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Molecular Identification and Characterisation of Acid Tolerant Microorganisms Isolated from Rivelin and Limb Valleys

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

Dedication

To my dear parents, my loving wife 'Sarah' and my sweet
daughters 'Layan, Layali and Lora'
To my brothers and sisters

<u>Acknowledgements</u>

Abstract

The Rivelin and Limb Valleys in Sheffield have a long history of Industrial activity and were chosen as acidic environments. The first aim was to analyse the microbial diversity present in the collected water samples from both sites using batch cultures. Two bacterial strains (*Bacillus cereus* and *Micrococcus luteus*) and two yeast strains (*Aureobasidium pullulans* and *Debaryomyces hansenii*) were successfully isolated and identified using 16S and 18S rDNA molecular identification techniques. Physiological characterisations were carried out on all four strains to examine their response to different pH values and high salinity. On the basis of these results, *D. hansenii* and *M. luteus* were chosen for further study based on their growth at high salinity at pH 3. Further physiological studies showed that *D. hansenii* was well adapted to grow at different, extreme conditions in M9 minimal and rich YPD media, while *M. luteus* required rich LB medium to successfully adapt to combined acid and salt stress. NMR spectroscopy showed when subject to high salinities, *M. luteus* accumulated betaine as the main compatible solute while *D. hansenii* accumulated glycerol. Most importantly, glycine betaine was identified as an additional compatible solute in *D. hansenii*. This is the first report of glycine betaine acting as a compatible solute in a yeast cell. *D. hansenii* was shown to maintain an internal pH of 6.7 when grown in pH 3 medium and unlike most acid tolerant microorganisms, their membrane potential remained negative when grown at pH 3. pH shock experiments (varying external pH between 3 and 7) suggested that it takes longer than 30 minutes for the *D. hansenii* cells to return their internal pH to pre-stress levels.

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__Abbreviations__

bp	Base pair (s)
BSA	Bovine serum albumin
^{o}C	Centigrade
CFE	Cell free extract
Caps	3-(cyclohexylamino)-1-propanesulfonic acid
dH$_2$O	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

CHAPTER ONE

1 **<u>INTRODUCTION AND AIMS</u>**

1.1 **Microbial Life**

When a biosphere existed and long before plants and animals evolved on Earth, the planet teemed with microbial life. Microorganisms continue to thrive, evolve and make all other life on Earth possible (Staley, 2002). Generally, microbial cells thrive in populations which are in association with other cells, but nevertheless, a number of biological, physical and chemical factors control the continued existence of a microorganism. In fact, the physical environmental factors sometimes deter adaptation and define an absolute limit beyond which no life can exist (Lin and Reysenbach, 2003).

The evidence indicates that there may be complex interactions between microbes growing in the environment. For example, when a particular organism starts metabolising a particular compound, it may reduce or remove inhibiting material and thus allow growth of other organisms. Kimura *et al.* (2006) have reported that, in the presence of acetic acid, sulphate reducing bacteria cannot grow. These bacteria require aerobic organisms to remove the acetic acid, a toxic by-product of their anaerobic sulphate reduction.

In 1998 the known organisms consisted of 1.5 million animal species, 0.3 million plant species (Cases and De Lorenzo, 2002) and half a million insects (Pace, 1997), compared to only 4500 prokaryotes (Torsvik *et al.,* 2002). This is significantly less than 1% of the total number of species on Earth, which is an astonishingly small percent when one considers that prokaryotes have been estimated to contain between 60-100 % of the total carbon contained in plants (Whitman *et al.,* 1998). This strongly suggests that there are a great

number of microorganism species over the three domains of life (Figure 1-1) still to be discovered and characterised (Pace, 1997). Therefore, recent research has included intensive studies of microbial life in unusual environments such as a stratosphere, space, and Mars in order to isolate novel species.

1.2   Extreme Environments and Extremophiles

Life can be found on Earth in a wide range of environments, some of which are normal from a human viewpoint and others are extreme in human terms (DasSarma, 2006). In this sense, normal environments are environments with a temperature between 20 and 40°C, pH near neutral, with sufficient levels of accessible water, essential nutrients, air pressure at 1 atmosphere and relatively low salt concentration. Therefore, any environmental condition that varies significantly from the normal condition can be considered as an extreme condition (Satyanarayana *et al.*, 2005).

There are various types of extreme environments such as extreme (acidity i.e. environments that have pH values lower than 4) or high alkalinity environments that have pH values above 9, high temperature between 55 to 121°C (volcanoes and hot springs) or extreme cold environments between -10 to 0°C (polar ice), and high-salt environments containing 2 – 5 M NaCl (saline lakes) (Hough and Danson, 1999, van den Burg, 2003, Gomes and Steiner, 2004). There are also high pressure environments that have hydrostatic pressures up to 1400 atmospheres (Satyanarayana *et al.,* 2005). Additionally, there are manmade, extreme conditions including cool houses, steam heated buildings and acid mine waters (Satyanarayana *et al.,* 2005).

