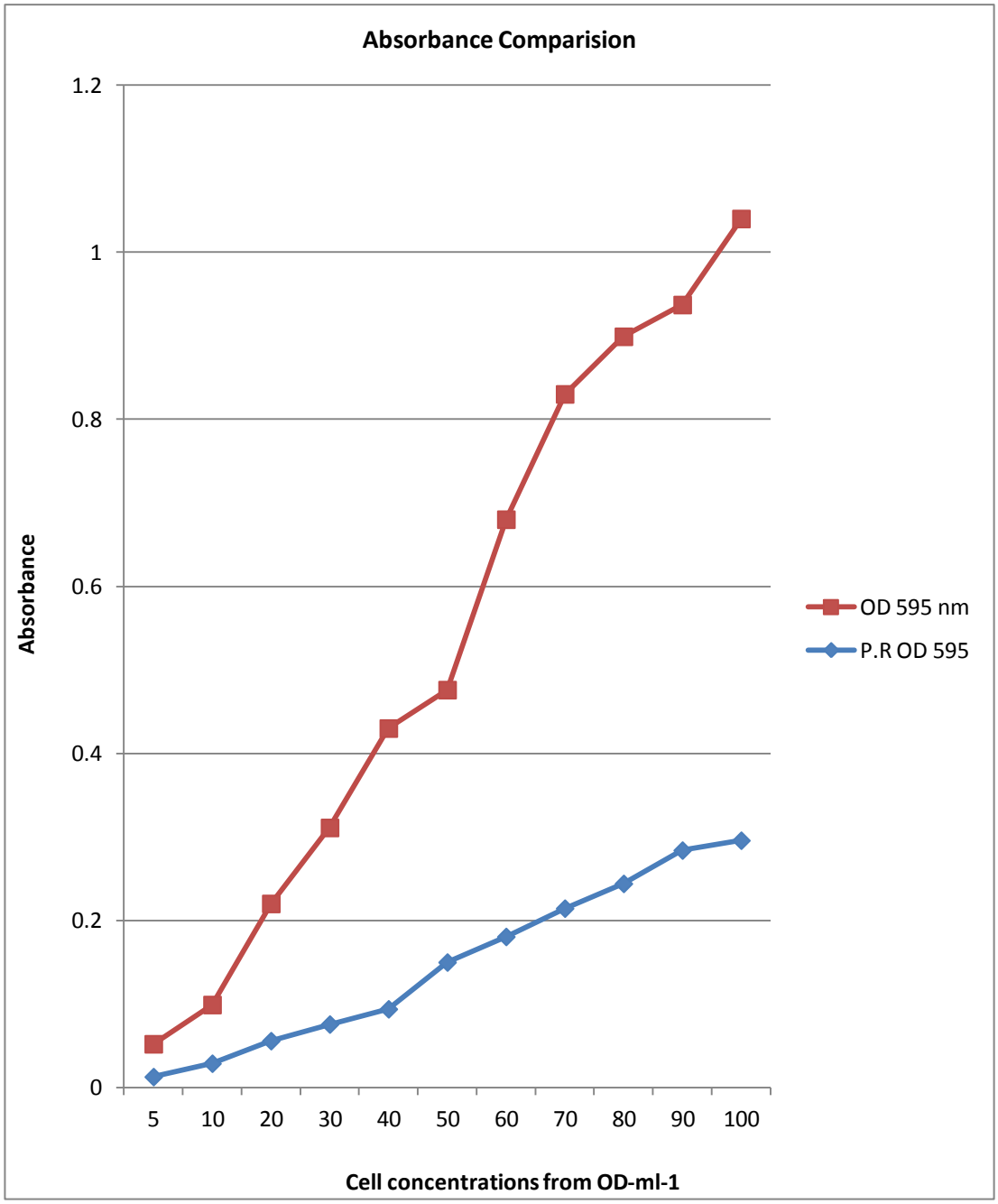


Figure 6.20: Comparison between OD595 measurements by spectrophotometer or plate reader for *Navicula* strain.

Figures 6.21 to 6.28 show measurements of neutral lipid production for *Navicula* on the basis of average NR fluorescence for a population of cells or on a per cell basis. Figures 6.21 to 6.24 show the effect of changing the concentration of silica in f/2 medium on neutral lipid production. The lowest values for neutral lipid were found at 50% silica and the highest values were found at 25% silica, but the latter values were not much different from 100 and 200% neutral lipid values. Therefore, no clear trend was evident.

Figures 6.25 to 6.28 show the effect of changing the pH of f/2 medium on the neutral lipid production by *Navicula.* The trend on population of cells basis shows pH 9.25 as the best condition for neutral lipid production (Figures 6.25 and 6.27). However, when expressed on a per cell basis, neutral lipid values were lowest at pH 9.25 (Figures 6.26 and 6.28).

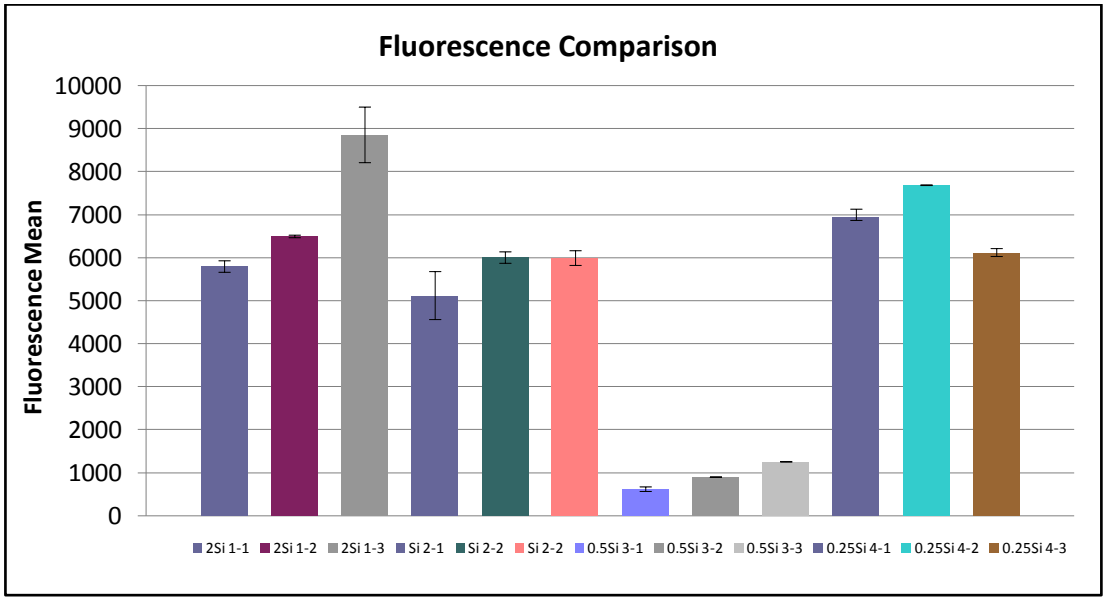


Figure 6.21: Neutral lipid content (as measured by NR Fluorescence) of Navicula strain in f/2 medium with different concentrations of silica from 25%(right hand side) to 200% (left hand side).

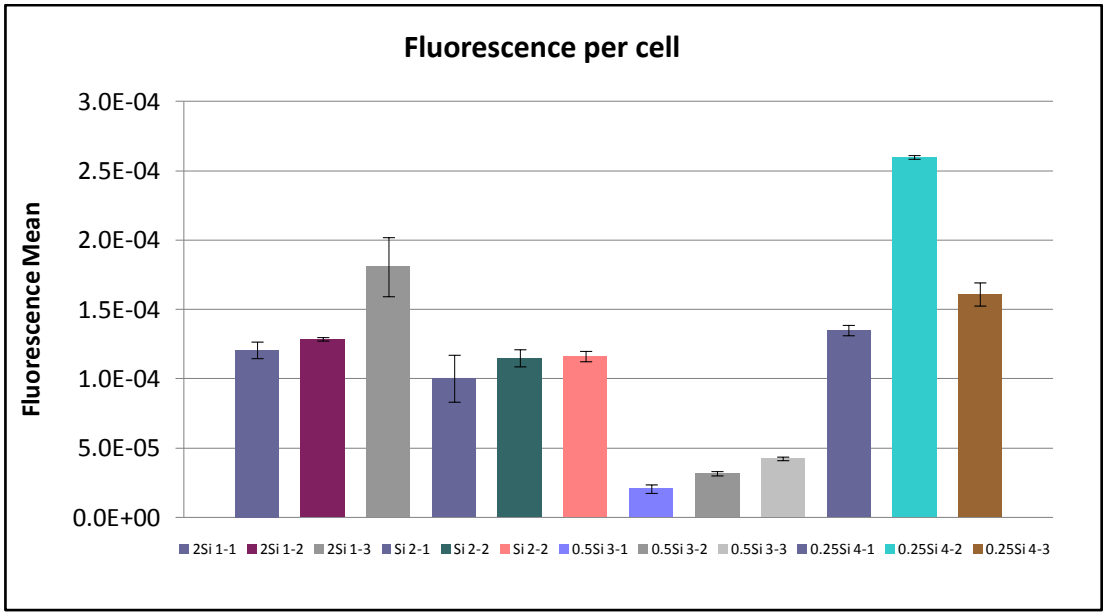


Figure 6.22: Neutral lipid content (as measured by NR fluorescence) of Navicula strain in f/2 medium with different concentrations of silica from 25% (right hand side) to 200% (left hand side). The results are expressed as fluorescence per cell.

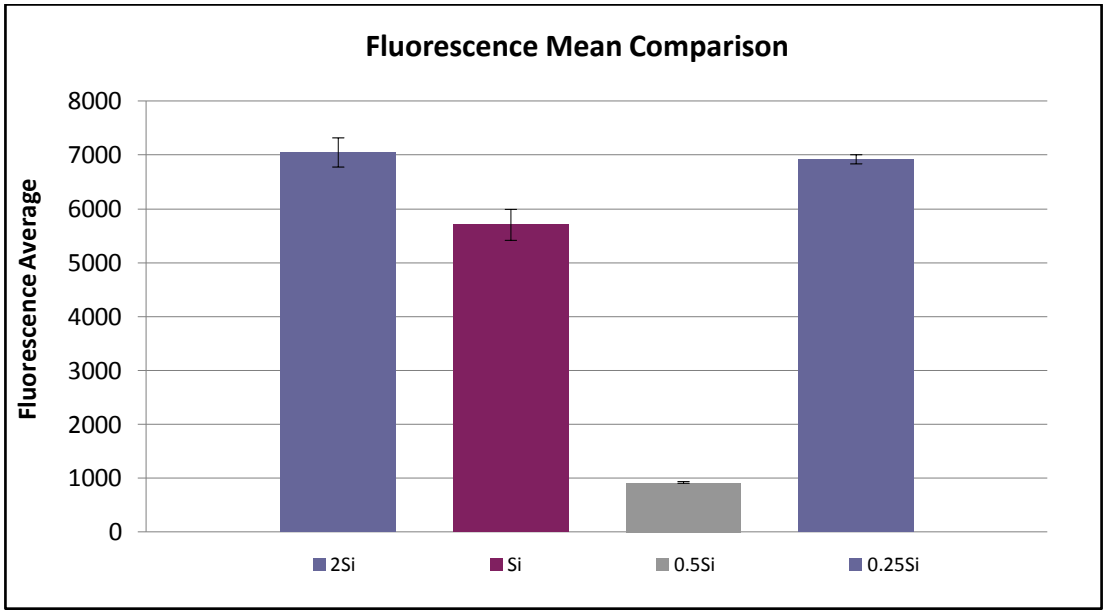


Figure 6.23: Mean neutral lipid content (as measured by NR fluorescence) of Navicula strain in f/2 medium with different concentrations of silica, data from Figure 6.21.

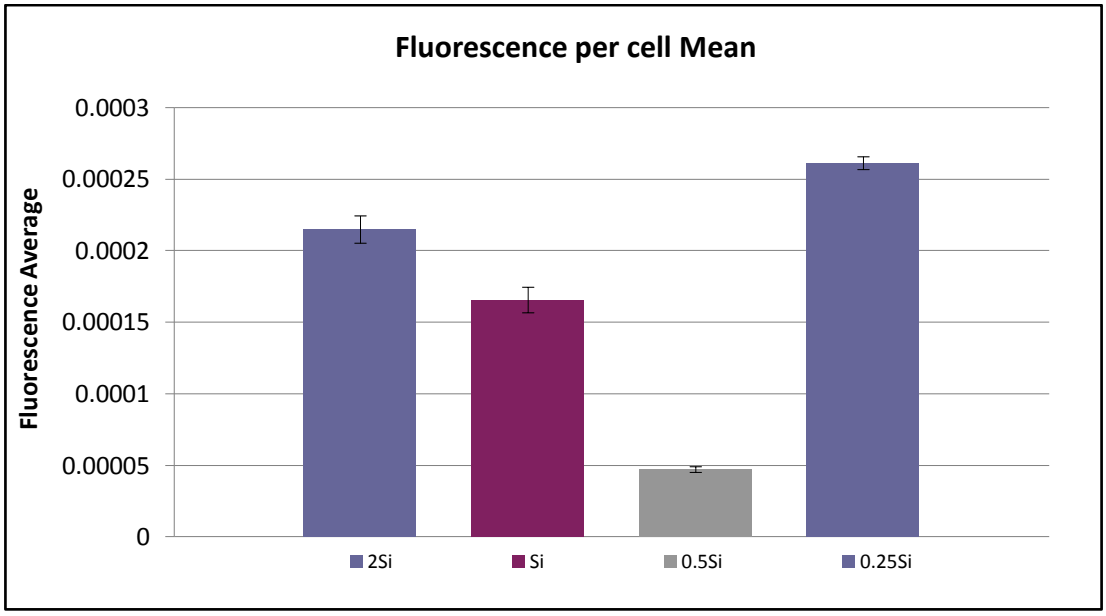


Figure 6.24: Mean neutral lipid content (as measured by NR fluorescence) of Navicula strain in f/2 medium with different concentrations of silica. Data presented per cell and taken from Figure 6.22.

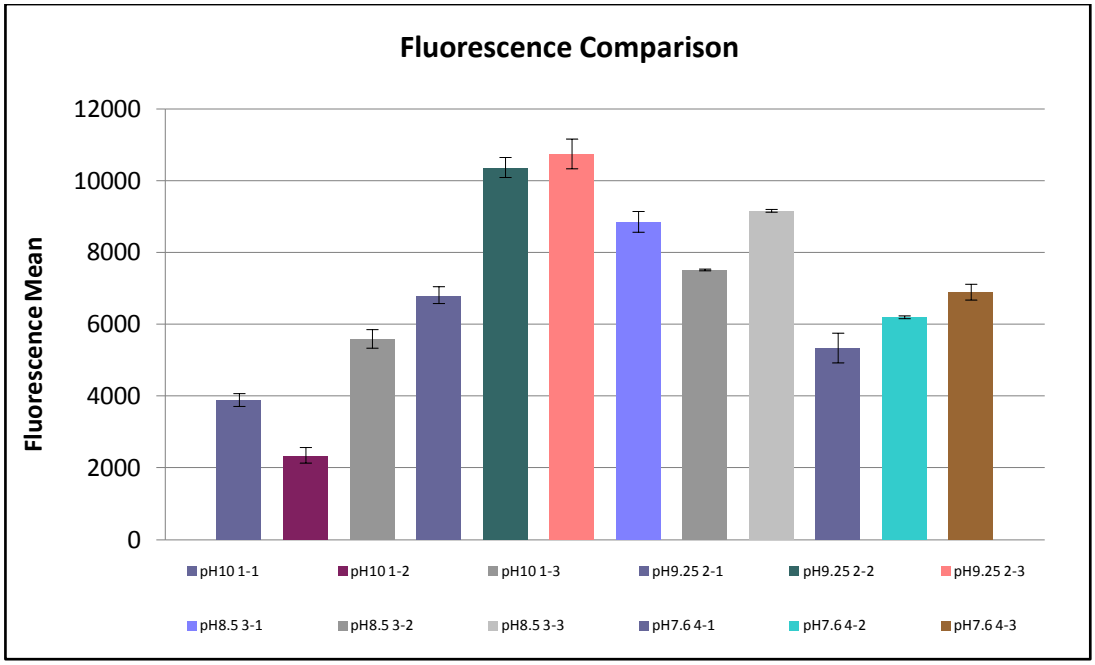


Figure 6.25: Neutral lipid content (as measured by NR fluorescence) of Navicula strain in f/2 medium at different pH levels.

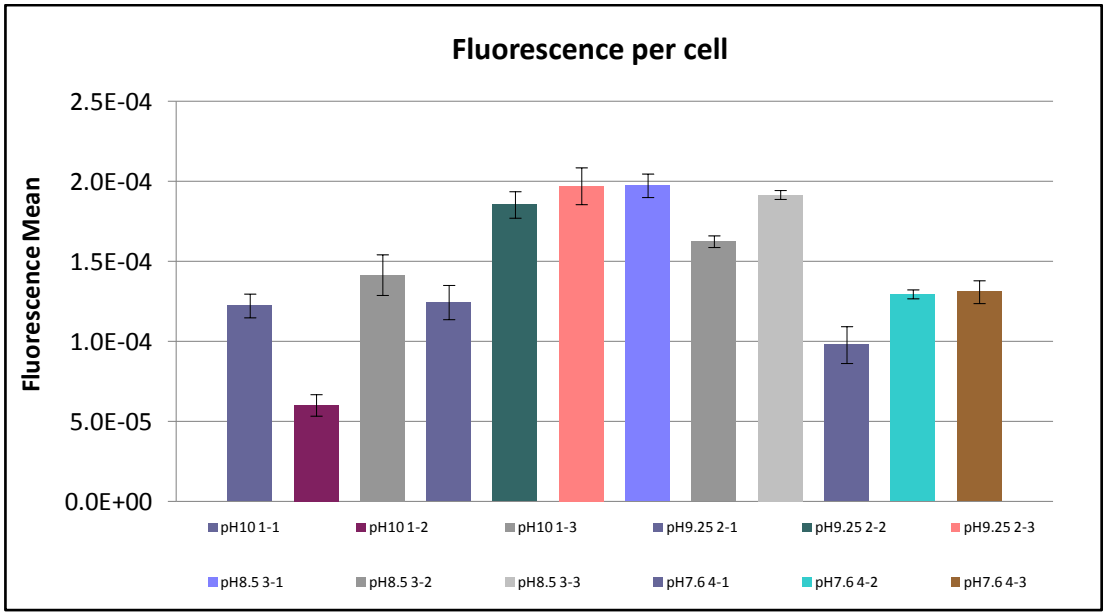


Figure 6.26: Neutral lipid content (as measured by NR fluorescence) of Navicula strain in f/2 medium at different pH values. The results are expressed as fluorescence per cell.

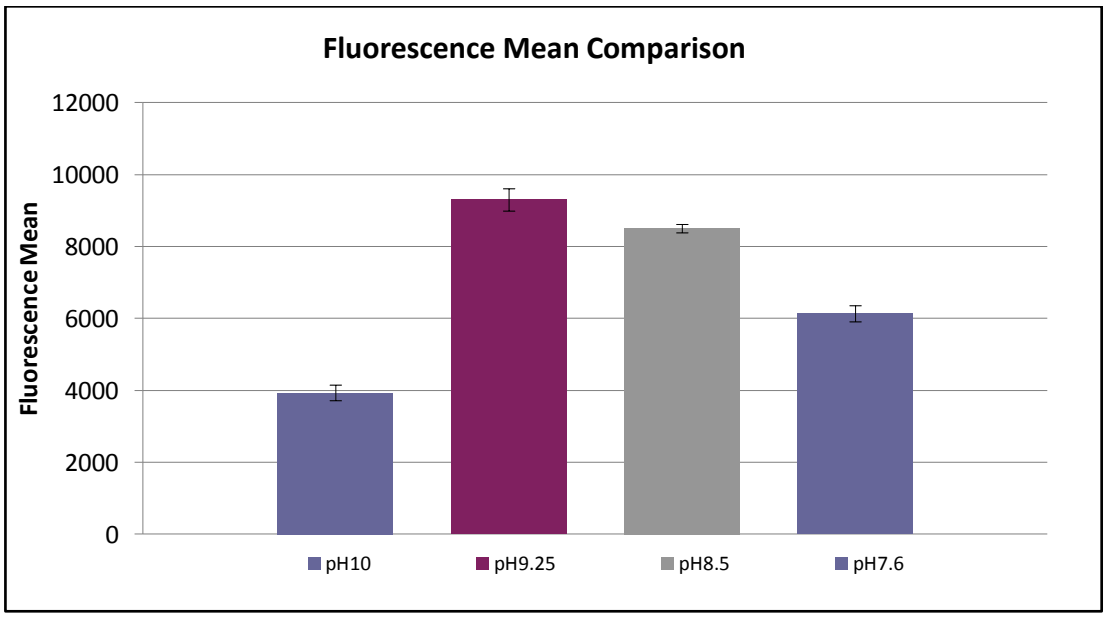


Figure 6.27: Mean neutral lipid content (as measured by NR fluorescence) of Navicula strain in f/2 medium at different pH values, data from Figure 6.25.

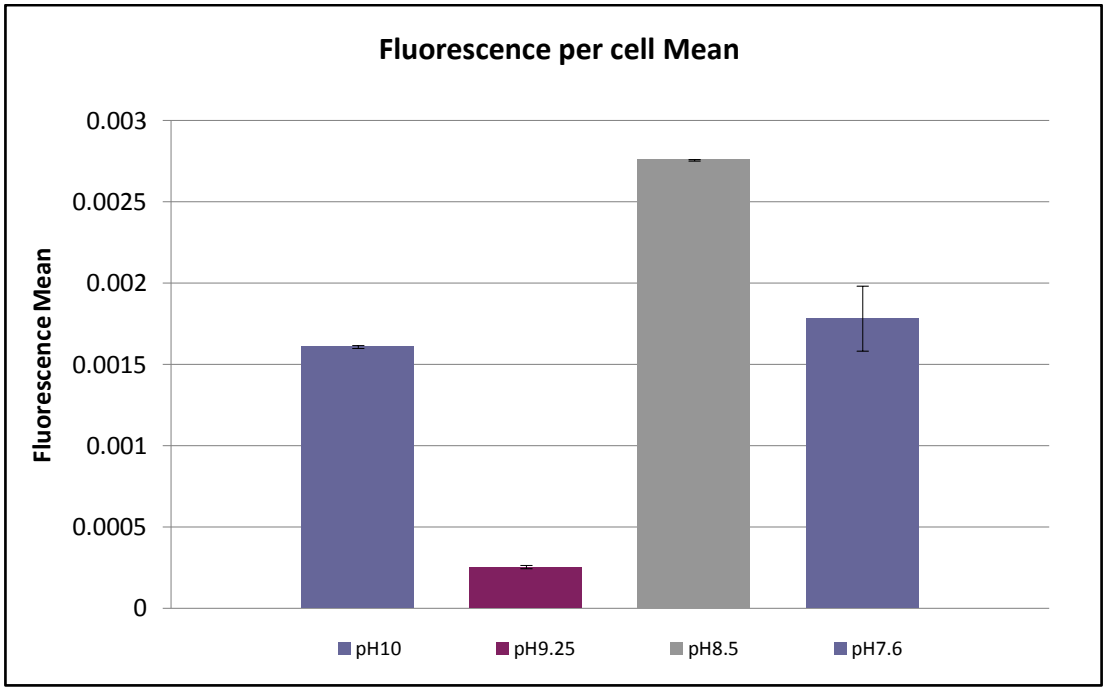


Figure 6.28: Mean neutral lipid content (as measured by NR fluorescence) of Navicula strain in f/2 medium at different pH values. Data presented per cell and taken from Figure 6.26.

Figures 6.29 to 6.32 show the effect of increasing the salt concentration of BBM medium on neutral lipid production by *Navicula.* It is clear that increasing the salinity increases the production of neutral lipid at 0.8 and 1 M NaCl. On a per cell basis, 0.8 M NaCl was best for neutral lipid production (Figure 6.32).

Figures 6.33 to 6.36 show the effect of changing the pH of BBM medium on neutral lipid production by *Navicula*. On both a population of cell basis and per cell basis, pH 9 was the best pH for driving neutral lipid synthesis.

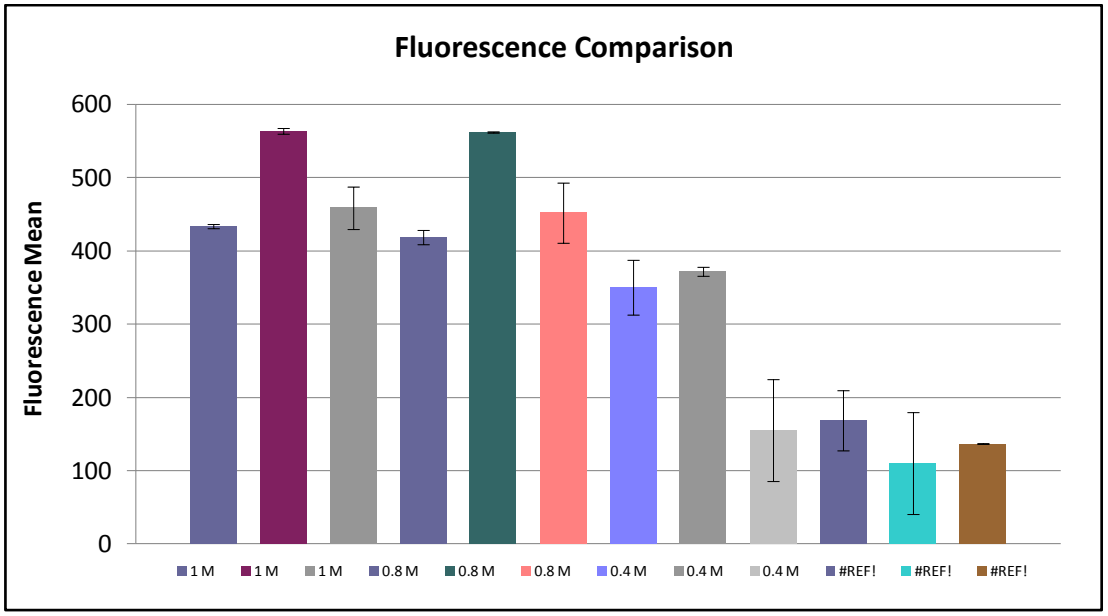


Figure 6.29: Neutral lipid content (as measured by NR Fluorescence) of Navicula strain in BBM medium with different concentrations of NaCl from 0.4 M (right hand side) to 1 M (left hand side).

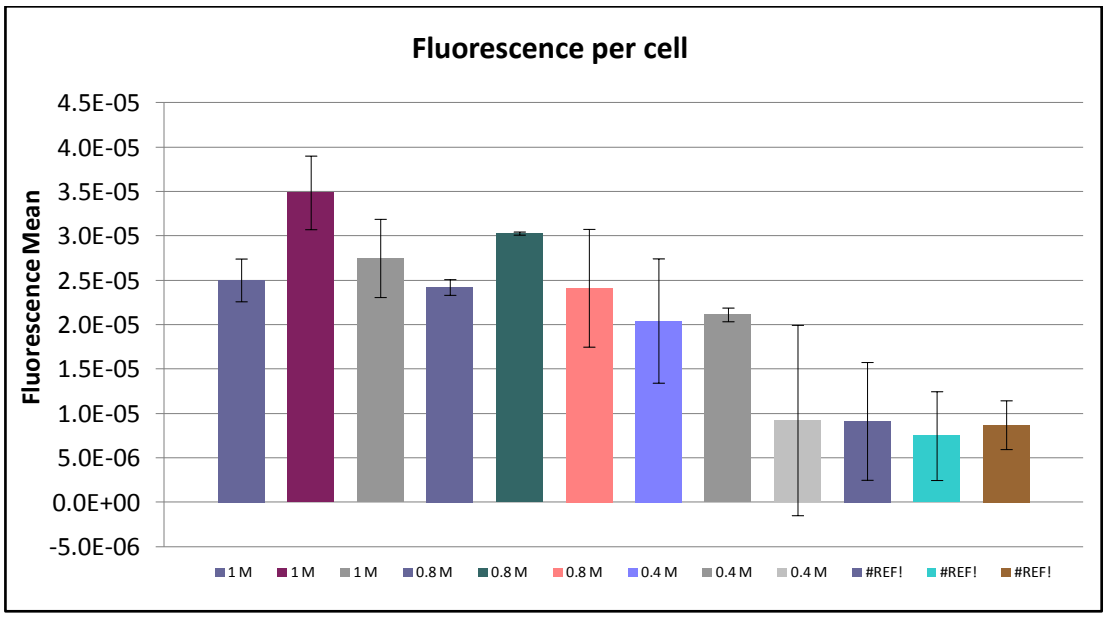


Figure 6.30: Neutral lipid content (as measured by NR fluorescence) of Navicula strain in BBM medium with different concentrations of NaCl from 0.4 M (right hand side) to 1 M (left hand side). The results are expressed as fluorescence per cell.

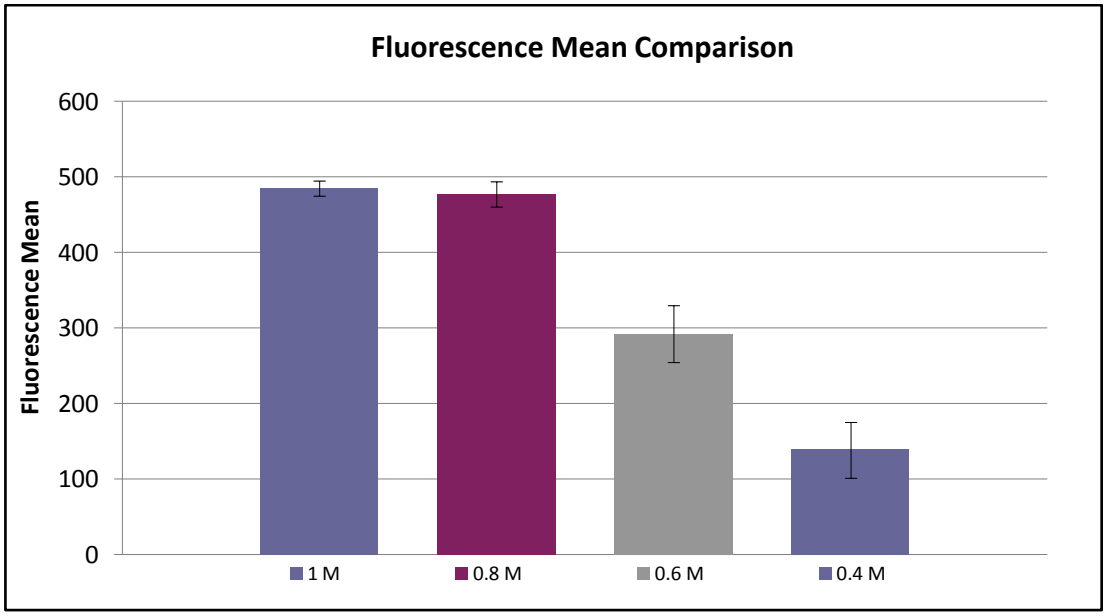


Figure 6.31: Mean neutral lipid content (as measured by NR fluorescence) of Navicula strain in BBM medium with different concentrations of NaCl, data from Figure 6.29.

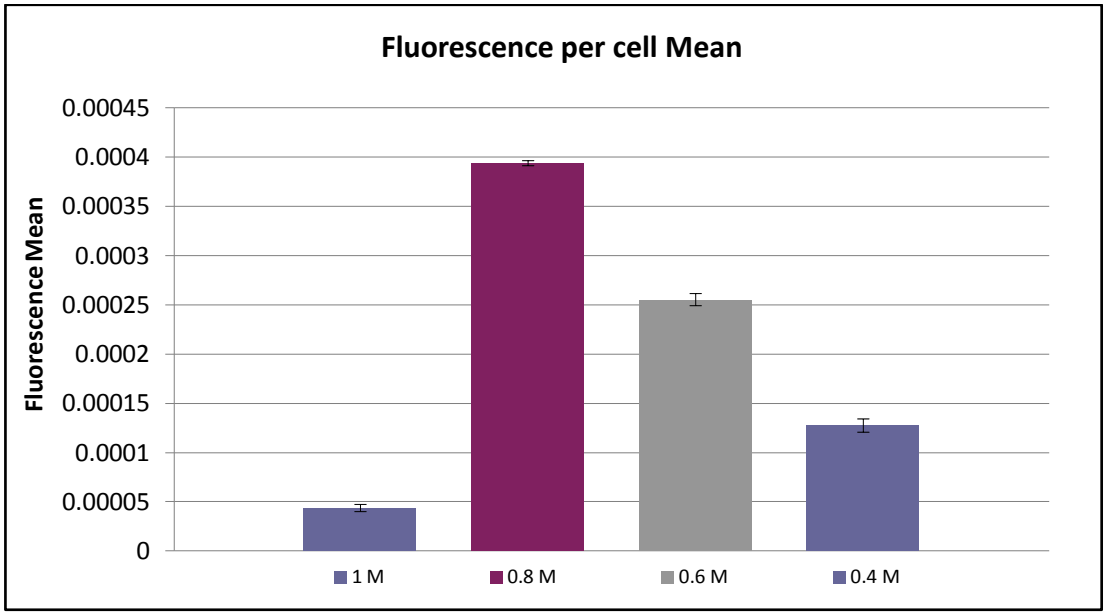


Figure 6.32: Mean neutral lipid content (as measured by NR fluorescence) of Navicula strain in BBM medium with different concentrations of NaCl. Data presented per cell and taken from Figure 6.30.

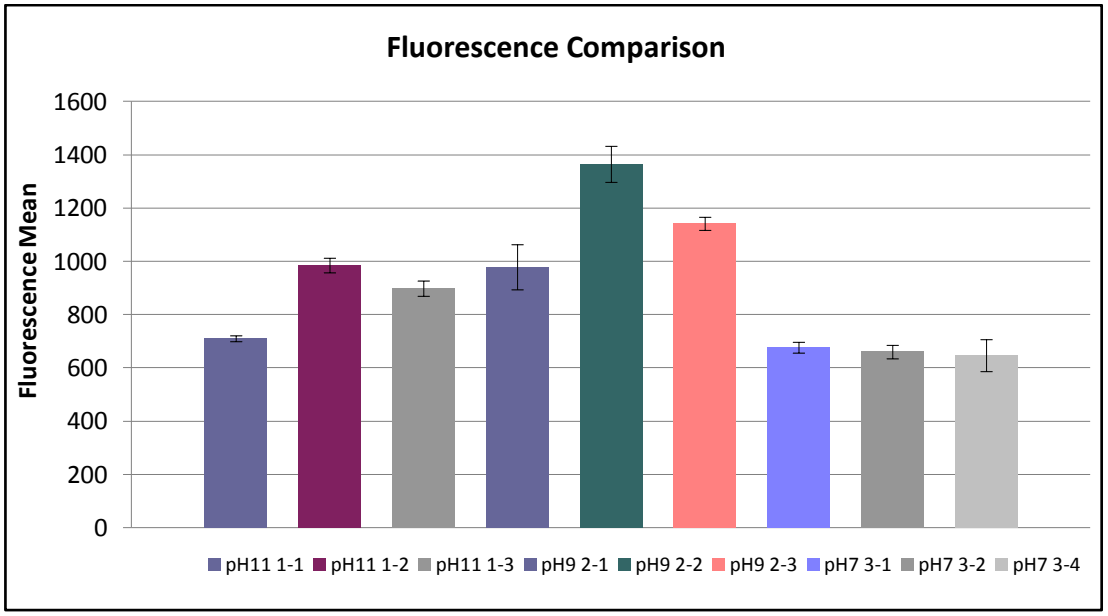


Figure 6.33: Neutral lipid content (as measured by NR fluorescence) of Navicula strain in 0.4 M NaCl BBM medium at different pH levels from pH 7 (right hand side to pH 11 (left hand side).

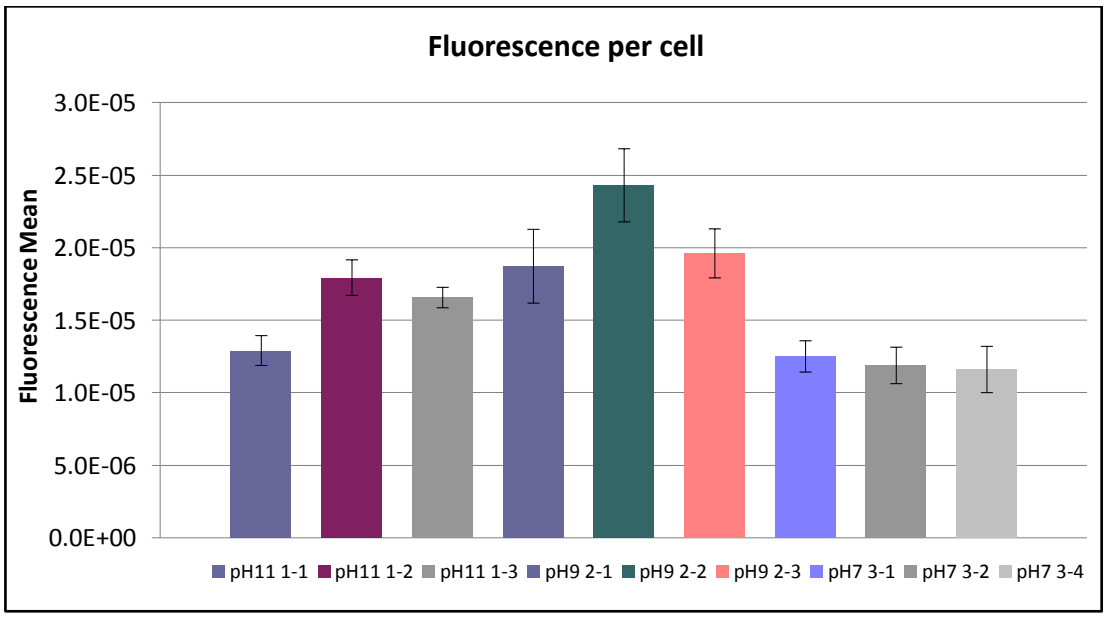


Figure 6.34: Neutral lipid content (as measured by NR fluorescence) of Navicula strain in 0.4 M NaCl BBM medium at different pH values from pH 7 (right hand side) to pH 11 (left hand side). The results are expressed as fluorescence per cell.