Life in extreme environments has been studied intensively, focusing attention on the diversity of organisms and the molecular and regulatory mechanisms involved. Extremophiles are structurally adapted at the molecular level to resist these extreme conditions (Gomes and Steiner, 2004). 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 (Figure 1-1) (Albers *et al.,* 2001, Konings *et al.,* 2002). Unlike many microorganisms that cannot grow and survive under extreme conditions, extreme microorganisms can develop and grow optimally when one or several stress conditions are in the extreme range (Edwards, 1990, Horikoshi and Grant, 1991, Albers *et al.,* 2001).

Furthermore, some extremophiles are polyextremophiles that are able to withstand multiple extremes (i.e. adapted to more than one type of extreme environment). Examples are the acidothermophiles *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius* that have been grown at high acidity and 80°C (Gomes and Steiner, 2004, Irwin and Baird, 2004). Thermophilic alkalitolerant bacteria have also been isolated, such as *Anaerobranca spp.* (Engle *et al.,* 1995).

Additionally, the extremozymes (biocatalysts) produced by extreme microorganisms are proteins that can work under extreme conditions via their extreme stability, they suggest new opportunities for biotransformations and biocatalysis (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 (Russell, 2000, Goto *et al.,* 2005).

Extremophilic microorganisms are classified according to the environmental conditions required for optimum growth e.g. acidophiles (pH < 4), alkaliphiles (pH > 9), halophiles (NaCl > 2M), thermophiles (temperature > 55°C), psychrophiles (temperature < 10°C) and osmophiles (water activity less than 0.8) (Edwards, 1990, Gilmour, 1990, Jennings, 1990, Horikoshi and Grant, 1991, ve Habitatlar, 2002, Gomes and Steiner, 2004). Moreover, it is fascinating 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).

On interesting line of recent work has focused on isolating extremophilic microorganisms from non extreme environments to demonstrate their ubiquitous presence in many so-called normal habitats (Staley and Gosink, 1999, Ma *et al.,* 2010)
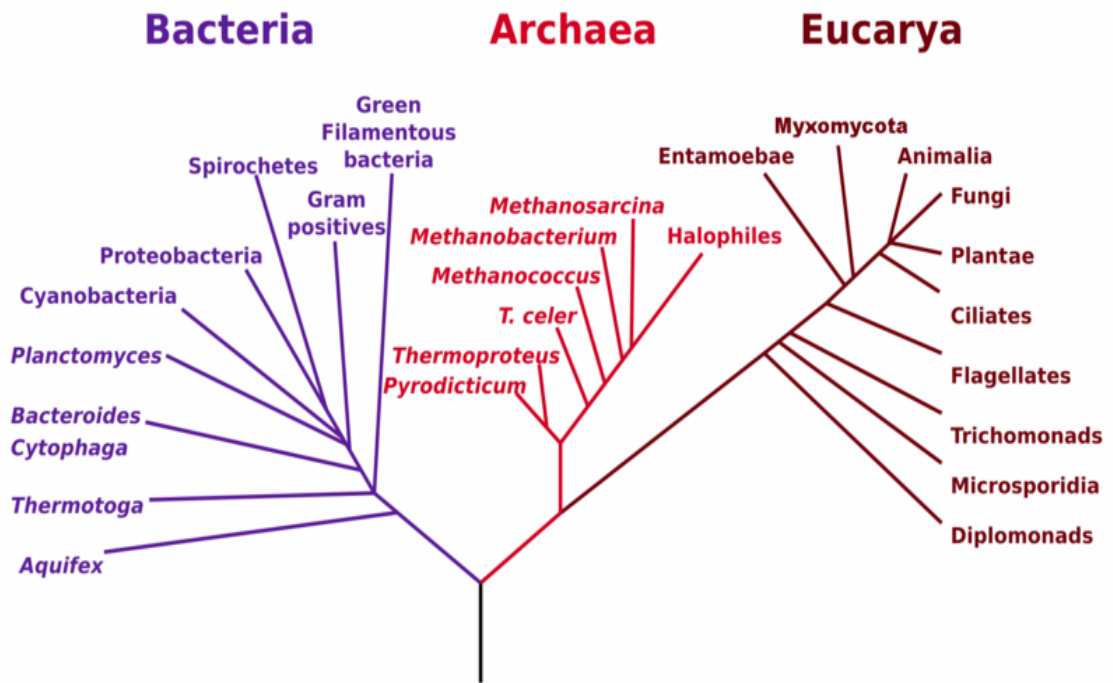
Figure 1-1: The universal phylogenetic tree, constructed from rRNA sequence comparisons. Branches representing the three domains (Archaea, Bacteria, Eukarya) are indicated (Woese *et al.*, 1990).