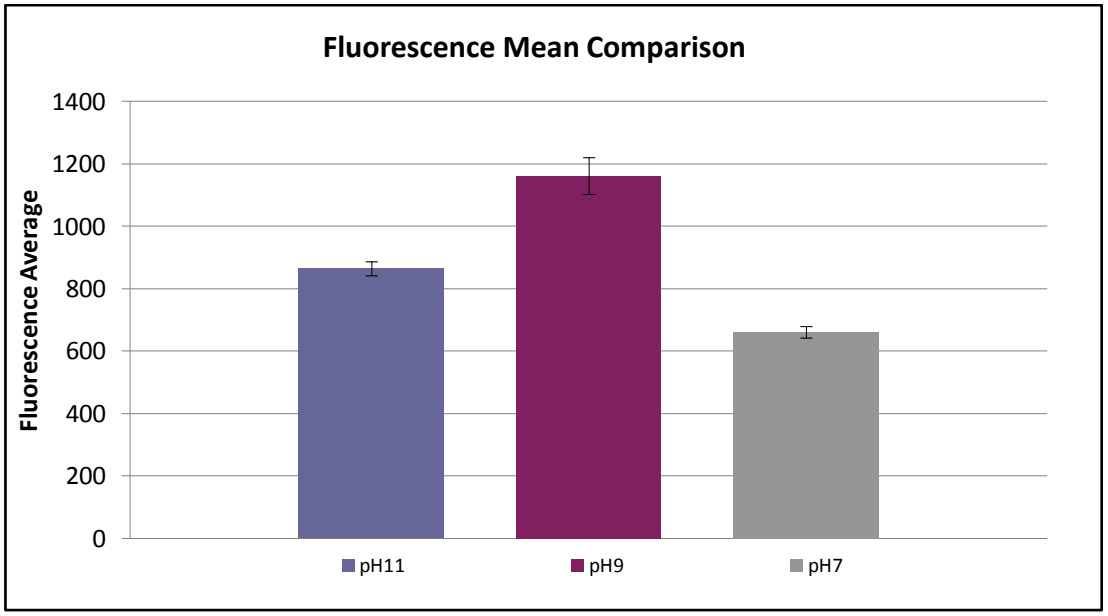


Figure 6.35: Mean neutral lipid content (as measured by NR fluorescence) of Navicula strain in 0.4 M NaCl BBM medium at different pH values, data from Figure 6.33.

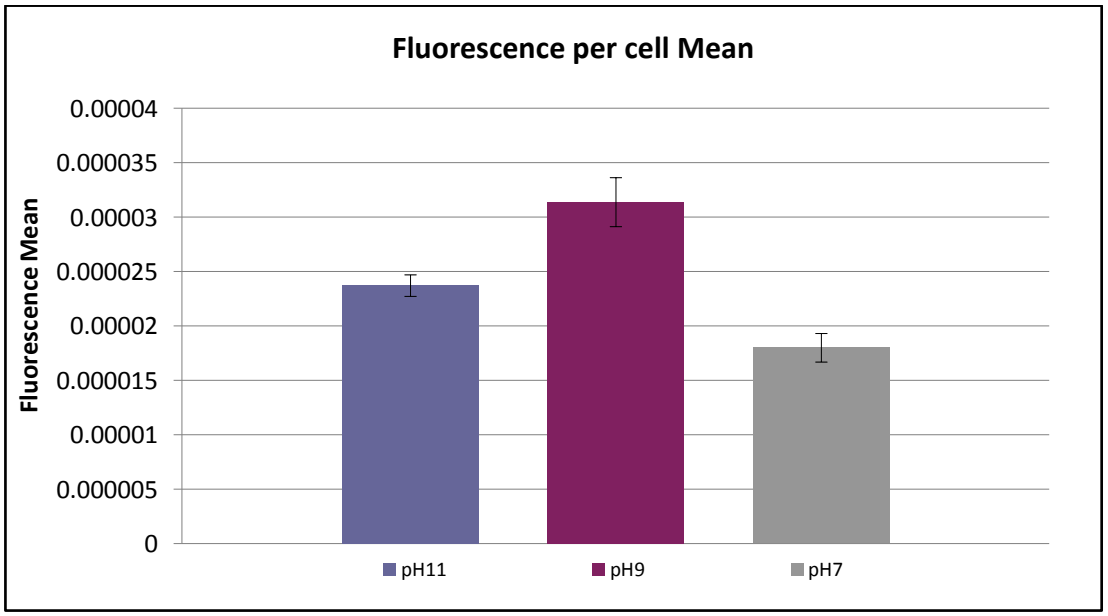


Figure 6.36: Mean neutral lipid content (as measured by NR fluorescence) of Navicula strain in 0.4 M BBM medium at different pH values. Data presented per cell and taken from Figure 6.34.

6.2.6 Determination of compatible solutes (osmolytes) by Nuclear Magnetic Resonance (NMR) spectroscopy

The aim of this part of the investigation was to identify the compatible solutes accumulated by *Navicula pelliculosa* when exposed to a range of external salinities in two different media (f/2 and BBM). Figure 6.37 shows that the major compatible solute found in *Navicula* cells at high salinity is glucosylglycerol, which is more commonly found in cyanobacterial cells grown at high salinities (Hagemann and Erdman, 1994). Recently, Scholz and Liebezeit (2012) examined compatible solute composition in another *Navicula* species (*N. phyllepta*) and found that glucose and glycerol were the compounds accumulated. Interestingly, Scholz and Liebezeit (2012) used chemical analysis to determine the compatible solutes present and not NMR and may have missed the fact that the glucose and glycerol were not separately accumulated, but were actually present as glucosylglycerol. Figures 6.38 and 6.39 further confirm the presence of glucosylglycerol in *Navicula* cells grown at high salinities in both BBM and f/2 medium.

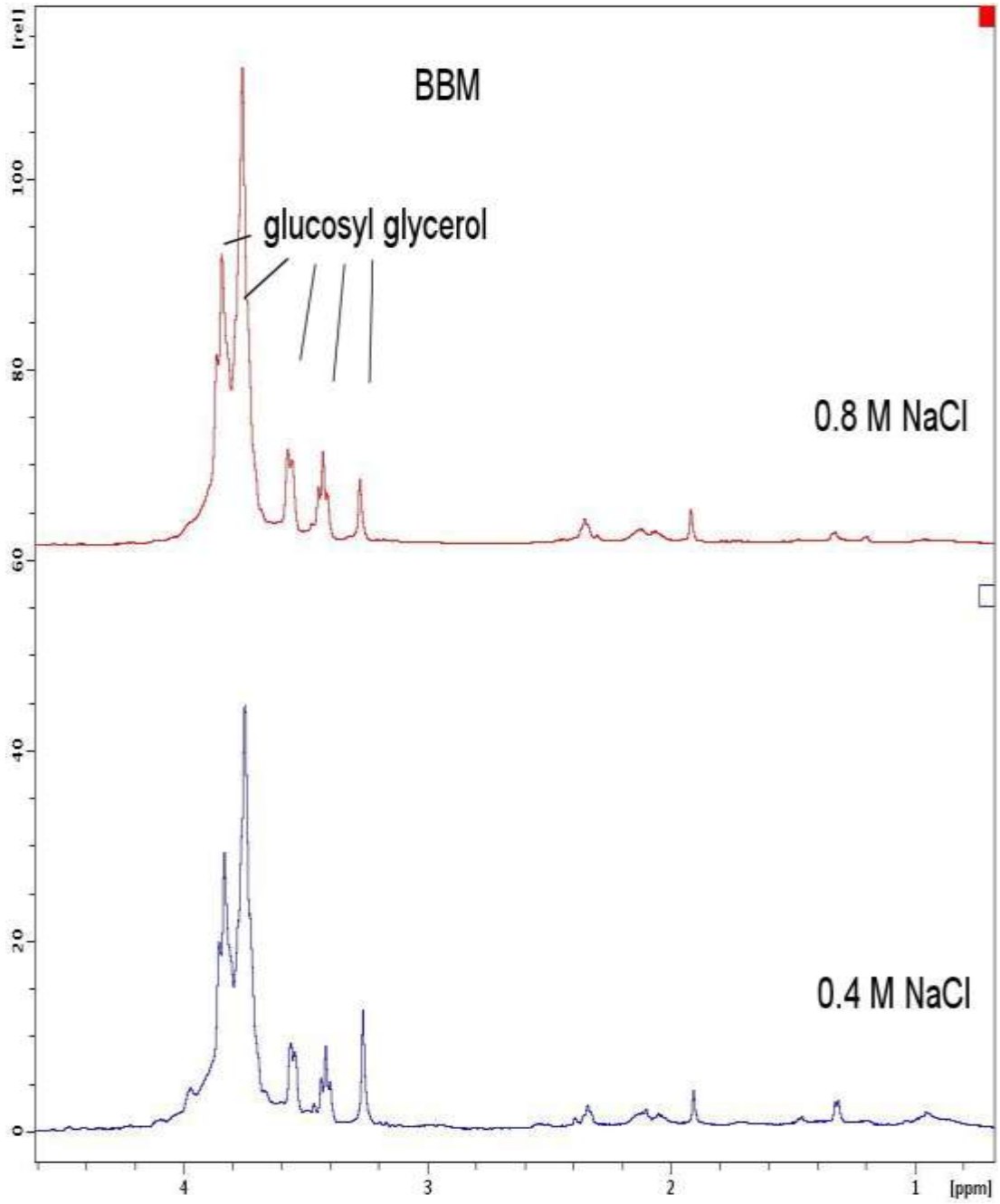


Figure 6.37: Figure 6.37: One-dimensional 1H-NMR spectra of cell extracts derived from Navicula cells grown in BBM medium supplemented with either 0.4 or 0.8 M NaCl. The NMR peaks that represent the compatible solute glucosylglycerol are indicated.

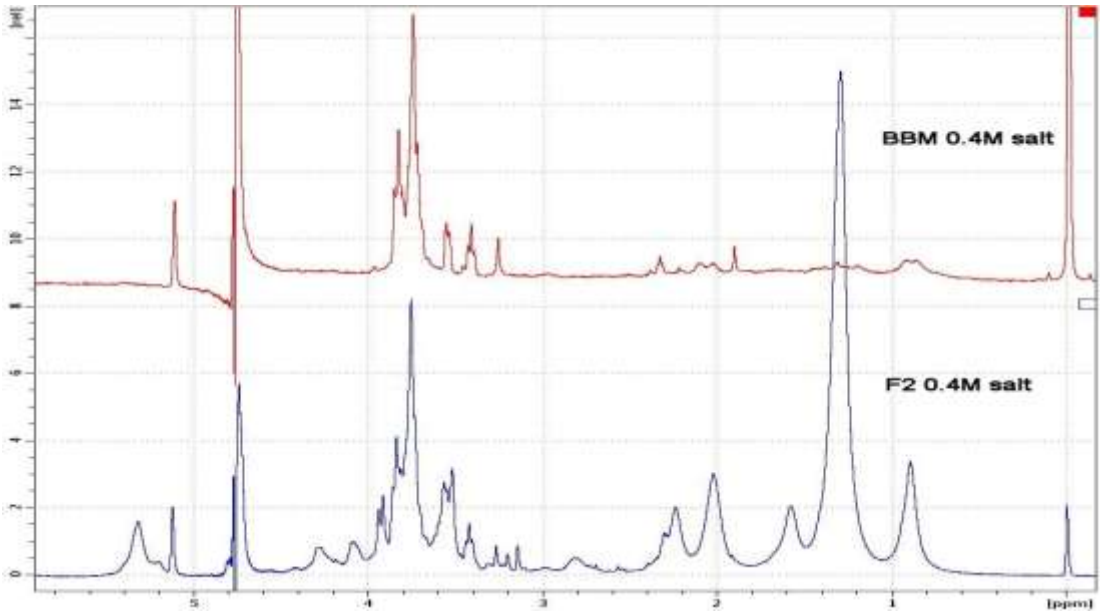


Figure 6.38: One-dimensional 1H-NMR spectra of cell extracts derived from Navicula cells grown in BBM medium supplemented with 0.4 M NaCl or in f/2 medium.

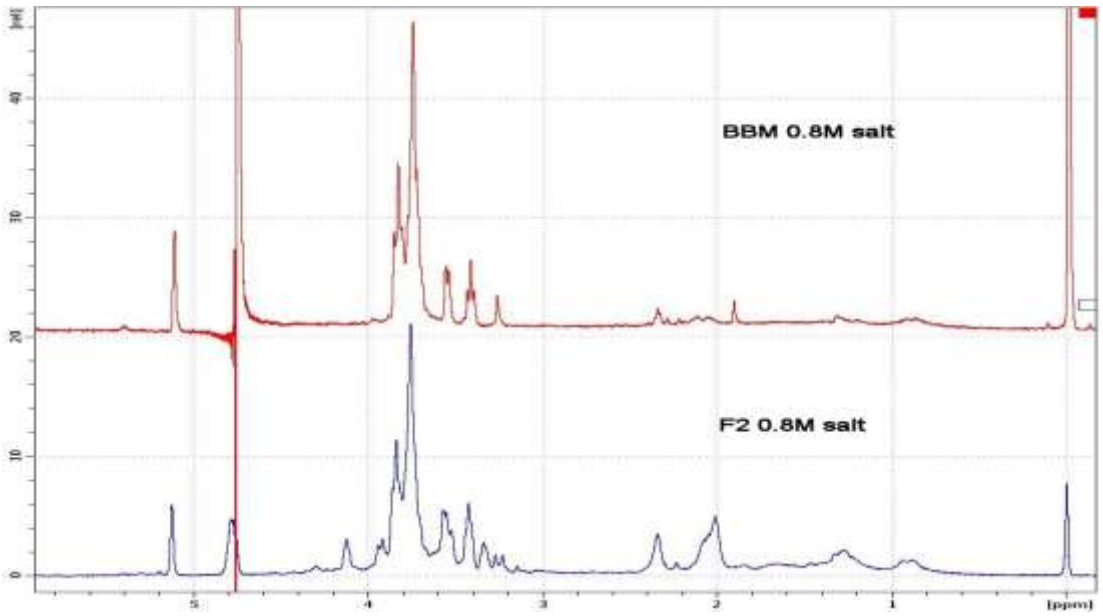


Figure 6.39: One-dimensional 1H-NMR spectra of cell extracts derived from Navicula cells grown in BBM and f/2 medium supplemented with 0.8 M NaCl.

6.2.7 Cell composition of Navicula pelliculosa

As part of a long standing collaboration with Dr Andrew Ross and Dr Patrick Biller at the University of Leeds, the opportunity arose for the cell composition of *Navicula* to be determined using Pyrolysis Gas Chromatography/Mass Spectrometry (Py-GC/MS). *Navicula* cells were grown in f/2 medium (0.4 M NaCl) with 100% silica (0.1 mM), 50% (0 .05 mM) and 25% (0.025) mM and hexane extracts were sent to Leeds for Py-GC/MS analysis. Figures 6.40, 6.41 and 6.42 show the relative amount of protein, chlorophyll and C17 lipid respectively as the silica level was decreased. In all cases, the level of protein, chlorophyll and C17 lipid decreased with decreasing silica suggesting that other component(s) of the cell that were not determined were increasing in response to silica limitation.

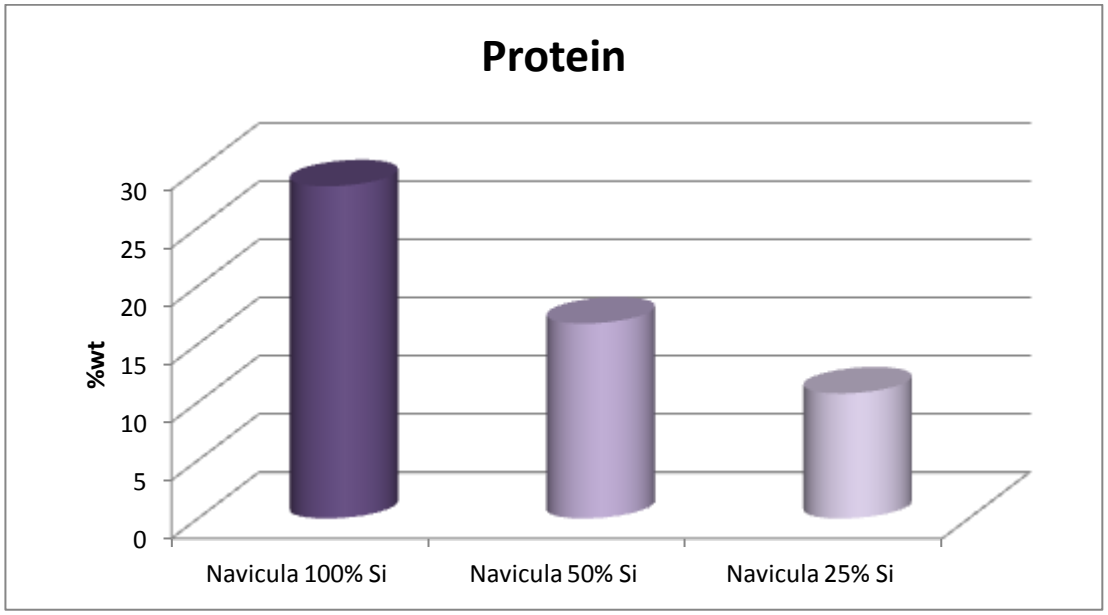


Figure 6.40: Relative concentrations of protein in Navicula cells grown in different concentrations of silica (100% = 0.1 mM, 50% = 0.05 mM and 25% = 0.025 mM). Measurements carried out on hexane extracts by Py-GC/MS

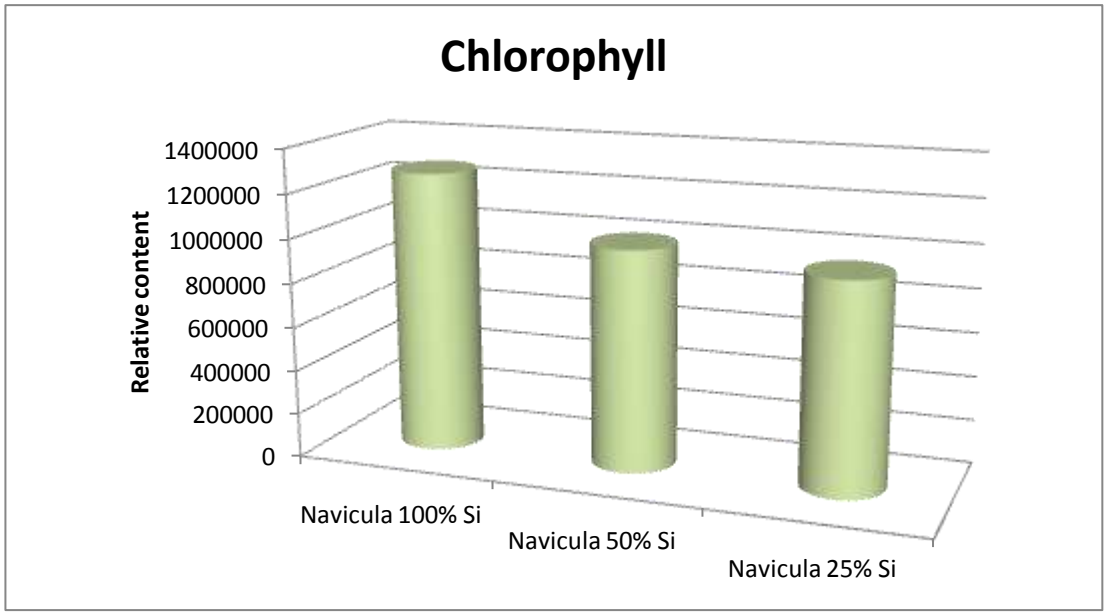


Figure 6.41: Relative concentrations of chlorophyll in Navicula cells strain grown in different concentrations of silica (100% = 0.1 mM, 50% = 0.05 mM and 25% = 0.025 mM). Measurements carried out on hexane extracts by Py-GC/MS.

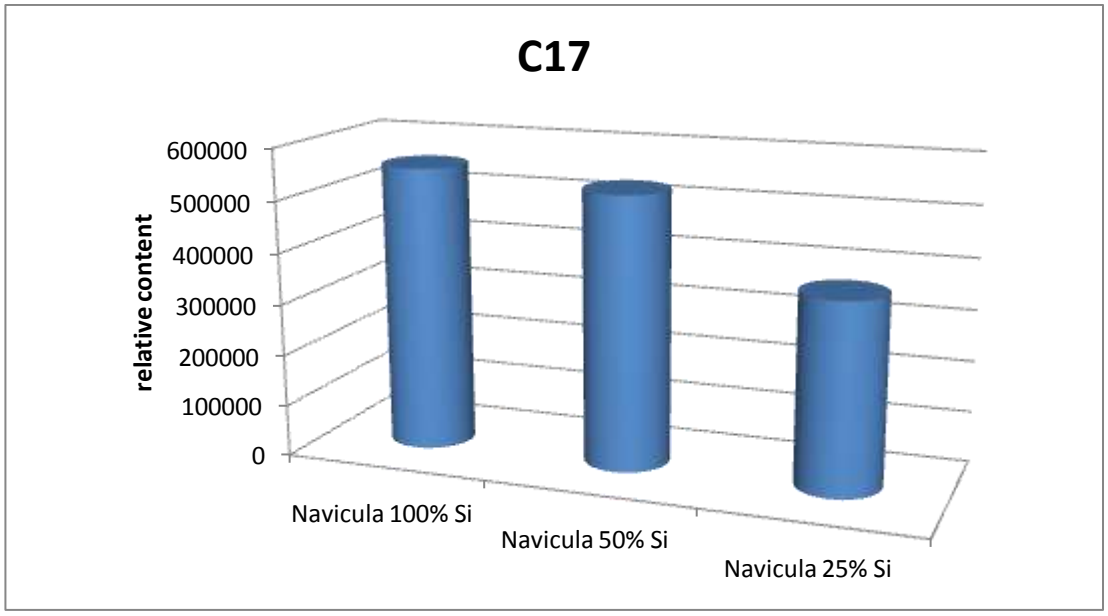


Figure 6.42: Relative concentrations of C17 lipid in Navicula cells grown in different concentrations of silica (100% = 0.1 mM, 50% = 0.05 mM and 25% = 0.025 mM). Measurements carried out on hexane extracts by Py-GC/MS.

6.3 Conclusion

The results described in this chapter show that *Navicula pelliculosa* is a promising strain for biofuel production. The ability of this strain of *Navicula* to grow at salinities up to 0.8 M and pH values up to at least pH 10, means that it can be grown in outdoor ponds without fear of contamination (Chisti, 2007). Total lipid accumulation was measured at about 20% of dry weight, which is at the lower threshold of viability for biofuel production (Chisti, 2008). However, experiments using NR fluorescence to measure neutral lipid indicated that stress conditions could increase neutral lipid accumulation. This raises the possibility of finding ideal stress conditions to drive a higher level of neutral lipid synthesis.

As part of the process of examining potential biofuel production by *Navicula*, the cells were adapted to higher salinities and compatible solute synthesis was examined. Very few reports of compatible solute synthesis in diatoms exist and it was only very recently that glucose and glycerol were found to be accumulated by *Navicula phyllepta* (Scholz and Liebzeit, 2012). As mentioned above chemical analysis was used by these authors to determine compatible solute composition and it appears that they missed the fact that the glucose and glycerol were not separately accumulated, but in fact as shown in this thesis are accumulated together as the well-known compatible solute glucosylglycerol.

Chapter 7

GENERAL CONCLUSIONS AND FUTURE WORK

7.1 General Conclusions

The first two aims of the project (section 1.6) involved the isolation, identification and characterization of salt tolerant bacteria and microalgae from river and pond water. The work described in Chapters 3, 4 and 5 showed that these aims were achieved and two salt tolerant bacteria (*Enterococcus amnigenus* (WP) and *Pseudomonas fluorescens* (DP)) and two salt tolerant microalgae (*Navicula pelliculosa* and *Chlorella* sp.) were isolated and characterized.

E. amnigenus is an unusual member of the *Enterococcus* genus and it has recently been suggested that it should be moved to the newly created genus *Lelliottia* (Brady *et al*., 2013). Like many enterococci, it is a potential pathogen (Bollet *et al*., 1991), but it also has uses in industry as a producer of bacterial cellulose (Hungund and Gupta, 2010).

On the other hand, *P. fluorescens* is an extremely well characterized organism that is important in biofilm research (Mastropaolo *et al*., 2012) and as a rhizobacterium (Dominguez *et al*., 2012).

The two algal species isolated, *Navicula* and *Chlorella,* are members of two of the major groups of algae – the diatoms and green algae, respectively. Initial work measuring total lipid concentrations suggested that the diatom *Navicula* was the most promising organism for biofuel production due to having a total lipid concentration of around 20%. The further characterization of *Navicula* described in Chapter 6 showed that it could grow under conditions of high pH and high salinity, making it a candidate species for growth in outdoor raceway ponds (Chisti, 2007). One potential drawback of

using diatom species for biofuel production is the requirement for silica in the growth medium and also possible problems with lipid extraction from the silica-rich biomass. In the laboratory experiments described here no obvious problems were found with extracting lipid from *Navicula* cells.

Relatively little is known about the mechanisms used by diatoms to grow in high salinities. It was assumed that diatoms would produce so-called compatible solutes in line with other algal groups (Oren, 2008). As noted in section 6.3, a recent publication by Scholz and Liebzeit, in 2012 showed that glucose and glycerol were the compatible solutes in *Navicula phyllepta* based on chemical analysis. This disagrees with the findings of the current work, where the cyanobacterial compatible solute glucosylglycerol was found instead. It is possible that the glucosylglycerol has been broken down to its constituent parts during the chemical analysis treatments of Scholz and Liebzeit (2012). Whereas, the NMR analysis described here does not subject the cell material to harsh chemical conditions. Furthermore, it is now known that the central carbon metabolism of diatoms is more closely related to cyanobacteria than to green algae (Hockin *et al*., 2012). This provides support for the presence of glucosylglycerol in *Navicula*.

7.2 Future Work

The main avenues for future work relate to the microalgae species characterized in this thesis. As described in section 7.1, *Navicula pelliculosa* is a potential candidate for growth in outdoor ponds and future grant proposals will seek funding for growing this organism in raceway ponds in greenhouse facilities in the UK.

The best characterized *Chlorella* strain (1B.4BBM) is already the subject of another PhD project in the Gilmour laboratory (student Richard Smith). This *Chlorella* strain grows exceptionally well under mixotrophic conditions (i.e. where a fixed carbon source (acetate or glucose) is added to the medium, but the cells are still incubated under illuminated conditions).

Therefore, both main strains of microalgae isolated, identified and characterized in this project are being studied further to attempt to make one or both of them commercially viable strains to produce biodiesel.

Chapter 8

REFERENCES

Albers, S. V., de Vossenberg, J. L.C.M., Driessen, A. J. M. and Konings, W. N. (2001). Bioenergetics and solute uptake under extreme conditions. *Extremophiles*, 5: 285-294.

Alonzo, F. and Mayzud, P. (1999). Spectrofluorometric quantification of neutral and polar lipids in zooplankton using Nile red. *Marine Chemistry*, 67: 289-301.

Amann, R., Gloëckner, F. and Neef, A. (1997). Modern methods in subsurface microbiology: *in situ* identification of microorganisms with nucleic acid probes. *FEMS Microbiology Reviews*, 20: 191-200.

Anton, J., Llobet-Brossa, E., Rodriguez-Valera, F. and Amann, R. (1999). Fluorescence *in situ* hybridization analysis of the prokaryotic community inhabiting crystallizer ponds. *Environmental Microbiology*, 1: 517-523.

Anton, J., Rossello-Mora, R., Rodriguez-Valera, F. and Amann, R. (2000). Extremely halophilic bacteria in crystallizer ponds from solar salterns. *Applied and Environmental Microbiology*, 66: 3052-3057.

Atlas, R. M. (1984). Advances in Microbial Ecology, K. C. Marshall Ed. Plenum, New York 7:1-47.

Banerjee, A, Sharma, R. and Chisti, Y. (2002) *Botryococcus braunii*: a renewable source of hydrocarbons and other chemicals. Critical Reviews in Biotechnology 22: 245-279

Barclay, B. Nagle, N., Terry, K. and Roessler, P. (1985) Collecting and screening microalgae from shallow, inland saline habitats. SERI/CP-23-2700, pp 52-68.

Basavaraj, S., Hungund, S. and Gupta, G. (2010) Production of bacterial cellulose from *Enterobacter amnigenus* GH-1 isolated from rotten apple. *World Journal Microbial Biotechnology* 26: 1823-1828

Becker, B. (2012) Snow ball earth and the split of Streptophyta and Chlorophyta. *Trends in Plant science*, 18: 180–183.

Becker, D. J. and Lowe, J. B. (2003). Fucose: biosynthesis and biological function in mammals. *Glycobiology,* 13: 41R–53R.

Beijerinck, M. W. (1890) Kulturversuche mit Zoochloren, Lichenen-gonidien und anderen niederen Algen. *Botanisches Zeitchnift,* 48:125-185.

Benemann, J. and Oswald, W (1996) Systems and economic analysis of microalgae ponds for conversion of CO_2 to biomass. Final report (other information: PBD: 21 Mar 1996). pp. 214.

Benito, N., Mirelis, B., Luz Galvez, M., Vila, M., Lopez-Contreras, J., Cotura, A., Pomar, V., Mach, F., Coll, P., and Gurgui, M. (2012) Outbreak of *Pseudomonas fluoreacens* bloodstream infection in a coronary car unit. *Journal of hospital infection,* 82: 286-289.

Benlloch, S., Lopez, A., Casamayor, E., Ovreas, L. and Goddard, V. (2002). Prokaryotic genetic diversity throughout the salinity gradient of a coastal solar saltern. *Environmental Microbiology*, 4**:** 349-360.

Bertozzini, E., Galluzzi, L., Penna, A. and Magnani, M. (2011). Application of the standard addition method for the absolute quantification of neutral lipids in microalgae using Nile red. *Journal of Microbiological Methods*, 87: 17-23.

Berzano, M., Marcheggiani, S., Rombini, S. and Spurio, R., (2012). The application of oligonucleotide probes and microarrays for the identification of freshwater diatoms. *Hydrobiologia,* 695: 57–72.

Bligh, E. G. and Dyer, W. J. (1959). A rapid method for total lipid extraction and purification. *Canadian Journal of Biochemistry Physiology*, 37: 911-917.

Bockelmann, U., Manz,W., Neu, T. R. and Szewzyk, U. (2000). Characterization of the microbial community of lotic organic aggregates (river snow) in the Elbe River of Germany by cultivation and molecular methods. *FEMS Microbiology Ecology***,** 33: 157-170.

Bollet, C., **Elkouby, A., Pietri, P. and de Micco, P. (1991)** Isolation of *Enterobacter amnigenus* from a Heart Transplant RecipientEur. *Journal of Microbiology and Infectious. Disease,* 10: 1071-1072

Bowers, J. K., Mesbah, M. N. and Wiegel, J. (2009). Biodiversity of poly-extremophilic Bacteria: Does combining the extremes of high salt, alkaline pH and elevated temperature approach a physico-chemical boundary for life. *Saline Systems*, 5: 9-16.

Brady, C.,, **Cleenwerck, I., Venter, S., Coutinho, T. and De Vos, P. (2013).** Taxonomic evaluation of the genus *Enterobacter* based on multilocus sequence analysis (MLSA). *Systemic and Applied Microbiology* 36: 309-319

Burlew, J. S. (ed) (1953a) Algae culture: from laboratory to pilot plant. Carnegie Institution of Washington, Washington, DC, pp 1-357

Burlew, J. S. (1953b) Current status of large-scale culture of algae.In: Burlew JS (ed) Algal culture: from laboratory to pilot plant.Carnegie Institution, Washington, DC, pp 3-23.

Cai, H., Archambault, M. and Prescott, J. F. (2003). 16S ribosomal RNA sequence–based identification of veterinary clinical bacteria. *Journal of Veterinary Diagnostic Investigation*, 15: 465-469.

Chen, W., Zhang, C., Song, L., Sommerfeld, M. and Hu, Q. (2009). A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *Journal of Microbiological Methods*, 77: 41-47.

Chisti, Y (2007) Biodiesel from microalgae. *Biotechnology Advances*, 25: 294–306.

Chisti, Y (2008) Biodiesel from microalgae beats bioethanol. *Trends in Biotechnology,* 26: 126–131.

Cooksey, K. E., Guckert, J. B., Williams, S. A. and Callis, P. R. (1987). Fluorometric-determination of the natural lipid-content of microalgal cells using Nile Red *Journal of Microbiological Methods,* 6: 333-345.

Chung, C-C., Chang, J., Gong, G-C., Hsu, S-C, Chiang, K-P., Liao, C-W (2011) Effects of Asian dust storms on synechococcus populations in the subtropical Kuroshio current. *Marine Biotechnol*ogy, 13:751–763.

Clarridge, J. E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria in clinical microbiology and infectious diseases. *Clinical Microbiology Reviews*, 17: 840-862.

Cohn, F (I850). Zur Naturgeschichte des Protocotrus plurlalls Ktitzing. *Nova Acta Academia Leopoldensis Caroliensis,* 22: 607.

Cook, P. M. (I950) Large-scale culture of C/r lorella.ln Brunel J, Prescott GW (eds) The culture of algae. Charles F. Kettering Foundation, Dayton, pp 53-11.

Cox, E. J. (1996) Identification of freshwater Diatoms from living Material; Chapman and Hall: London.

Crump, B. C., Armbrust, E. V. and Baross, J. A (1999). Phylogenetic Analysis of Particle-Attached and Free-living Bacterial comities in the Columbia River, it's Estuary and the adjacent Coastal Ocean. *Applied and Environmental Microbiology* 65: 3192-3204.

Cunningham, J. (2007) Biofuel joins the jet set. Prof Eng 20: 32–32.

Cyplik, P., Grajek, W., Marecik, R. and Kroliczak, P. (2007). Effect of macro/micro nutrients and carbon source over the denitrification rate of *Haloferax denitrificans* archaeon. *Enzyme and Microbial Technology*, 40: 212-220.

Day, S. A., Wickham, R. P., Entwisle, T. J. and Tyler, P. A. (1995) Bibliographic check-list of non-marine algae in Australia. *Flora Australia Supplement Series,* 4: 1-276

De Stefano, L.; Rea, l., Rendina, l., De Stefano, M. and Moretti, L., (2007) Lensless light focusing with the centric marine diatom *Coscinodiscus walesii Optical Express,* 15: 18082-18088.

Dominguez, J. A., Martin, A., Anriquez, A. And Albanesi, A. (2012) The combined effects of *Pseudomonas fluorescens* and *Tuber melanosporum* on the quality of *Pinus halepensis* seedlings. Mycorrhiza, 22: 429-436

Doronina, N. V., Darmaeva, T. D. and Trotsenko, Y. A. (2001). Novel aerobic methylotrophic isolates from the Soda Lakes of the Southern Transbaikal Region. *Microbiology*, 70: 398-404

Drancourt, M. and Raoult, D. (2005). Sequence-Based Identification of New Bacteria: a Proposition for creation of an Orphan Bacterium Repository. *Clinical Microbioloy-Minireview,* 43: 4311-4315.

Echigo, A., Hino, M., Fukushima, T., Mizuki, T., Kamekura, M. and Usami1, R. (2005). Endospores of halophilic bacteria of the family Bacillaceae isolated from non-saline Japanese soil may be transported by Kosa event (Asian dust storm). *Saline Systems*, 8: 1-13**.**

Edwards, C. (1990). Thermophiles. In Microbiology of extreme environments. Ed. Edwards, C, *Open University Press*, Milton Keynes, U.K. pp. 1- 32.

Elsey, D., Jameson, D., Raleigh, B. and Cooney, M. J. (2007). Fluorescent measurement of microalgal neutral lipids. *Journal of Microbiological Methods*, 68: 639-642.

Engle, M., Li, Y., Woese, C. and Wiegel, J. **(1995).** Isolation and characterization of a novel alkalitolerant thermophile, *Anaerobranca horikoshii* gen. nov., sp. Nov. *International Journal of Systematic Bacteriology*. 45: 454-461.

Ettl, H. and Gärtner, G. (1995) Syllabus der Boden-, Luft- und Flechtenalgen. Gustav Fischer Verlag, Stuttgart, p 721

Fanrintzin, A (1871) Die anorganischen Salze als ausgezeichneted Hiilfsninel zum Studium der Entwicklun-e niederer chlorophyll-haltiger Organismen. *Bulletin of the Academy of Science St Petersburg* 17: 31-70

Fenollar, F., Roux, V., Stein, A., Drancourt, M. and Raoult, D. (2006). Analysis of 525 samples to determine the usefulness of PCR amplification and sequencing of the 16S rRNA gene for diagnosis of bone and Joint Infections. *Journal of Clinical Microbiology*, 44: 1018-1028.