Table 1-1: Characteristics of different groups of extremophiles and their biocatalysts (extremozymes), which could be applicable in industrial processes, *Genus of the domain Bacteria; all others are Archaea. Modified from, Hough and Danson (1999) and van den Burg,(2003).

Phenotype	Environment	Typical genera (Bacteria and Archaea)	Extremozymes
Thermophilic	55 – 80°C	*Methanobacterium*, *Thermoplasma*, *Thermus**, some *Bacillus** species	Amylases, Pullulanase,Glucoamylases, Glucosidases, Cellulases, Xylanases, Chitinases, Lipases, and Esterases
Hyper-thermophilic	80 –113°C	*Aquifex**, *Archaeoglobus*, *Hydrogenobacter**, *Methanothermus*, *Pyrococcus Pyrodictium*, *Pyrolobus*, *Sulfolobus*, *Thermococcus*, *Thermoproteus*, *Thermotoga*	Proteases
Psychrophilic	– 2 to 10°C	*Alteromonas**, *Psychrobacter*	DNA polymerases, Dehydrogenases, Proteases, Amylases, Cellulases, Dehydrogenases and Lipases

Table 1-1: *(Continued)*

Phenotype	Environment	Typical genera (Bacteria and Archaea)	Extremozymes
Halophilic	2–5 M NaCl	*Haloarcula, Halobacterium, Haloferax, Halorubrum*	Proteases, Dehydrogenases
Acidophilic	pH<4	*Acidianus, Desulfurolobus, Sulfolobus, Thiobacillus**	Amylases, Glucoamylases Proteases and Cellulases
Alkaliphilic	pH>9	*Natronobacterium, Natronococcus,* some *Bacillus* species	Proteases and Cellulases

1.3 Acidic Environments

Acidic environments are environments which have a pH value less than 5. Extremely low pH value environments are not very abundant, and are often due to large amounts of pyrite and sulphur being exposed to oxygen causing several chemical processes to lower pH values (Figure 1-2) (Rainey and Oren, 2006). Other less typical environments, such as those contaminated by heavy metals or other pollutants, are also extreme. Prokaryotes (both bacteria and archaea) are the dominant microorganisms in most extreme environments, but some extremophilic eukaryotes are known.

Acidic environments capable of sustaining life are usually dominated by sulphate anions, and there is usually only a low concentration of dissolved organic substances, with as little as 20 mg l^{-1} dissolved carbon in some environments (Johnson, 1998). Extremely acidic environments may be formed by processes that are entirely natural. However, anthropogenic influences (both direct and indirect) have become increasingly important in creating such environments, particularly since the onset of the industrial revolution. Indeed, the majority of extremely acidic sites now in existence worldwide have their origin in one particular human activity, the mining of metals and coal.

Figure 1-2: Acidic (sulfur-enriched geothermal) environment in Yellowstone National Park. Taken from Rawlings and Johnson (2002).

A variety of microbial activities create net acidity. These include nitrification, and the formation and accumulation of organic acids either during fermentation or as products of aerobic metabolism. Most relevant, however, to the genesis of extremely acidic environments is the microbial dissimilatory oxidation of elemental sulfur, reduced sulfur compounds (RSCs), and ferrous iron.

Elemental sulphur is found in geothermal areas (e.g. around the margins of fumaroles) where it forms by the compression of sulfur dioxide and hydrogen sulfide:

$$SO_2 + 2H_2S \longrightarrow 2H_2O + 3S^0$$
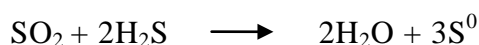
Oxidation of sulphur by autotrophic and heterotrophic microorganisms produces sulfuric acid.

$$S^0 + H_2O + 1.5O_2 \longrightarrow H_2SO_4$$
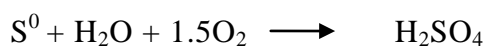
Which, if not neutralised by carbonates or other fundamental minerals present, can result in a dramatic lowering of pH within microsites or on the macro scale. Of larger environmental significance, however, is the generation of acidity which results from the microbial oxidation of sulphide minerals. Many metals occur as sulphides (Johnson, 1995); indeed, sulphides are the major mineralogical form of many commercially important metals, such as copper, lead and zinc. Iron sulphides (most notably pyrite) are the most abundant sulphide minerals. In the past, pyrite has been mined (for its sulphur, rather than for its iron content) but this is no longer commercially viable. However, iron sulphides are often associated with other metal sulfides in ore deposits, and as such are accidentally processed during the mining

process, ending up as waste materials (in mineral tailings etc.). Pyrite and other iron sulphides are also present in coal deposits (range: < 1 to > 20%) and, inevitably, in coal spoils. The mechanisms involved in the oxidation of pyrite have been subject to significant amounts of discussion (e.g. Sand *et al.,* 1995, Evangelou, 1995). Current agreement is that ferric iron acts as the major oxidant of the mineral, as:

$$FeS_2 + 6Fe(H_2O)_6 + 3H_2O \longrightarrow Fe^{2+} + S_2O_2^{3-} + 6Fe(H_2O)_6^{2+} + 6H^+$$