Friedlingstein, P. and Solomon, S. (2005) Contributions of past and present human generations to committed warming caused by carbon dioxide. *Proceedings of the National Academy of Sciences of the United States of America,* 102**:** 10832-10836.

Gardner R, Peters P, Peyton B and Cooksey K (2011) Medium pH and nitrate concentration effects on accumulation of triacylglycerol in two members of the Chlorophyta. *Journal* of *Applied Phycology*, 23: 1005–1016.

Gardner, R. D., Cooksey K. E., Mus, F., Macur, R., Moll, K., Eustance, E., Carlson, R. P., Gerlach, R. and Fields, M. W. (2012). Use of sodium bicarbonate to stimulate triacylglycerol accumulation in the chlorophyte Scenedesmus sp. and the diatom Phaeodactylum tricornutum. *Journal of Applied Phycology,*.24: 1311-1320

Geoghegan, M. J. (1951) Unicellular algae as food. *Nature*, 168: 426-427

Gilmour, D. J. (1990). Halotolerent and halophilic microorganisms. In Microbiology of extreme environments. Ed. Edwards, C., *Open University Press*, Milton Keynes, U.K. pp. 147-177.

Gilmour, D. J. and Zimmerman, W. B. (2012) Can algal biofuels play a major role in meeting future energy needs? *Biofuels,* 3: 511-53

Gomes, J. and Steiner, W. (2004). The biocatalytic potential of extremophiles and extremozymes. *Food Technology and Biotechnology,* 42: 223-235.

Gordon, R., Losic, D., Tiffany, M. A., Nagy, S. S. and Sterrenburg, F. A. S. (2009). The Glass Menagerie: Diatoms for Novel Applications in nanotechnology. *Trends Biotechnology,* 27: 116-121.

Gordon. R.: Sterrenburg. F. A. S. and Sandhage. K. A., (2005) Special issue on diatom nanotechnology. *Journal Nanoscience. Nanotechnology,* 5: 1-4.

Goto T., Matsuno T, Hishinuma-Narisawa M, Yamazaki, K, Matsuyama, H., Inoue N, and Yumoto, I. (2005). Cytochrome *c* and bioenergetic hypothetical model for alkaliphilic *Bacillus* spp. *Journal of Bioscience and Bioengineering*, 100: 365-379.

Grant, W. D., Mwatha, W. E., and Jones, B. E. (1990). Alkaliphiles: ecology, diversity and applications. *Federation of European Microbiological Societies* (*FEMS) Microbiology Reviews*, 75: 255-270.

Guiry, M. D. (2012) how many species of algae are there. *Journal of Phycology,*. 48: 1057–1063.

Gurr, M. I., Harwood, J. L. and Frayn K. N. (2002) Lipid biochemistry. An introduction, 5th edn. Blackwell, Oxford, 120 pp.

Hagemann, M. and Erdmann, N. (1994) activation and pathway of glucosylglycerol synthesis in the cyanobacterium *Synechocytis* sp. PCC 6830. *Microbiology,* 140: 1427-1431

Harder R and von Witsch, H. (1942a) Bl:richt iiber Versuche zur Fetrsynthese mittels autotropher Microorganismen. *Forschungsdienst Sonderhefr,* 16: 270-215

Harder R. and von Witsch, H. (1942b) Die massenkultur von diatomeen. *Berichte Deutsch Botanische Gessellschaft,* 60:146-152.

Hezayen, F. F., Younis, M. A. M., Hagaggi, N. S. A. and Shabeb, M. S. A. (2010). *Oceanobacillus aswanensis* Strain FS10 sp. Nov., an extremely halotolerant bacterium isolated from salted fish sauce in Aswan City, Egypt. *Global Journal of Molecular Sciences*, 5: 1-6.

Hill, J., Nelson, E., Tilman, D., Polasky, S. and Tiffany, D. (2006) Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. *Proceeding National Academy Science* USA, 103:11206–11210.

Hockin, N. L., Moch, T., Mulholland, F., Kopriva, S. and Malin, G. (2012) The response of diatom central carbon metabolism to nitrogen starvation Is different from that of green algae and higher plants. *Plant Phycology*, 158: 229-312

Horikoshi, K. (1991a). General view of alkaliphiles and thermophiles. In Superbugs: microorganisms in extreme environments. Eds. Horikoshi, K. and Grant, W. D., *Springer -Verlag*, Berlin, pp. 3 -14.

Horikoshi, K. (1991b). Microorganisms in alkaline environments. *Kodansha, VCH,* Tokyo, Weinhem, New York. pp. 6-7.

Horikoshi, K. (1996). Alkaliphiles-from an industrial point of view. *Federation of European Microbiological Societies (FEMS) Microbiology Reviews*, 18: 259 - 270.

Horikoshi, K. (1999). Alkaliphiles: Some applications of their products for biotechnology. *Microbiology and Molecular Biology Reviews*, 63: 735 -750.

Hoshina, R. and Fujiwara, Y. (2013) Molecular characterization of *Chlorella* cultures of the National Institute for Environmental Studies culture collection with description of *Micractinium inermum* sp. Nov., *Didymogenes sphaerica* sp. nov., and *Didymogenes soliella* sp. Nov. (Chlorellaceae, Trebouxiopfyceae). *Phycological Research,* 61: 124-132

Hou, W., Wang, S., Dong, H., Jiang, H., Briggs, B. R., Peacock, J. P., Huang, Q., Huang, L., Wu, G., Zhi, X., Li, W., Dodsworth, J. A., Hedlund, B. P., Zhang, C., Hartnett, H. E., Dijkstra, P. and Hungate, B. A. (2013) A comprehensive census of microbial diversity in hot springs of Tengchong, Yunnan Province China using 16S rRNA gene pyrosequencing. PLoS One 8:e53350.

Hough, D. W. and Danson, M. J. (1999). Extremozymes. *Current Opinion in Chemical Biology,* 3: 39- 46.

Hozzein, W. N., Li, W., Ali, M. I., Hammouda, O., Mousa, A. S., Xu, L. and Jiang, C. (2004). *Nocardiopsis alkaliphila* sp. nov., a novel alkaliphilic actinomycete isolated from desert soil in Egypt. *International Journal of Systematic and Evolutionary Microbiology,* 54: 247-252.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M. and Darzins, A. (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant Journal,* 54: 621–639.

Hungund, B. S. and Gupta, S. G. (2010) Production of bacterial cellulose from *Enterobacter amnigenus* GH-1 isolated from rotten apple*. World Journal Microbiology Biotechnology*,. 26: 1823-1828

Hung, Y. L., Chi, Y. S., Hung, C. L., Jeng, C., Ying, L. C., Yet, R. C., Han, T. L., Yu, Y. C., Chun, H. H. and Han, J. L. (2013) Identification and Characterization of an Extracellular Alkaline Phosphatase in the Marine Diatom *Phaeodactylum tricornutum*. Springer Science and Business Media New York 10.1007/s10126-013-9494-3.

Huss, V. A. R., Frank, C., Hartman, E. C., Hirmer, M., Kloboucek, A., Seidel, B. M., Wenzeler, P. and Kessler, E. (1999) Biochemical Taxonomy and molecular phylogeny of the genus *Chlorella* sensu lato (Chlorophyta). *Journal of. Phycology.* 35: 587-598

Imhoff, F. J. (1986). Survival strategies of microorganisms in extreme saline environments, *Advances in Space Research*, 6: 299-306.

Inagaki, F., Sakihama, Y., Inoue, A., Kato, C. and Horikoshi1, K. (2002). Molecular phylogenetic analyses of reverse-transcribed bacterial rRNA obtained from deep-sea cold seep sediments. *Environmental Microbiology,* 5: 277-286.

Irwin, J. A. and Baird, **A. W. (2004).** Extremophiles and their application to veterinary medicine. *Irish Veterinary Journal*, 57: 348-354.

Jennings, D. H. (1990). Osmophiles. In Microbiology of extreme environments, ed. Edwards, C., *Open University Press*, Milton Keynes, U.K. pp. 117-146.

Jiang, H., Dong H., Yu, B., Liu, X., Li, Y., Ji, S. and Zhang, C. (2007). Microbial response to salinity change in Lake Chaka, a hypersaline lake on Tibetan plateau. *Environmental Microbiology*, 9: 2603-2621.

Kikuchi, R., Gerardo, R. and Santos, S. M. (2009) Energy lifecycle assessment and environmental impacts of ethanol biofuel. *International. Journal. Energy Research*, 33: 186-193.

Killops, S. D. and Killops, V. J. 2005 *introduction to Organic geochemistry,* 2nd Edition: Blackwell Publishing: Malden. MA.

Kim, S. and Dale, B. E. (2004) Global potential bioethanol production from wasted crops and crop residues. *Biomass and Bioenergy,* 26: 361-375.

Kitada, M., Kosono, S. and Kudo, T. (2000). The Na^+/H^+ antiporter of alkaliphilic *Bacillus* sp. *Extremophiles,* 4: 253-258.

Konings, W. N., Albers, S.V., Van de Vossenberg, J, Koning, S and. Driessen, A. J. M. **(2002).** The cell membrane plays a crucial role in survival of bacteria and archaea in extreme environments. *Antonie van Leeuwenhoek,* 81: 61-72.

Konstantinidis, K., Tebbe, A., Klein, C., Scheffer, B., Aivaliotis, M., Bisle, B., Falb, M., Pfeiffer, F., Siedler, F. and Osterhelt, D. (2007). Genome wide proteomics of *Natronomonas pharaonis*. *Proteome Research*, 6: 185-193.

Koojstra, W. H. and Medlin, L. K. (1996) Evolution of the diatoms (*Bacil lariophyta*) lV. A reconstrucrion of their age irom small subunit rRNA coding regions and the fossil record. *Molecular. Phylogenetics,* 6: 391-407.

Kroll, R. G. (1990). Alkalophiles. In Microbiology of Extreme Environments, Ed. Edwards, C., *Open University Press*, Milton Keynes, U.K. pp. 55-92.

Kushner, D. J. (1978). Life in high salt and solute concentrations: Halophilic bacteria. In Microbial Life in Extreme Environments, ed. by Kushner, D. J., Academic Press, London, New York and San Francisco, pp. 318-346.

Kushner, D. J. (1985). The Halobacteriaceae. In Woese CR, Wolfe RS (eds) The Bacteria, vol. 8. Academic Press, New York, pp 171-214.

Joo, W. A. and Kim, C. W. (2005). Proteomics of halophilic archaea. *Chromatography B, Analytical Technologies in the Biomedical and Life Sciences*, 815: 237-250.

Larsen, H. (1986). Halophilic and halotolerant microorganisms-an overview and historical perspective. *FEMS Microbiology Reviews*, 39: 3-7.

Lexa, M., Horak, J. and Brzobohaty, B. (2001). Virtual PCR, application note. *Bioinformatics*, 17: 192-193

Li, D-M. and Qi, Y-Z. (1997) Spirulina industry in China: Present status and future prospects. *Journal of Applied Phycology*, 9: 25-28.

Ma, Y., Galinski, E. A., Grant, W. D., Oren, A. and Ventosa, A. (2010). Halophiles 2010: Life in saline environments**.** *Applied and Environmental Microbiology*, 76: 6971-6981.

Ma, Y., Xue, Y., Grant, W. D., Collins, N. C., Duckworth, A. W., van Steenbergen, R. P. and Jones B. E. (2004a). *Alkalimonas amylolytica* gen. nov., sp. nov., and *Alkalimonas delamerensis* gen. nov., sp. nov., novel alkaliphilic bacteria from soda lakes in China and East Africa. *Extremophiles*, 8:193-200.

MacElroy, R. D. (1974). Some comments on the evolution of extremophiles, *Biosystems*, 6: 74-75.

Madern, D. and Zaccai, G. (2004). Molecular adaptation: the malate dehydrogenase from the extreme halophilic bacterium *Salinibacter ruber* behaves like a non-halophilic protein*. Biochimie*, 86: 295-303.

Madigan, M. T., Martinko, J. M., Parker, J. and Brock, T. D. (2003) Brock, biology of microorganisms, New. Jersey, Prentice Hall

Margesin, R., and Schinner, F. (2001). Potential of halotolerant and halophilic microorganisms for biotechnology*. Extremophiles*, 5: 73-83.

Mastropaolo, M. D., Silby M. W., Nicoll, J. S. and Levy S. B. (2012) Novel genes Involved in *Pseudomonas fluorescens* Pf0-1 motility and biofilm formation. *Applied and Environmental microbiology,* 78: 4318-4329

McGenity, T. J., Gemmell, R. T., Grant, W. D. and Stan-Lotter, H. (2000). Origins of halophilic microorganisms in ancient salt deposits. *Environmental Microbiology*, 2: 243-250

Metzger, J. and Hüttermann, A. (2009) Sustainable global energy supply based on lignocellulosic biomass from afforestation of degraded areas. *Naturwissenschaften,* 96: 279-288.

Mignard, S. and Flandrois, J. P. (2006). 16S rRNA sequencing in routine bacterial identification: A 30-month experiment. *Journal of Microbiological Methods*, 67: 574-581.

Milner, H. W. (1951) Possibilities in photosynthetic methods for production of oils and proteins. JAOCS 28:363-367

Miquel, P. (1892). De la culture artificielle des Diatomes. *Comp Rend Academy Science Paris,* 94:780 782

Mullakhanbhai, M. F. and Larsen H. (1975). *Halobacterium volcanii* spec. nov. a Dead Sea halobacterium with a moderate salt requirement. *Archives of Microbiol*ogy, 104: 207-214.

Olivera, N., Sineriz, F. and Breccia, J. D. (2005). *Bacillus patagoniensis* sp. nov., a novel alkalitolerant bacterium from the rhizosphere of *Atriplex lampa* in Patagonia, Argentina. *International Journal of Systematic and Evolutionary Microbiology*, 55: 443-447.

Ochsenreiter, T., Pfeifer, F., and Schleper, C. (2002). Diversity of Archaea in hypersaline environments characterized by molecular-phylogenetic and cultivation studies. *Extremophiles*, 6: 267-274.

Ollivier, B., Caumettte, P., Garcia, J. L., and Mah, R. (1994). Anaerobic bacteria from hypersaline environments. *Microbiological Reviews*, 58: 27-38.

Olsen, G. J., Lane, D. J., Giovannoni, S. J. and Pace, N. R. (1986). Microbial ecology and evolution: A ribosomal RNA approach. *Annual Reviews Microbiology*, 40: 337-365.

Oren, A. (1994). The ecology of extremely halophilic archaea. *FEMS Microbiology Reviews*, 13: 415-440.

Oren, A. (2006). Life at high salt concentrations. Ecophysiological and biochemical aspects. In The Prokaryotes, A Handbook on the Biology of Bacteria. Third Edition, 2: 263-282.

Oren, A. (2008). Microbial life at high salt concentrations: phylogenetic and metabolic diversity. *Saline Systems,* 4: 1-13.

Pace, N. R. (1997). A molecular view of microbial diversity and the biosphere. *Science*, 276: 734-740.

Padan, E., Bibi, E., Ito, M. and Krulwich, T. A. (2005). Alkaline pH homeostasis in bacteria: New insights. *Biochimica et Biophysica Acta,* 1717: 67-88.

Padan, E., Venturi, M., Gerchman, Y. and Dover, N. (2001). Na+/H+ antiporters. Biochimica et Biophysica Acta, 1505: 144-157.

Patrick, R. (1961). A study of the numbers and kinds of species found in the rivers of Eastern United States. *Proceedings of the Academy of Natural Sciences of Philadelphia*, 113: 215–258.

Peddie, C. J., Cook, G. M., and Morgan, H. W. (1999). Sodium-dependent glutamate uptake by an alkaliphilic, thermophilic *Bacillus* strain, TA2.A1. *Journal of Bacteriology,* 181: 3172-3177.

Percent, S., Frischer, M., Duffy, F., Milano, V., Melellan, M., Stevens, B., Boylen, C., Sandra, A. and Baure, A. (2008). Bacterial Community Structure of Acid-Impacted lake. *Applied and Environmental Microbiology,* 74: 1856-1868.

Pernthaler, J. and Amann, R. (2005). Fate of heterotrophic microbes in pelagic habitats: Focus on populations. *Microbiology and Molecular Biology Reviews*, 69: 440-461.

Phukan, M. M., Chutia, R. S., Konwar, B. K. and Kataki, R. (2011) Microalgae *Chlorella* as potential bio-energy feedstock. *Applied Energy*, 88: 3307-3312

Pick, U. and Rachutin-Zalogin, T. (2012). Kinetic anomalies in the interactions of Nile red with microalgae. *Journal of Microbiological Methods*, 88: 189-196.

Pimentel, D. and Patzek, T. (2005) Ethanol Production Using Corn, Switchgrass and Wood; Biodiesel Production Using Soybean. *Biofuels, Solar and Wind as Renewable Energy Systems.*

Purdy, K. J., Cresswell-Maynard, T. D., Nedwell, D. B., McGenity, T. J., Grant, W. D., Timmis, K. N. and Embley, T. M. (2004). Isolation of haloarchaea that grow at low salinities. *Environmental Microbiology, 6: 591-595.*

Reed, R. D. (1998). Practical Skills in Bimolecular Sciences, Harlow, Prentice Hall.

Rodriguez-Valera, F. (1986). The ecology and taxonomy of aerobic chemoorganotrophic halophilic eubacteria. *FEMS Microbiology Reviews*, 39: 17-22.

Romano, I., Lama, L., Orlando, P., Nicolaus, B., Assunta, G. and Gambacorta, A. (2007). *Halomonas sinaiensis sp*. nov., a novel halophilic bacterium isolated from a salt lake inside Ras Muhammad Park, Egypt. *Extremophiles*, 11: 789-796.

Rosello, M. R. and Amann, R. (2001). The Species concept for prokaryotes. *FEMS Microbiology Reviews,* 25: 39-67.

Roszak, D. B. and Colwell, R. R. (1987). Survival strategies of bacteria in the natural environment. *Microbiology Reviews,* 51: 365-379.

Rothschild, L. J. and Mancinelli, R. L. (2001). Life in extreme environments. *Nature*, 409: 1092-1101.

Round, F. E. (1973) The Biology of the algae 2nd Edition

Round, F. E.; Crawford, R. M. and Mann, D. G. (1990) The Diatoms, Biology and Morphology of the Genera; Cambridge University Press: Cambridge, U.K.

Russell, N. J. (2000). Toward a molecular understanding of cold activity of enzymes from psychrophiles. *Extremophiles,* 4: 83-90.

Saeki, K., Hitomi, J., Okuda, M., Hatada, Y., Kageyama, Y., Takaiwa, M., Kubota H., Hagihara, H., Kobayashi, T., Kawai, S.and Ito, S. (2002). A novel species of alkaliphilic *Bacillus* that produces an oxidatively stable alkaline serine protease. *Extremophiles,* 6: 65-72.

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. and Arnheim, N. (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of Sickle Cell Anemia. *Science*, 230: 1350-1354.

Satyanarayana, T., Raghukumar, C. and Shivaji, S. (2005). Extremophilic microbes: Diversity and perspectives, *Current Science*, 89: 78-90.

Scow, K. M. Egbert, S., Mara, J., Johnson and Jennifer, L. M. (2001). Microbial Biodiversity, Measurement. *Encyclopaedia of Biodiversity,* 4: 177-217.

Schieper, C., Holben, W. and Klenk H. (1997). Recovery of Crenarchaeotal ribosomal DNA sequences from freshwater-lake sediments. *Applied and Environmental Microbiology*, 63: 321-323.

Schieber, J., Krinsley, D. l. and Riciputi, L., (2000). Diagenetic origin of quartz silt in mudstones and implications for silica cycling. *Nature*, 406: 981-985.

Scholz, B. and liebezeit, G (2012) Compatible solutes in three marine intertidal microphytobenthic Wadden Sea diatoms exposed to different salinities. *European. Journal. Phycology.* 47: 393-407

Schreiber, B. C. (1986). Arid shorelines and evaporites. *In Sedimentary Environments and Facies*. Reading, H.G. (ed.). Oxford: Blackwell Scientific Publications, pp. 189-228.

Sheehan, J., Dunahay, T., Benemann, J., Roessler, P. (1998) A look back at the U.S. Department of Energy's aquatic species program - biodiesel from algae. National Renewable Energy Laboratory, Golden, pp 1-328

Skood, D. A., Holler, F. J. and Crouch, S. R. (2007) Principles of instrumental analysis, Belmont, C. A., Thomson/brooks-Cole

Sorokin, D. Y., Lysenko, A. M., Mityushina, L. L, Tourova, T. P., Jones, B. E., Rainey, F. A., Robertson, L. A. and Kuenen, G. J. (2001a). *Thioalkalimicrobium aerophilum* gen. nov., sp. nov., and *Thioalkalimicrobium sibericum* sp. nov., and *Thioalkalivibrio versutus* gen. nov., sp. nov., and *Thioalkalivibrio nitratis* sp. nov., and *Thioalkalivibrio denitrificans* sp. nov., novel obligately alkaliphilic and obligately chemolithoautotrophic sulfur-oxidizing bacteria from soda lakes. *International Journal of Systematic and Evolutionary Microbiology*, 51: 565-580.

Sousa, L. D. C., Chundawat, S. P. S., Balan, V. and Dale, B. E. (2009) Cradle to grave assessment of exisiting lignocelluloses pretreatment technologies. *Current. Opinion. Biotechnology*, 20: 339-347.

Spoehr, H. A. and Milner H. W. (1948) Chlorella as a source of food. Carnegie Institution Washington Yearbook 47:100 103

Spoehr, H. A. And Milner H. W. (1949) The chemical composition of *Chlorella*; effect of environmental conditions. *Plant Physiology*, 24: 120-149.

Spratt, D. A. (2004). Significance of bacterial identification by molecular biology methods. Endodontic Topics 9: 5-14.

Taylor, G. (2008) Biofuels and the biorefinery concept. *Energy Policy,* 36**:** 4406-4409.

Tiago, I., Mendes, V., Pires, C., Morais, P. V. and Verissimo, A. (2006). *Chimaereicella alkaliphila* gen. nov., sp. nov., a gram-negative alkaliphilic bacterium isolated from a nonsaline alkaline groundwater. *Systematic and Applied Microbiology,* 29: 100-108.

Toepel, J., Langner, U. and Wilhelm, C. (2005) Combination of flow cytometry and single cell absorption spectroscopy to study the phytoplankton structure and to calculate the chi a specific absorption coefficients at the taxon level. *Journal Phycology,* 41:1099–1109

Torsvik, V. and Tron, F. T. (2002). Prokaryotic diversity magnitude, dynamics and controling factors. *Environmental Microbiology,* 296: 1064-1065.

Ulukanli, Z. and Digrak, M. (2002). Alkaliphilic microorganisms and habitats. *Turkish Journal of Biology,* 26: 181-191.

Urbach, E. and Vergin, K. L. (2001). Unusual bacterioplankton community structure in ultra oligotrophic Crater Lake. *Limnology and Oceanography* 46: 557-572.

Urbach, E., Vergin, K. L., Larson, G. L. and Giovannoni, S. J. (2007). Bacterioplankton communities of Crater lake, OR: dynamic changes with euphotic zone food web structure and stable deep water populations. *Hydrobiologia,* 574: 161-177.

Van den Burg, B. (2003). Extremophiles as a source for novel enzymes. *Current Opinion in Microbiology*, 6: 213-218.

Van den Hoek, C. (1995) ALGAE *An introduction to phycology*. Cambridge University Press.

Van de Vijver, B., and Beyens, L. (1999) Freshwater diatoms from Ile de la Possession (Crozet Archipelago, sub-Antarctica): an ecological assessment. *Polar Biology,* 22:178–188.

Van Handel, E. (1985) Rapid determination of total lipids in mosquitoes*. Journal of American Mosquito Control Association,* 1: 302-304

Ventosa, A., Marquez, C., Garahito, M. and Arahal, D. (1998). Moderately halophilic gram-positive bacteria diversity in hypersaline environments. *Extremophiles*, 2: 297-304.

Vreeland, R. H. and Huval, J. H. (1991). Phenotypic characterization of halophilic bacteria from ground water sources in the United States*. In General and Applied Aspects of Micro-Organisms*. New York: Plenum Press, pp. 53-60.

Warnecke, F., Amann, R. and Pernthaler, J. (2004) Actinobacterial 16S rRNA genes from freshwater habitats cluster in four distinct lineages. *Environmental Microbiology,* 6: 242–253.

Weaver, R. (2005). Molecular Biology 3rd edetion. Boston: McGraw Hill.

Werner, D. (1977). The Biology of Diatoms. Blackwell Scientific Publications, Oxford.

Whitman, W. B., Coleman, D. C. and Wiebe, W. J. (1998). Prokaryotes: the unseen majority. *Proceedings of National Academy of Sciences USA,* 95: 6578-6583.

Woese, C. R., Kandler, O. and Wheelis, M. L. (1990). Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria and Eucarya. *Proceedings of the National Acadeny of Science USA,* 87: 4576- 4579.

Woo, P. C. Y., Leung, P. K. L., Leung, K. W. and Yuen, K. Y. (2000). Identification by 16S ribosomal RNA gene sequencing of an Enterobacteriaceae species from a bone marrow transplant recipient. *Journal of Clinical Pathology incorporating Molecular Pathology,* 53: 211-215.

Yang, V. W., Zhuang, Z., Elegir, G. and Jeffries, T. W. (1995). Alkaline-active xylanase produced by an alkaliphilic *Bacillus* sp isolated from kraft pulp, *Journal of Industrial Microbiology,* 15: 434- 441.

Yeung, S. N., Butler, A. and Mackenzie, P. J. (2009). Applications of the polymerase chain reaction in clinical ophthalmology. Canadian Journal of Ophthalmology, 44: 23-30.

Yoon, J. H., Kang, K. H., Oh, T. K. and Park, Y. H. (2004). *Halobacillus locisalis sp*. nov., a halophilic bacterium isolated from a marine solar saltern of the Yellow Sea in Korea. *Extremophiles*, 8: 23-28.

Yumoto, I. (2002). Bioenergetics of alkaliphilic *Bacillus* spp. *Journal of Bioscience and Bioengineering*, 93: 342-353.

Yumoto, I., Yamazaki, K., Hishinuma, M., Nodasaka, Y., Inoue, N. and Kawasaki, K. (2000). Identification of facultatively alkaliphilic *Bacillus* sp. strain YN-2000 and its fatty acid composition and cell-surface aspects depending on culture pH. *Extremophiles*, 4: 285-290.

Zahran, H. H. (1997). Diversity, adaptation and activity of the bacterial flora in saline environments. *Biology and Fertility of Soils*, 25: 211-223.

Zimmerman, W. B., Zandi, M., Bandulasena, H. C. H., Tesar, V., Gilmour, D. J. and Ying, K. (2011) Desgin of an airlift loop bioreactor and pilot scales studies with fluidic oscillator induced microbubbles for growth of microalgal *Dunaliella salina. Applied Energy,* 88: 3357-3369

Appendix

A- Nile Red Concentration Test 96 Well Microplate Method

A.1- Materials Needed:

- Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one)
- Acetone
- Standard 96 Well plate (Black walled preferably)
- 2ml Eppendorf tubes (x8)
- Reagent reservoir for multipipette
- 1ml plastic cuvettes (x2)
- Timer

All other chemicals and solvents of analytical grade were purchased from Sigma or other commercial suppliers.

A.2- Stock solution

A.2.1- Nile-red

Six stock solutions are needed for this procedure, one primary stock and five secondary stocks to give Nile red concentrations in the range of 0.25-3 μM/ml from a 20μl aliquot:

A.2.2- Primary Stock:

0.0025g of Nile Red is added to 10ml of Acetone, making the first stock of 0.25 mg/mL (or 250μg/ml).

A.3- Secondary Stocks:

NR µM/ml	From Primary (µl)	Acetone (µl)
0.25	15.9	984.1
0.5	31.8	968.2
1	63.7	936.3
2	127.3	872.7
3	191	809

Note: *Final stock concentration i.e. 1 µM/ml, is only achieved when 20µl of the stock is added to 1ml of an algal sample.*

At each step the mixture should be whirlimixed and after the stock its complete the containers need to be wrapped in tin foil to stop photo-degradation.

A.4- Cell Concentration

A cell concentration large enough to produce a good fluorescent signal, whilst avoiding self shading needs to be selected for this procedure. This selection should be based on the results from a peak fluorescence test. Every strain has a different optimal value, due to different pigmentation and cell geometry. This needs to be calibrated for to ensure the accuracy of the test. Once a value is established (For reference D.Salina 19/30 optimum is approx. $1x10^6$ cells /ml), the following needs to be performed:

1. Remove 10ml of culture from growth vessel and adjust the optimal OD (A) at 595nm (make sure to blank using appropriate medium).
2. Centrifuge the adjusted culture for 5 mins at 3000 rpm and discard the supernatant immediately when finished.

3. Replace the supernatant with an equivalent volume of fresh media and mix until algal pellet is re-suspended.
4. The pipette the culture into 5 separate 2ml Eppendorf tubes, one for each Nile Red concentration 0.25-3 µM/ml. For best results cultures must be in stationary phase.

A.5- Plate Reader Settings:

Before carrying out the procedure make sure the plate reader is set to a temperature of constant 25°C and filters for excitation and emission wavelengths 490 nm and 580 nm are installed. The following software settings also need to be set:

Fluorometry Label Properties:

Name:	Nile Red Stain (0.1s) Ex490 Em580
CW-Lamp Energy:	9032
CW-Lamp Control:	Stabilised Energy
CW-Lamp Filter:	P490
Emission Filter:	F580
Emission Aperture:	Normal
Counter Position:	Top
Counting Time:	0.1 sec

Photometry Label Properties:

Name:	Absorbance @ 595 (1.0s)
CW-Lamp Filter:	P595
Reading Time:	1 sec

A.6- Protocol Settings

1. First Step: Shaking – *Settings*; Duration: 5 sec, Speed: Fast, Diameter: 1mm, Type: Double Orbit.

2. Second Step: Reading (using label settings (Nile Red Stain (0.1s) Ex490 Em580) and plate layout (below)), Readings taken from A1-D5.

A.7- Protocol Settings (Shaken OD @ 595 (1.0s)):

3. First Step: Shaking – *Settings*; Duration: 5 sec, Speed: Fast, Diameter: 1mm, Type: Double Orbit.
4. Second Step: Reading (using label settings (Absorbance @ 595 (1.0s)) and plate layout (below)), Readings taken from A1-H12.
5. Repeats: 0.

A.7.1- Plate Layout:

NR Conc (µM/ml):			3	2	1	0.5	0.25	Empty Wells						
			1	2	3	4	5	6	7	8	9	10	11	12
Nile Red Stained Cells	R1	A	200	200	200	200	200	0	0	0	0	0	0	0
	R2	B	200	200	200	200	200	0	0	0	0	0	0	0
	R3	C	200	200	200	200	200	0	0	0	0	0	0	0
	R4	D	200	200	200	200	200	0	0	0	0	0	0	0
Unstained Cells	R1	E	200	200	200	200	200	0	0	0	0	0	0	0
	R2	F	200	200	200	200	200	0	0	0	0	0	0	0
	R3	G	200	200	200	200	200	0	0	0	0	0	0	0
	R4	H	200	200	200	200	200	0	0	0	0	0	0	0

Note: Rows R1 to R4 are replicates from the same concentration

B- Nile Red Peak Fluorescence 96 Well Microplate Method

B.1- Materials Needed:

- Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one)
- Acetone
- Standard 96 Well plate (Black walled preferably)
- 2ml Eppendorf tubes (x8)
- Reagent reservoir for multipipette
- 1ml plastic cuvettes (x2)

- Timer

All other chemicals and solvents of analytical grade were purchased from Sigma or other commercial suppliers.

B.2- Stock solutions:

B.2.1- Nile Red

The concentration of Nile red dye needed in the final culture is 1 μM/ml from 20μl, to get this concentration two stocks need to be made:

1. 0.0025g of Nile Red is added to 10ml of Acetone, making the first stock of 0.25 mg/mL (or 250μg/ml).
2. 63.7μl of the previous stock is added to 936.3μl of Acetone, making a second stock of 15.9 μg/mL. 20μl of this stock is equal to 0.318 μg/mL or 1 μM/ml.

At each step the mixture should be whirlmixed and after the stock its complete the containers need to be wrapped in tin foil to stop photo-degradation.

B.2.2- Cell Concentration

1. Remove culture from growth vessel (at least 10ml) and adjust the OD to 1 (A) at 595nm (make sure to blank using appropriate medium).
2. Centrifuge the adjusted culture for 5 mins at 3000 rpm and discard the supernatant immediately when finished.
3. Replace the supernatant with an equivalent volume of fresh media and mix until algal pellet is re-suspended.

4. Pipette the culture using the following concentrations into 2ml Eppendorf tubes:

Percentage:	100	87.5	75	62.5	50	37.5	25	12.5	Total (ml)
Culture (µl)	2000	1750	1500	1250	1000	750	500	250	9
Medium (µl)	0	250	500	750	1000	1250	1500	1750	7

Note: For best results cultures must be in stationary phase.

B.3- Plate Reader Settings:

Before carrying out the procedure make sure the plate reader is set to a temperature of constant 25°C and filters for excitation and emission wavelengths 490 nm and 580 nm are installed. The following software settings also need to be set:

Fluorometry Label Properties:

Name:	Nile Red Stain (0.5s) Ex490 Em580
CW-Lamp Energy:	9032
CW-Lamp Control:	Stabilised Energy
CW-Lamp Filter:	P490
Emission Filter:	F580
Emission Aperture:	Normal
Counter Position:	Top
Counting Time:	0.5 sec

Photometry Label Properties:

Name:	Absorbance @ 595 (1.0s)
CW-Lamp Filter:	P595
Reading Time:	1 sec

B.4- Protocol Settings (1):

1. First Step: Shaking – *Settings*; Duration: 5 sec, Speed: Fast, Diameter: 1mm, Type: Double Orbit.
2. Second Step: Reading (using label settings (Nile Red Stain (0.5s) Ex490 Em580) and plate layout (below)), Readings taken from A1-D8.
3. Repeats: 60, approx. time per repeat 50 secs.

B.5- Protocol Settings (2):

1. First Step: Shaking – *Settings*; Duration: 5 sec, Speed: Fast, Diameter: 1mm, Type: Double Orbit.
2. Second Step: Reading (using label settings (Nile Red Stain (0.5s) Ex490 Em580) and plate layout (below)), Readings taken from E1-H8.
3. Repeats: 1.

B.6- Protocol Settings (Shaken OD @ 595 (1.0s)):

1. First Step: Shaking – *Settings*; Duration: 5 sec, Speed: Fast, Diameter: 1mm, Type: Double Orbit.
2. Second Step: Reading (using label settings (Absorbance @ 595 (1.0s)) and plate layout (below)), Readings taken from A1-H12.
3. Repeats: 1.

B.6.1- Plate Layout:

Dilution from 1(A) @ OD 595 (%):			100	87.5	75	62.5	50	37.5	25	12.5	Empty Wells			
			1	2	3	4	5	6	7	8	9	10	11	12
Nile Red Stained Cells	R1	A	200	200	200	200	200	200	200	200	0	0	0	0
	R2	B	200	200	200	200	200	200	200	200	0	0	0	0
	R3	C	200	200	200	200	200	200	200	200	0	0	0	0
	R4	D	200	200	200	200	200	200	200	200	0	0	0	0
Unstained Cells	R1	E	200	200	200	200	200	200	200	200	0	0	0	0
	R2	F	200	200	200	200	200	200	200	200	0	0	0	0
	R3	G	200	200	200	200	200	200	200	200	0	0	0	0
	R4	H	200	200	200	200	200	200	200	200	0	0	0	0

Note: Rows R1 to R4 are replicates from the same concentration

C- Nile Red Triolein Concentration Test 96 Well Microplate Method

C.1- Materials Needed:

- Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one)
- Acetone
- Triolein (TO) or 1,2,3-Tri-[(cis)-9-octadecenoyl]glycerol, C57 H104 O6 ~99%) (44895-U Supelco) – Neutral Lipid
- Isopropanol
- Standard 96 Well plate (Black walled preferably)
- 2ml Eppendorf tubes (x8)
- Reagent reservoir for multipipette
- 1ml plastic cuvettes (x2)
- Timer

All other chemicals and solvents of analytical grade were purchased from Sigma or other commercial suppliers.

C.2- Stock solutions:

C.2.1- Nile Red

The concentration of Nile red dye needed in the final culture is 1 μM/ml from 20μl (or in the range of 0.25-3 μM/ml depending on the results of the previous experiment). To get this concentration two stocks need to be made:

1. 0.0025g of Nile Red is added to 10ml of Acetone, making the first stock of 0.25 mg/mL (or 250μg/ml).
2. 63.7μl of the previous stock is added to 936.3μl of Acetone, making a second stock of 15.9 μg/mL. 20μl of this stock is equal to 0.318 μg/mL or 1 μM/ml.

At each step the mixture should be whirlmixed and after the stock its complete the containers need to be wrapped in tin foil to stop photo-degradation.