The amount of thiosulphate formed depends on environmental pH; in circum-neutral environments this reduced sulfur compound (RSC) is chemically stable, but in acidic liquors it hydrolyses to form a variety of polysulphides, as well as elemental sulphur and sulphate (Sand *et al.,* 1995). Ferrous iron and RSCs are potential energy sources for some acidophilic chemolithotrophic prokaryotes (described below). The regeneration of the ferric iron oxidant may be brought about biologically or abiotically; however, oxygen is required in both cases, so that the continued oxidation of pyrite requires the provision of both air and water. This requirement is met when coal spoils and mineral wastes are stored on the land surface, and when water accumulates in exposed deep mine shafts following the cessation of active mining (Johnson, 1998).

Concentration of soluble metals is another important physico-chemical feature of extremely acidic environments and their concentrations tend to be much larger than in neighbouring areas of higher pH. The solubilities of metal oxyanions (such as molybdate) tend to be lower in acidic than in neutral solutions, those of cationic metals (such as aluminium and many heavy metals) are generally much larger. Heavy

metals types and concentrations which present in any specific extremely acidic environment are much dictated by the local geochemistry; metals may originate directly from the oxidation of sulphide minerals (various chalcophilic metals) or from the accelerated mineral weathering which occurs under conditions of high acidity (e.g. aluminium from the weathering of clay minerals). Also high concentrations of soluble metalloid elements may occur in extremely acidic environments, of which the most important (from the point of view of ecotoxicology) is arsenic, which occurs in several sulphide minerals such as arsenopyrite (FeAsS) and realgar (AsS) (Johnson, 1998).

1.4   Acidophiles and Diversity

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

Therefore, most acidophilic microorganisms have been isolated from extreme environments that present an inaccessible physical barrier which reduces the colonization potential of other microorganisms that grow at or around neutral pH (neutralophilic). These extreme environments include both man-made and natural acidic environments that occur in the biosphere such as acid mine drainage and geothermal vents (Futterer *et al.,* 2004, Johnson and Hallberg, 2003). True

acidophiles such as the archaea *Picrophilus torridus* and *P. oshimae* have been grown optimally at pH 0.7 and at 60°C (Gomes and Steiner, 2004). The biodiversity of extreme acidophiles has recently attracted considerable attention with regard to their physiology and phylogenetic affiliations (Table 1-2). The ability to use inorganic chemicals as electron donors is common among extreme acidophiles, as ferrous iron and sulfur represent two major energy sources in many extremely acidic environments (Johnson and Hallberg, 2008). Reduction of iron and sulfur by acidophilic microorganisms is driven by their use as electron acceptors under oxygen limited conditions and in turn these reactions drive major biogeochemical processes in low pH environments. Acidophiles show considerable diversity in how they assimilate carbon, some are obligate autotrophs and others are obligate heterotrophs, whereas a great number use either organic or inorganic carbon, depending on the availability of the former (Johnson and Hallberg, 2008).

However, acidic effluents associated with acid mine drainage can cause huge environmental pollution such as the contamination of drinking water. Therefore, the reduction of this pollution is one of the important biotechnological applications of acidophiles, in addition to their role in metal extraction from ores (Rohwerder *et al.,* 2003, Golyshina and Timmis, 2005). Furthermore, acidophilic microorganisms can be used as a source of acid-stable enzymes with applications as lubricants and catalysts (van den Burg, 2003). These enzymes included, pullulanases, amylases glucosidases and glucoamylases (Gomes and Steiner, 2004).

According to their carbon source and method of energy generation and further subdivided based on their growth optimum temperature acidophiles are most commonly grouped as shown in (Table 1-2).

Most extremely acidic environments contain relatively low concentrations (< 20 mg l^{-1}) of dissolved organic carbon, and may therefore be classed as oligotrophic. Primary production in sites which do not receive sunlight (e.g. abandoned deep mines) is based exclusively on chemolithoautotrophy, and is inexorably linked to the oxidation of ferrous iron and reduced sulphur compounds. The main focus of research in this area of microbiology is chemolithotrophic acidophiles, and much is known of the detailed physiology and biochemistry of some of these prokaryotes, most notably the iron/ sulphur-oxidising bacterium *Acidothiobacillus ferrooxidans* (Leduc and Ferroni, 1994). Most iron- and sulfur-oxidising acidophiles are regarded as autotrophic, though the ability to assimilate organic carbon has been demonstrated with some of these (e.g. utilisation of formic acid by *At. ferrooxidans* (Pronk *et al.,* 1991)). Either mixotrophic (i.e. may assimilate organic and inorganic carbon) or obligately heterotrophic are other prokaryotes which catalyse the dissimilatory oxidation of iron and/or RSCs.

In those extremely acidic environments that are illuminated, primary production may also be mediated by phototrophic acidophiles. The majority of these are eukaryotic microalgae, and include filamentous and unicellular forms, and diatoms (Gyure *et al.,* 1987, Lopez-Archilla *et al.,* 1995).

Table 1-2: Acidophilic prokaryotic microorganisms, adapted from *Johnson et al.* (2003).

Mineral-degrading acidophiles	Thermal classification*	Phylogenetic affiliation
1a. Iron-oxidizers		
Leptospirillum ferrooxidans	Meso	*Nitrospira*
L. ferriphilum	Meso	*Nitrospira*
L. thermoferrooxidans	Mod Thermo	*Nitrospira*
"*Thiobacillus ferrooxidans*" m-1	Meso	β-*Proteobacteria*
"*Ferrimicrobium acidiphilum*"	Meso	*Actinobacteria*
Ferroplasma acidiphilum	Meso	*Thermoplasmals*
"*Fp. acidarmanus*"	Meso	*Thermoplasmales*
1b. Sulfur-oxidizers		
Acidithiobacillus thiooxidans	Meso	β/γ-*Proteobacteria*
At. caldus	Mod Thermo	β/γ-*Proteobacteria*
Thiomonas cuprina	Meso	β-*Proteobacteria*
Hydrogenobacter acidophilus	Mod Thermo	*Aquifacales***
Metallosphaera spp.	Ext Thermo	*Sulfolobales*
Sulfolobus spp.	Ext Thermo	*Sulfolobales*
1c. Iron- and sulfur-oxidizers		
Acidithiobacillus ferrooxidans	Meso	β/γ-*Proteobacteria*
Acidianus spp.	Ext Thermo	*Sulfolobales*
Sulfolobus metallicus	Ext Thermo	*Sulfolobales*
1d. Iron-reducers		
Acidiphilium spp.	Meso	α-*Proteobacteria*
1e. Iron-oxidizers/reducers		
Acidimicrobium Ferrooxidans	Meso	*Actinobacteria*
1f. Iron-oxidizers/reducers and sulfur-oxidizers		
Sulfobacillus spp.	Meso and Mod Thermo	*Firmicutes*
2. Heterotrophic acidophiles (non mineral-degrading)		
Acidocella spp.	Meso	α-*Proteobacteria*
Acidisphaera rubrifaciens	Meso	α-*Proteobacteria*
Acidobacterium capsulatum	Meso	*Acidobacterium*
Acidomonas methanolica	Meso	α-*Proteobacteria*
Alicyclobacillus spp.	Meso	*Firmicutes*
Picrophilus spp.	Mod Thermo	*Thermoplasmales*
Thermoplasma spp.	Mod Thermo	*Thermoplasmales*
3. Obligate anaerobes		
Stygiolobus azoricus	Ext Thermo	*Sulfolobales*
Acidilobus aceticus	Ext Thermo	*Sulfolobales*

* Meso—mesophiles (Toptimum < 40 ◦C); Mod Thermo—moderate thermophiles (Toptimum 40–60 ◦C); Ext Thermo—extreme thermophiles (Toptimum > 60 ◦C).
** Inferred ability to oxidize minerals (via production of sulfuric acid).

Mesophilic acidophilic phototrophs include *Euglena spp.*, *Chlorella spp.*, *Chlamydomonas acidophila*, *Ulothrix zonata* and *Klebsormidium fuitans*. The unicellular rhodophyte *Galdieria sulphuraria* (formerly *Cyanidium caldarium*) has been isolated from geothermal acidic springs and streams in Yellowstone National Park and elsewhere (Brock, 1978). This moderate thermophile may grow as a heterotroph in the absence of light (as may *Euglena spp.*) and has been reported to grow at pH values around zero (Schleper *et al.,* 1995).