C.2.2- Cell Concentration

A cell concentration large enough to produce a good fluorescent signal, whilst avoiding self shading needs to be selected for this procedure. This selection should be based on the results from a peak fluorescence test. Every strain has a different optimal value, due to different pigmentation and cell geometry. This needs to be calibrated for to ensure the accuracy of the test. Once a value is established (For reference D.Salina 19/30 optimum is approx. $1x10^6$ cells /ml), the following needs to be performed:

1. Remove 16ml of culture from growth vessel and adjust the optimal OD (A) at 595nm (make sure to blank using appropriate medium).

2. Centrifuge the adjusted culture for 5 mins at 3000 rpm and discard the supernatant immediately when finished.
3. Replace the supernatant with an equivalent volume of fresh media and mix until algal pellet is re-suspended.

C.3- Lipid Standards

Eight different lipid standards need to be made using a mixture of Triolein, isopropanol and re-suspended culture. Add the isopropanol to a 2ml Eppendorf tube first , followed by the triolein and then finally the culture using the volumes specified (below). Make sure to whirlimix well after the culture is added and return the triolein to the fridge promptly.

Conc Triolein (mg/ml)	100	87.5	75	62.5	50	37.5	25	12.5	Total (ml)
Culture (μl)	1980	1980	1980	1980	1980	1980	1980	1980	15.84
Triolein (μl)	20	16	12	8	4	2	1	0	0.063
Isopropanol (μl)	0	4	8	12	16	18	19	20	0.097

Note: For best results cultures must be in stationary phase, over two weeks of growth.

C.4- Plate Reader Settings:

Before carrying out the procedure make sure the plate reader is set to a temperature of constant 25°C and filters for excitation and emission wavelengths 490 nm and 580 nm are installed. The following software settings also need to be set:

Fluorometry Label Properties:

Name:	Nile Red Stain (0.1s) Ex490 Em580
CW-Lamp Energy:	9032
CW-Lamp Control:	Stabilised Energy
CW-Lamp Filter:	P490
Emission Filter:	F580
Emission Aperture:	Normal
Counter Position:	Top
Counting Time:	0.1 sec

Photometry Label Properties:

Name:	Absorbance @ 595 (1.0s)
CW-Lamp Filter:	P595
Reading Time:	1 sec

C.5- Protocol Settings (NR Lipid curve):

1. First Step: Shaking – *Settings*; Duration: 5 sec, Speed: Fast, Diameter: 1mm, Type: Double Orbit.
2. Second Step: Reading (using label settings (Nile Red Stain (0.1s) Ex490 Em580) and plate layout (below)), Readings taken from A1-H8.

C.6- Protocol Settings (Shaken OD @ 595 (1.0s)):

1. First Step: Shaking – *Settings*; Duration: 5 sec, Speed: Fast, Diameter: 1mm, Type: Double Orbit.
2. Second Step: Reading (using label settings (Absorbance @ 595 (1.0s)) and plate layout (below)), Readings taken from A1-H12.
3. Repeats: 1.

C.6.1- Plate Layout:

Triolein Conc (mg/ml):			0.05	0.04	0.03	0.02	0.01	0.005	0.0025	0	Empty Wells			
			1	2	3	4	5	6	7	8	9	10	11	12
Nile Red Stained Cells	S1	A	200	200	200	200	200	200	200	200	0	0	0	0
	S2	B	200	200	200	200	200	200	200	200	0	0	0	0
	S3	C	200	200	200	200	200	200	200	200	0	0	0	0
	S4	D	200	200	200	200	200	200	200	200	0	0	0	0
Unstained Cells	S1	E	200	200	200	200	200	200	200	200	0	0	0	0
	S2	F	200	200	200	200	200	200	200	200	0	0	0	0
	S3	G	200	200	200	200	200	200	200	200	0	0	0	0
	S4	H	200	200	200	200	200	200	200	200	0	0	0	0

Note: Rows R1 to R4 are replicates from the same concentration

D- Nile Red Sample Measurement Test 96 Well Microplate Method v1.0

D.1- Materials Needed:

- Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one)
- Acetone
- Distilled water
- Standard 96 Well plate (Black walled preferably)
- 2ml Eppendorf tubes (x12)
- 1.5ml Eppendorf tubes (x12)
- Reagent reservoir for multipipette
- 1ml plastic cuvettes (x12 + more dependant on number of blanks)
- Timer
- Drying oven crucible [min 1ml capacity] (x12)

All other chemicals and solvents of analytical grade were purchased from Sigma or other commercial suppliers.

D.2- Nile Red stock solutions

The concentration of Nile red dye needed in the final culture is 1 μM/ml from 20μl (or in the range of 0.25-3 μM/ml depending on the results of previous optimisation experiments). To get this concentration two stocks need to be made:

1. 0.0025g of Nile Red is added to 10ml of Acetone, making the first stock of 0.25 mg/mL (or 250μg/ml).
2. 63.7μl of the previous stock is added to 936.3μl of Acetone, making a second stock of 15.9 μg/mL. 20μl of this stock is equal to 0.318 μg/mL or 1 μM/ml.

At each step the mixture should be whirlimixed and after the stock its complete the containers need to be wrapped in tin foil to stop photo-degradation.

D.3- Cell Concentration & Dry weight prep

A cell concentration large enough to produce a good fluorescent signal, whilst avoiding self shading needs to be selected for this procedure. This selection should be based on the results from a peak fluorescence test. Every strain has a different optimal value, due to different pigmentation and cell geometry. This needs to be calibrated for to ensure the accuracy of the test. Once a value is established (For reference D.Salina 19/30 optimum is approx. $1x10^6$ cells /ml).

D.4- Plate Reader Settings:

Before carrying out the procedure make sure the plate reader is set to a temperature of constant 25°C and filters for excitation and emission wavelengths 490 nm and 580 nm are installed. The following software settings also need to be set:

Fluorometry Label Properties:

Name:	Nile Red Stain (0.1s) Ex490 Em580
CW-Lamp Energy:	9032
CW-Lamp Control:	Stabilised Energy
CW-Lamp Filter:	P490
Emission Filter:	F580
Emission Aperture:	Normal
Counter Position:	Top
Counting Time:	0.1 sec

Photometry Label Properties:

Name:	Absorbance @ 595 (1.0s)
CW-Lamp Filter:	P595
Reading Time:	1 sec

D.5- Protocol Settings (NR Sample Reading (1)):

1. First Step: Shaking – *Settings*; Duration: 5 sec, Speed: Fast, Diameter: 1mm, Type: Double Orbit.
2. Second Step: Reading (using label settings (Nile Red Stain (0.1s) Ex490 Em580) and plate layout (below)), Readings taken from A1-C12 & F1-H12.

D.6- Protocol Settings (Shaken OD @ 595 (1.0s)):

3. First Step: Shaking – *Settings*; Duration: 5 sec, Speed: Fast, Diameter: 1mm, Type: Double Orbit.

4. Second Step: Reading (using label settings (Absorbance @ 595 (1.0s)) and plate layout (below)), Readings taken from A1-H12.

5. Repeats: 0.

D.6.1- Plate Layout:

Repeat Readings (R)		Nile Red Stained Samples (S)											
		S1-1	S2-1	S3-1	S4-1	S1-2	S2-2	S3-2	S4-2	S1-3	S2-3	S3-3	S4-3
		1	2	3	4	5	6	7	8	9	10	11	12
R1	A	200	200	200	200	200	200	200	200	200	200	200	200
R2	B	200	200	200	200	200	200	200	200	200	200	200	200
R3	C	200	200	200	200	200	200	200	200	200	200	200	200
	D	0	0	0	0	0	0	0	0	0	0	0	0
	E	0	0	0	0	0	0	0	0	0	0	0	0
R1	F	200	200	200	200	200	200	200	200	200	200	200	200
R2	G	200	200	200	200	200	200	200	200	200	200	200	200
R3	H	200	200	200	200	200	200	200	200	200	200	200	200
		S1-1	S2-1	S3-1	S4-1	S1-2	S2-2	S3-2	S4-2	S1-3	S2-3	S3-3	S4-3
		Un-Stained Samples (S)											

Note: R1 to R3 are repeat measurements on the plate, S1 to S4 are different concentration of Si and NaCl to be tested, each with triplicate repeat culture readings

E- Cell count and OD_{595} Method

E.1- Material needed

- Gram's Iodine
- Distilled water
- Standard 96 Well plate (Black walled preferably)
- 15 ml falcon tube (x12)
- 1.5ml Eppendorf tubes (x11)
- Reagent reservoir for multipipette
- 1ml plastic cuvettes (x12 + more dependant on number of blanks)
- Neubauer improved haemocytometer (with coverslip)

E.2- Plate reader Setting

Photometry Label Properties:

Name:	Absorbance @ 595 (1.0s)
CW-Lamp Filter:	P595
Reading Time:	1 sec

E.3- Protocol Settings (Shaken OD @ 595 (1.0s)):

1. First Step: Shaking – *Settings*; Duration: 5 sec, Speed: Fast, Diameter: 1mm, Type: Double Orbit.
2. Second Step: Reading (using label settings (Absorbance @ 595 (1.0s)) and plate layout (below)), Readings taken from A1-H12.
3. Repeats: 0.

E.3.1- Plate layout:

Repeat Readings (R)		Nile Red Stained Samples (S)												
		100	90	80	70	60	50	40	30	20	10	5	MED	
		1	2	3	4	5	6	7	8	9	10	11	12	
1	**A**	200	200	200	200	200	200	200	200	200	200	200	200	Blank Media
2	**B**	200	200	200	200	200	200	200	200	200	200	200	200	
3	**C**	200	200	200	200	200	200	200	200	200	200	200	200	
4	**D**	200	200	200	200	200	200	200	200	200	200	200	200	
5	**E**	200	200	200	200	200	200	200	200	200	200	200	200	Empty Wells
6	**F**	200	200	200	200	200	200	200	200	200	200	200	200	
7	**G**	200	200	200	200	200	200	200	200	200	200	200	200	
8	**H**	200	200	200	200	200	200	200	200	200	200	200	200	

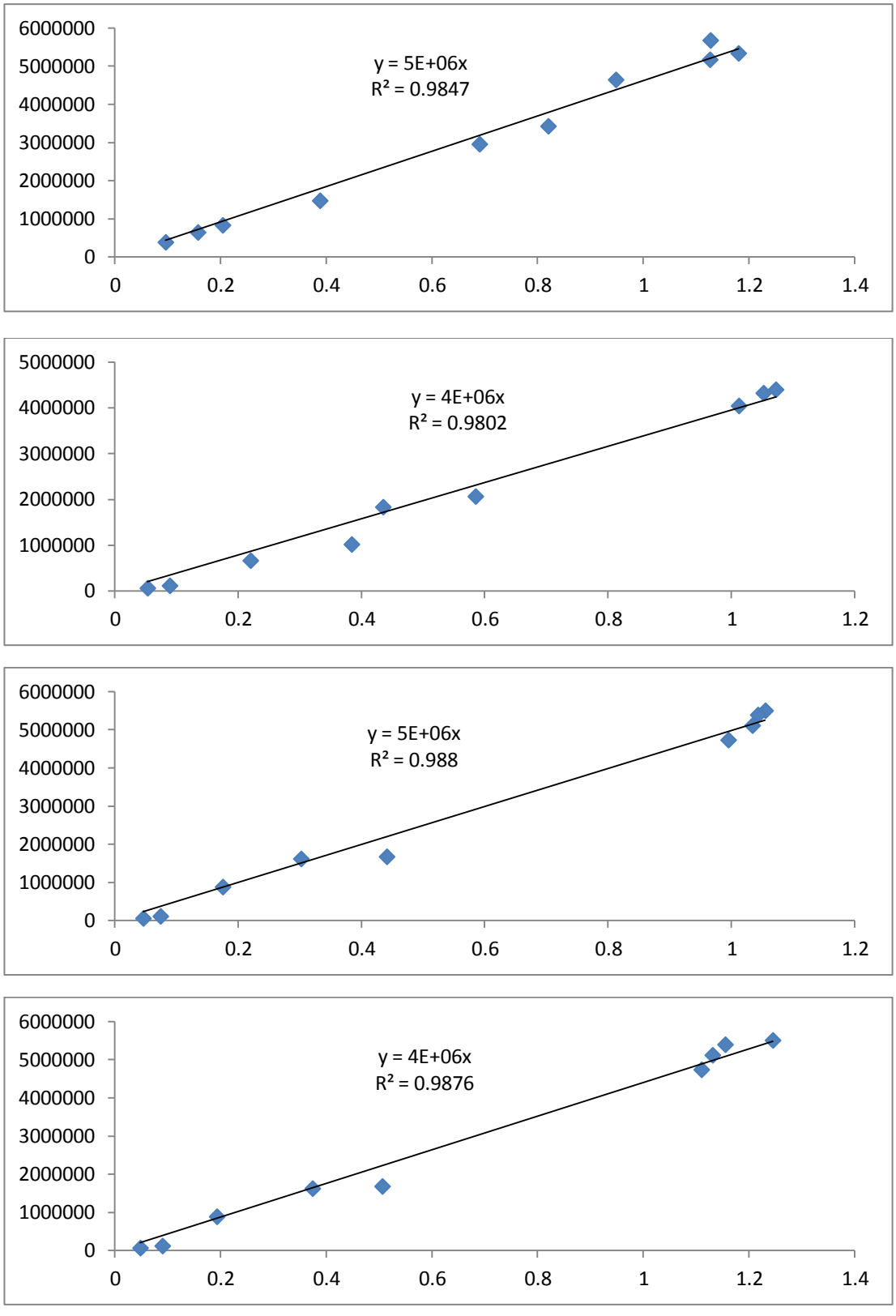


Figure 8.1: The correlation of cell counts and OD595 of Navicula strain in f/2 medium with deferent silica concentration (25%, 50%, 100% and 200%) respectively.

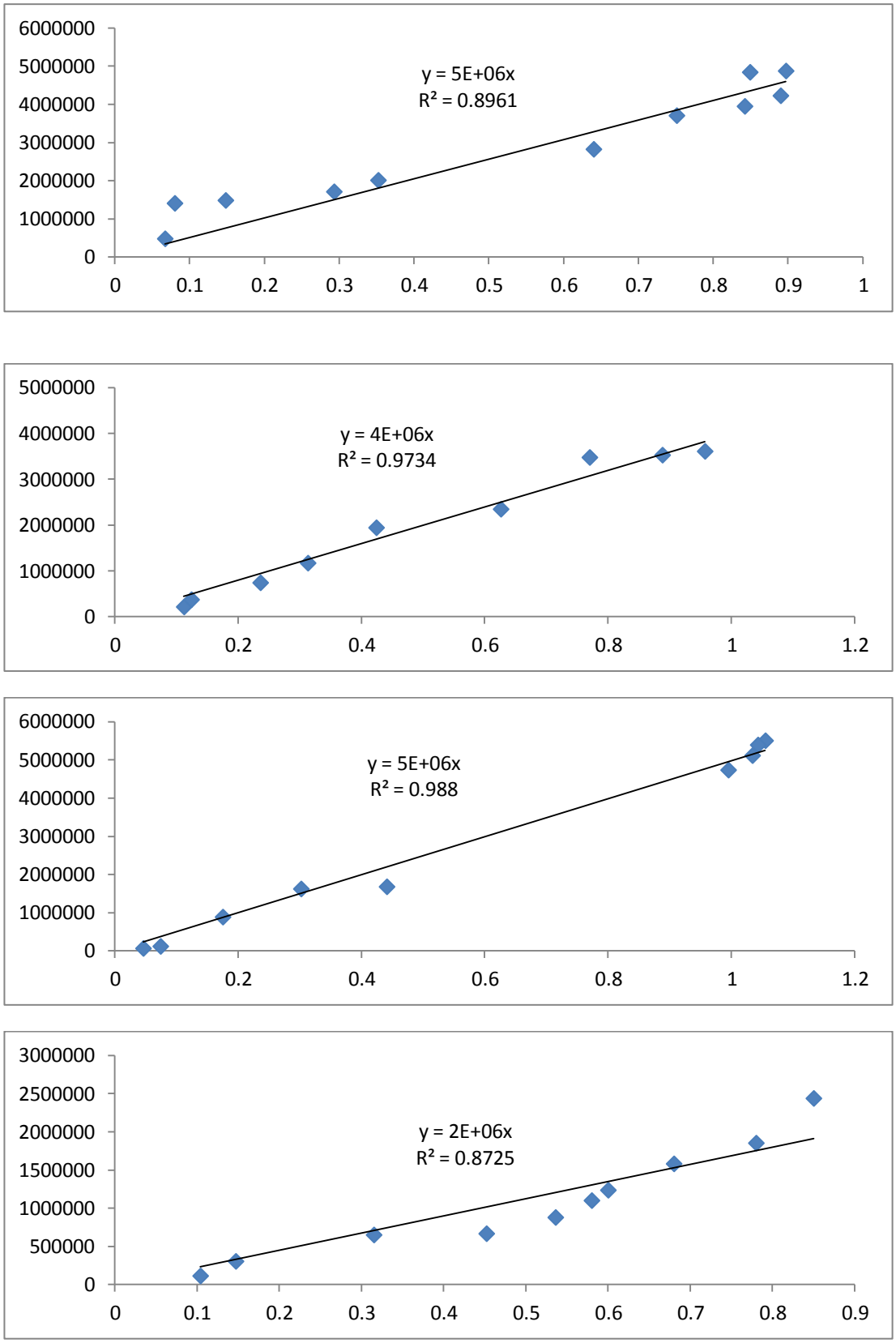
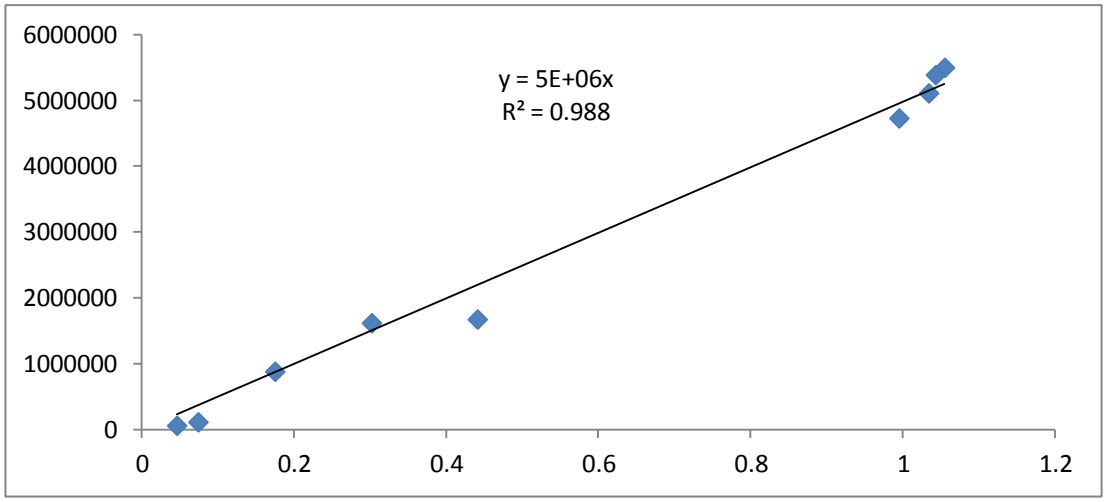
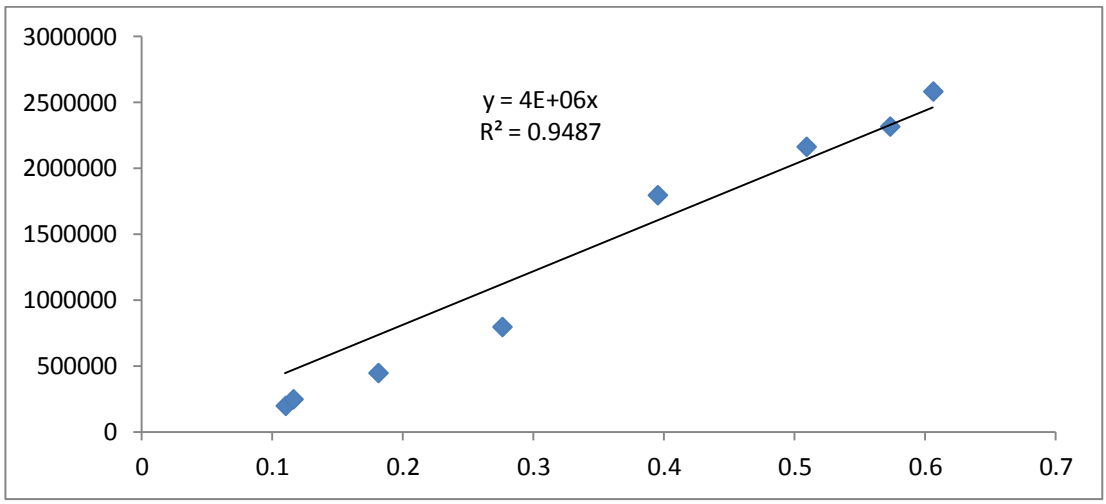


Figure 8.2: The correlation of cell counts and OD595 of Navicula strain in f/2 medium in defferent pH (7.6, 8.5, 9.25 and 10), respectively.