Heterotrophic microorganisms may easily be isolated from most extremely acidic environments. Many are adept scavengers and rely to a greater or lesser extent on carbon originating as leakage or lysis products from chemolithotrophic acidophiles. Obligately acidophilic heterotrophs include archaea, bacteria, fungi, yeasts and protozoa. Some prokaryotic acidophilic heterotrophs have a direct role in the dissimilatory oxido-reduction of iron (Pronk and Johnson, 1992). These include the iron-oxidiser `*Ferromicrobium acidophilus'* (Johnson, 1998) which appears to use the energy from iron-oxidation to support growth, and various *Acidiphilium*-like isolates which can use ferric iron as terminal electron acceptor. Many acidophilic archaea (Table 1-2) are obligate heterotrophs, including *Sulfolobus acidocaldarius*; early reports of this archaeon being a facultative chemolithotroph are now thought to be due to the inadvertent use of mixed cultures of *Sf. acidocaldarius* and another extreme thermophile (possibly *Sulfolobus metallicus* (Johnson, 1998)). The two characterized species of the moderately thermophilic heterotrophic archaeon *Picrophilus* have the lowest recorded pH optima for growth (ca. pH -0.7) of all known acidophilic microorganisms (Schleper *et al.,* 1995).

A number of yeasts have also been reported to inhabit extremely acidic environments. *Rhodotorula spp.* are frequently encountered (and readily isolated) in acid mine drainage waters, and isolates belonging to other genera (e.g. *Candida, Cryptococcus*) have also been described (Lopez-Archilla *et al.,* 1995). Among the filamentous fungi which have been isolated from acidic sites are some of the most acidophilic of all microorganisms; *Acontium cylatium*, *Trichosporon cerebriae* and a *Cephalosporium sp.* have all been reported to grow at ca. pH 0 (Schleper *et al.,* 1995). Protozoa are frequently encountered in acidic mineral leaching and related environments. A laboratory study of three flagellates (*Eutreptia/Bodo spp.*), a ciliate (*Cinetochilium sp.*) and an amoeba (*Vahlkampvia sp.*) showed that all were obligately acidophilic (growing in media poised at pH 1.6 and above) and that they grazed mineral-oxidising (and other) acidophilic bacteria (Johnson and Rang, 1993).

The basis of acidophilic microorganisms response to different temperatures is one of the more convenient ways of subdividing them (e.g. (Norris and Johnson, 1998)). Three groups have been recognised: mesophiles (T_{opt} ca. 20-40°C), moderate thermophiles (T_{opt} ca. 40-60°C) and extreme thermophiles (T_{opt} >60°C; Table 1-2). Moderately thermophilic acidophilic prokaryotes include archaea and bacteria (the majority of which are Gram-positive), while the extreme thermophiles group is made up exclusively of archaea. In contrast, mesophilic acidophiles (autotrophs and heterotrophs) are dominantly by rod-shaped, Gram-negative bacteria. Exceptions to this general tendency include `*F. acidophilus'* which, on the basis of 16S rDNA base sequence analysis, is located within the *Actinobacteria* (Johnson, 1998), and *Sulfobacillus disulfidooxidans*, a mesophilic spore-forming Gram-positive bacterium

which has been reported to use pyrite and elemental sulphur as sole energy sources or to grow heterotrophically on various organic substrates (Dufresne *et al.,* 1996). However, there is some uncertainty regarding the capacity of *S. disulfidooxidans* to grow chemolithotrophically, and the isolate is, in fact, more closely related to the obligately heterotrophic *Alicyclobacillus spp.* than to the iron/sulfur-oxidising *Sulfobacillus spp*. Relatively few studies have focused on psychrophilic and psychrotolerant acidophiles, even though many extremely acidic, low-temperature sites are known, such as subterranean mine waters in the mid-high latitudes.

Berthelot *et al.* (1994) isolated acidophilic bacteria from water draining a uranium mine in Ontario, and studied their ability to grow at between $4^{o}C$ and $37^{o}C$. Although 96% of the iron-oxidising isolates and 54% of the heterotrophic isolates were classed as psychrotolerant, none was shown to be truly psychrophilic. Water samples were collected in the winter months, when temperatures ranged from 0.5 to $5^{o}C$ and it is conceivable that the higher summer temperatures experienced at the mine may have precluded the establishment of psychrophilic strains.

Langdahl and Ingvorsen in 1997 reported the presence of *Thiobacillus*-like and heterotrophic acidophiles in an exposed sulphide ore deposit located in the High Arctic; the mean air temperature at this site was between ⊢ 15 and ⊢ $20^{o}C$ (range ⊢ 30 to $+10^{o}C$). Although autotrophic and heterotrophic carbon assimilation of microorganisms from the site were both recorded to be optimum at ca. $21^{o}C$, microbial ore dissolution at $0^{o}C$ was noted to be 30% of the maximum recorded (at $21^{o}C$). There is likely to be a potentially important biotechnological function (e.g. in

in situ mining) for mineral-mobilising acidophilic bacteria which are active at very low temperatures (Langdahl and Ingvorsen, 1997).