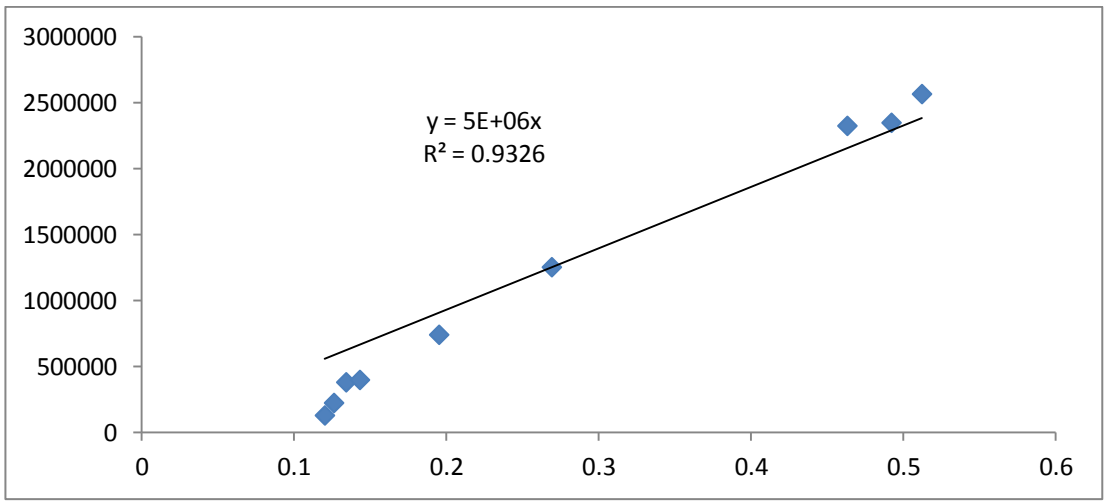


Figure 8.3: The correlation of cell counts and OD595 of Navicula strain in f/2 medium with defferent NaCl concentration (0.4, 0.6 and 0.8), respectively.

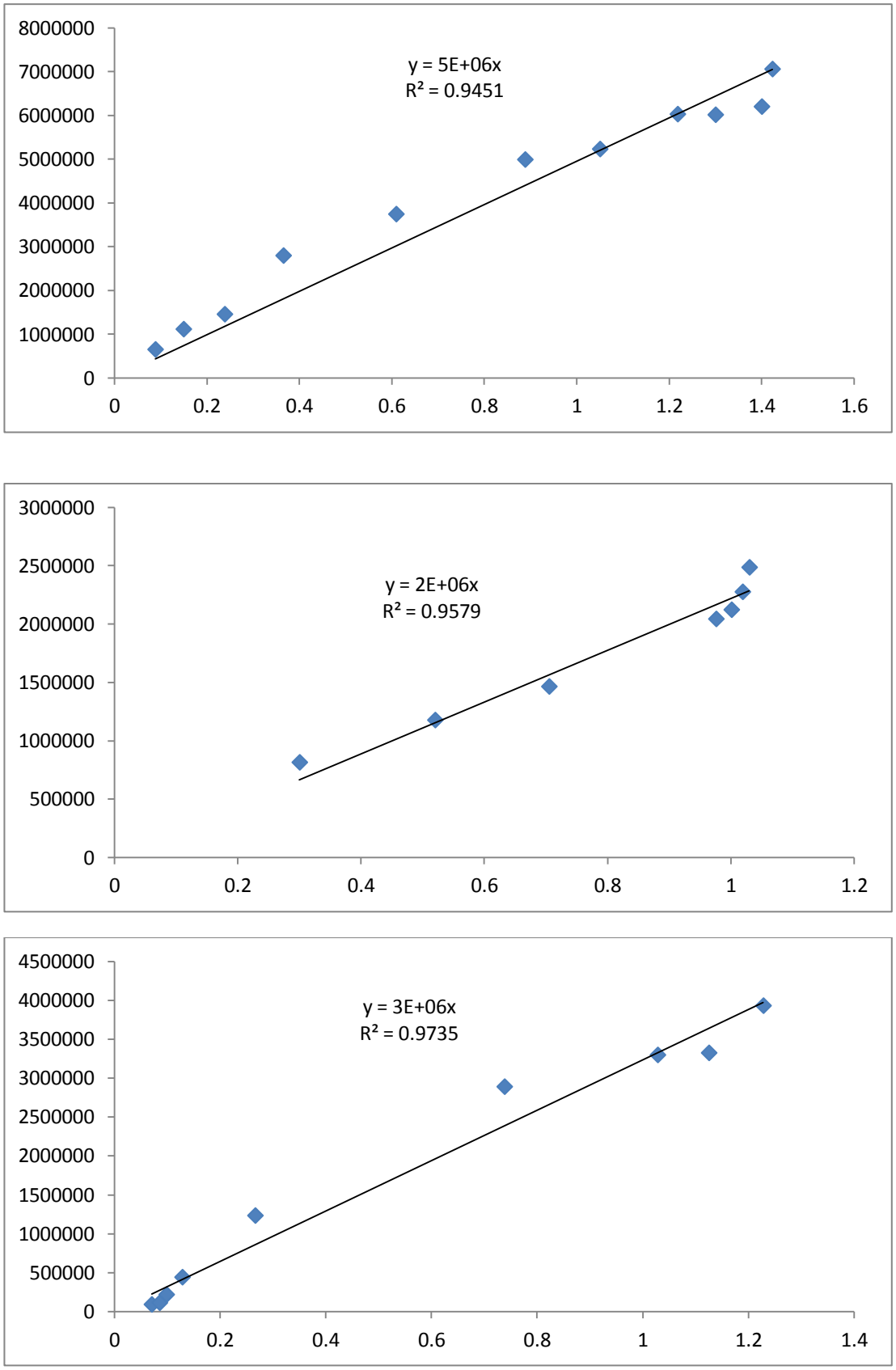
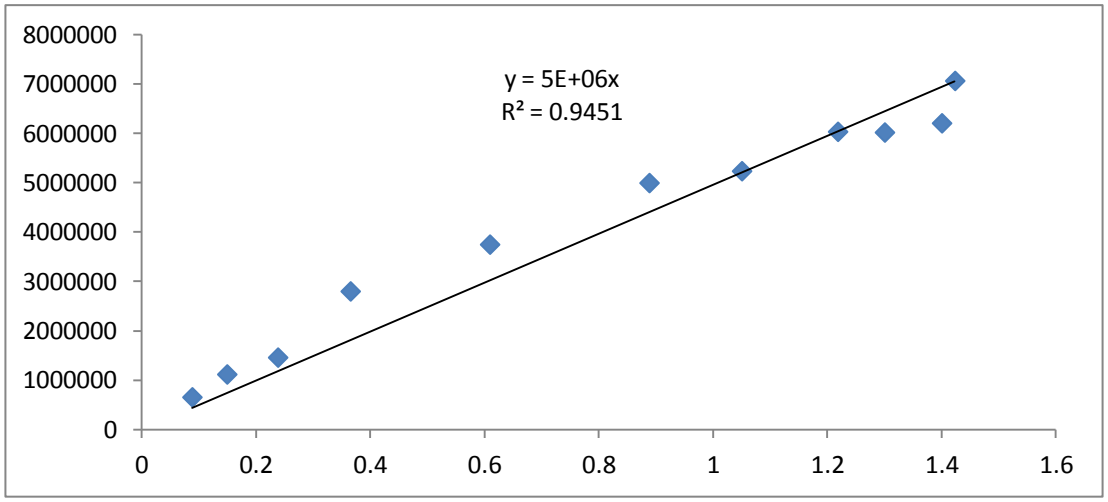
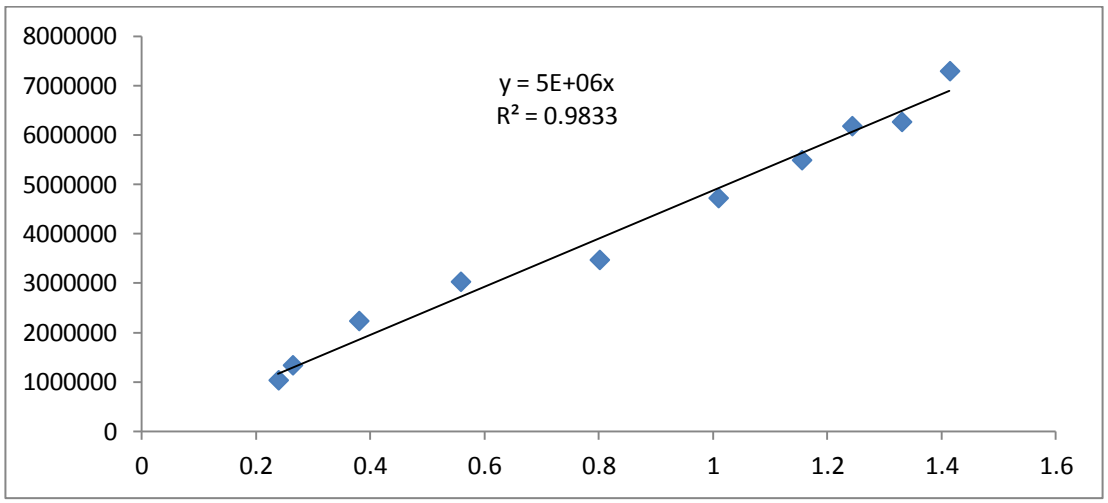


Figure 8.4: The correlation of cell counts and OD595 of Navicula strain in BBM medium in defferent pH (7, 9 and 11), respectively.





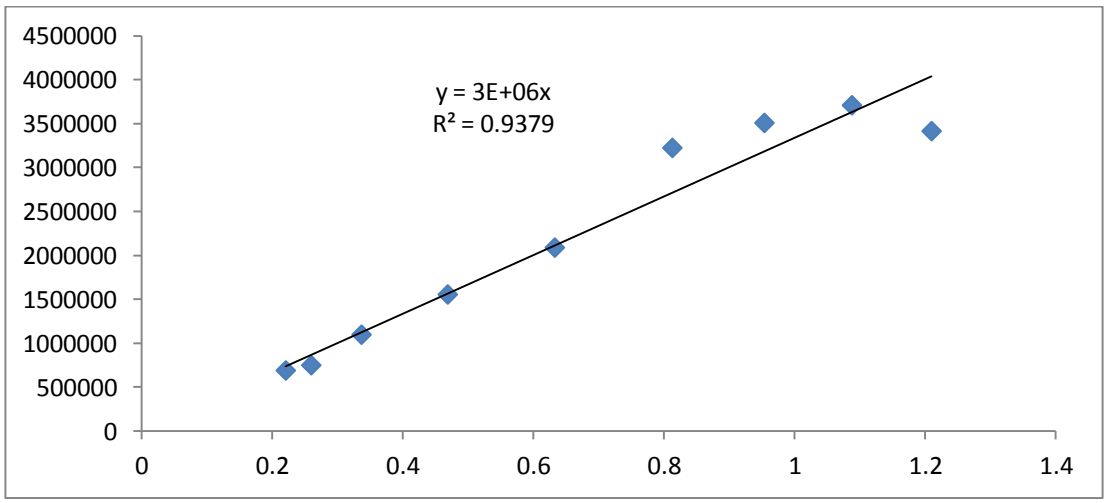
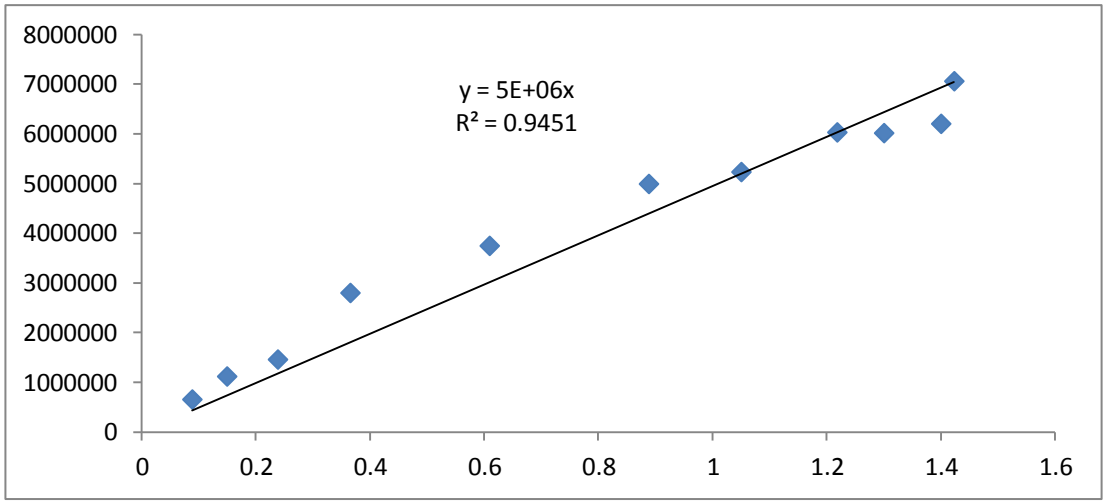
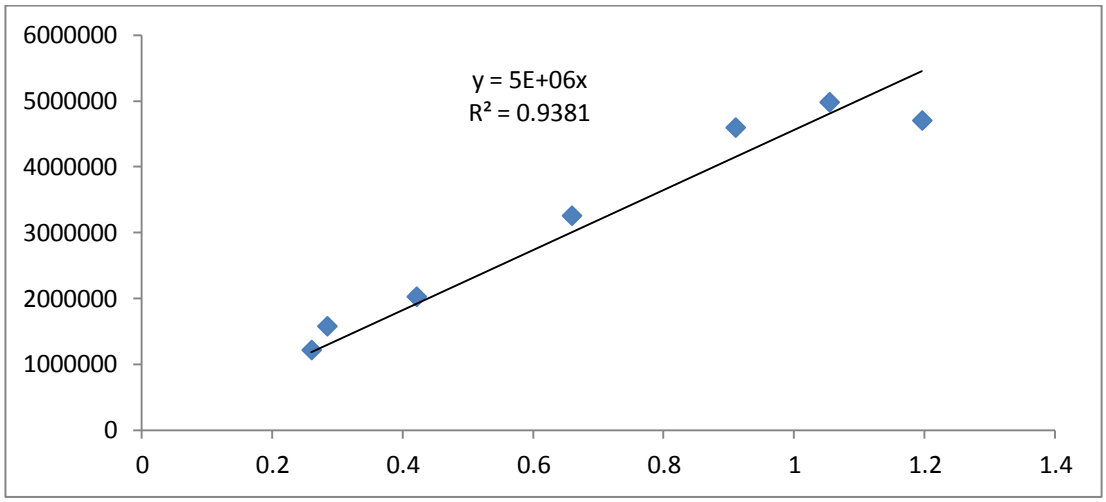


Figure 8.5: The correlation of cell counts and OD595 of Navicula strain in BBM medium with defferent NaCl concentration (0.4, 0.6 and 0.8), respectively.





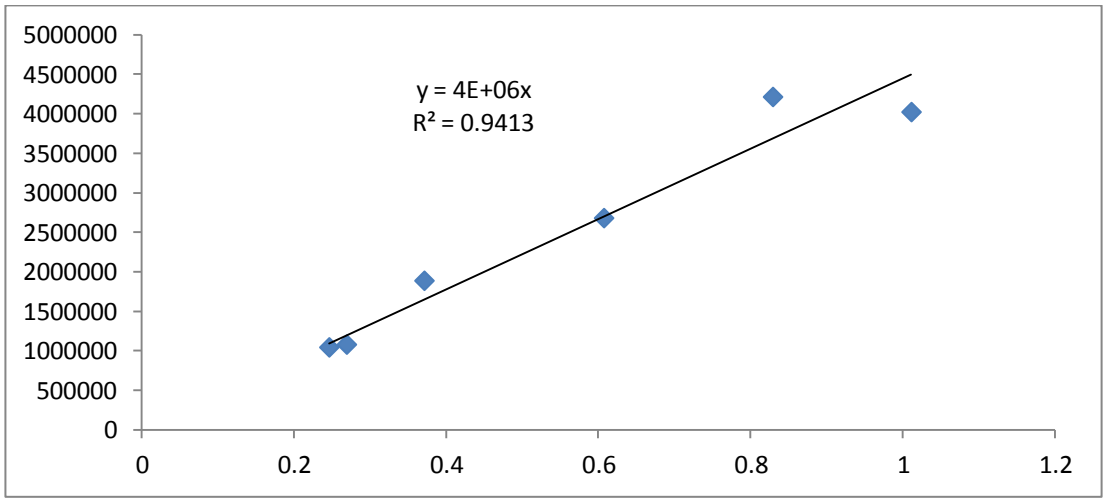


Figure 8.6: The correlation of cell counts and OD595 of Navicula strain in f/2 medium with defferent silica concentration (25%, 50% and 100%) respectively.