1.5   Mechanisms of Cytoplasmic pH Regulation in Acidophiles

There are many different mechanisms and complex processes which support the survival of microorganisms in acidic habitats. Intracellular pH homeostasis is one of the most important mechanisms for the survival of any cell in acidic environments, regardless of the external pH. Growth in external pH values as low as -0.7 requires stringent control and regulation of internal pH in order for the cell to continue to function normally. In most cases, acidophiles must maintain a near-neutral intracellular pH, which can be several pH units higher than the pH of the external environment (Matin, 1990).

Proton motive force (Δp) is a key factor in energy generation and pH homeostasis for all microorganisms. The Δp describes the energised state of the cell membrane, and is composed of membrane potential, $\Delta\Psi$, which is the difference in charge separation between the membrane and the external medium and ΔpH, the difference in pH between the internal (cytoplasm) and external environment ($\Delta pH = pH_{in} - pH_{out}$). Δp is calculated as follows:

$$\Delta p \text{ (mV)} = \Delta\Psi - 60\ \Delta pH \text{ (at } 25^{\circ}C).$$

In most neutralophiles, Δp is approximately -200 mV (inside negative), which results from a slightly negative ΔpH, in the approximate range -0.1 to -1.5 for most microorganisms, and a $\Delta\Psi$ around 100 mV. However, acidophiles have a much greater ΔpH. This could be seen as advantageous as it allows the formation of a larger Δp and therefore for a greater proton influx though F_0F_1-ATPases and consequently

20

increased ATP generation. However, uncontrolled influx of protons into a cell can be extremely harmful, potentially leading to cell death. An increased proton concentration within the cell inhibits protein function and stability of nucleic acids, impedes enzymatic activity, protein synthesis and transcription of DNA, ultimately resulting in the complete dissipation of ΔpH which would result in a much decreased Δp incapable of meeting the cells energy requirements (Baker-Austin and Dopson, 2007).

Acidophiles can minimise or prevent proton influx into their cells by several known mechanisms, as well as other mechanisms which allow the cell to tolerate and survive a small influx of protons into the cell. Mechanisms which inhibit or prevent proton influx into the cell include reversing the membrane potential ($\Delta\Psi$), possessing a highly impermeable cell membrane and proton pumping. Meanwhile, cytoplasmic buffering, along with the presence of chemicals and enzymes which sequester protons within the cell and an increased number of secondary transporters help the cell to cope with proton influx (Matin, 1990).

A reversal of membrane potential is one of the most commonly observed adaptations in acidophiles. All neutralophilic microorganisms have an inside negative $\Delta\Psi$ whereas most acidophiles possess an inside positive $\Delta\Psi$, a reversal of the normal membrane potential (Figure 1-3). A reversed membrane potential is a mechanism for reducing proton influx into the cell as it forms a chemiosmotic barrier which deflects positively charged protons, preventing them from entering the cell.
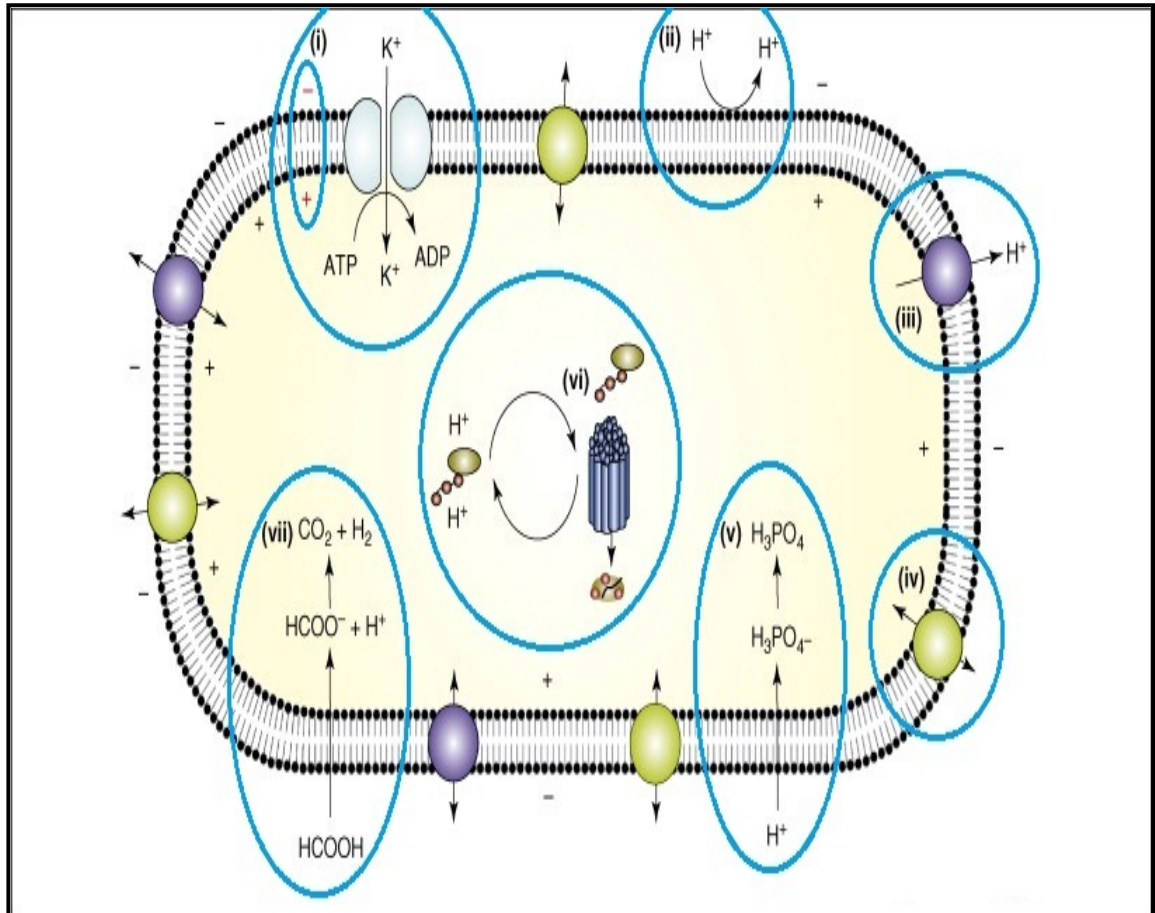
Figure 1-3: Diagrammatic representation of commonly occurring adaptations to low pH observed in acidophilic microorganisms. The blue circles highlight several adaptations including (From top left, clockwise) reversed membrane potential, increased K+ ion accumulation, impermeable cytoplasmic membrane, proton pumping, increased number of secondary transporters, cytoplasmic buffering, DNA and protein repair mechanisms and weak acid degradation pathways. Diagram adapted from Baker-Austin and Dopson (2007).

It is thought that the reversed $\Delta\Psi$ is generated by a Donnan potential (Donnan, 1924) of positively charged molecules, particularly accumulation of K^+ ions. The creation of a reversed $\Delta\Psi$ by an accumulation of K^+ ions is supported by evidence that there is an increased number of potassium-transporting ATPases found in acidophile genomes (Futterer *et al.,* 2004, Tyson *et al.,* 2004). Reversal of $\Delta\Psi$ results in a decrease in the overall proton motive force; however the reversed $\Delta\Psi$ detracts from such a large ΔpH that the final Δp is large enough to meet the cells energy demands.

As a result of the reversed membrane potential further difficulties are encountered. When the cell is in acidic media, the positive inside membrane potential results in the exclusion of permeant cations and the accumulation of permeant anions, which can begin to poison the cell. Whilst this is true there is one exception, SO_4^{2-} , the most common anion in acidic environments. Acidophiles are able to tolerate higher levels of SO_4^{2-} than other anions for two reasons. The first reason is that acidophiles have adapted systems to remove SO_4^{2-} from inside the cell, whilst the second is that SO_4^{2-} is less membrane permeable than other anions since it has additional polarity and carries a double charge (Johnson, 2006).

Research into the cytoplasmic membranes of some acidophiles has discovered a cytoplasmic membrane which is highly resistant to proton entry, particularly in archaeal species which synthesise tetraether lipids (van de Vossenberg *et al.,* 1998) (Figure 1-3). A delicate balance of membrane proton permeability is required, since the need to minimise proton influx is offset by the cells need to transport other ions and nutrients into the cell and pump protons out of the cell. This balance will determine if a cell can cope with a particular Δp. *In silico* shotgun sequencing of

several acidophilic bacterial species' genomes from a biofilm revealed a large and varied set of genes for cell membrane biosynthesis (Tyson *et al.,* 2004). These data imply that a complex structure, with an as yet unknown function, may be formed and could be an intrinsic component in acid tolerance.

Certainly the cell cannot exclude all protons from the cytoplasm, therefore active proton pumping may be utilised in order to maintain a near-neutral intracellular pH (Figure 1-3) (Michels and Bakker, 1985). However, there is no definitive evidence which shows induced or increased expression of systems which exclude protons from the cytoplasm as yet. Putative proton efflux systems have been identified in all of the acidophilic genomes sequenced to date (Baker-Austin and Dopson, 2007) including the *Leptospirillum* species in the biofilm present in acid mine drainage at Iron Mountain, California (Tyson *et al.,* 2004). An increased number of secondary transporters have also been noted in the genomes of some acidophiles, which is thought to be a further adaptation to growth in extremely low pH, since cells will utilise the Δp generated for metabolic purposes (Figure 1-3).

The ability of the cytoplasm to buffer the intracellular pH of the cell has been observed and shown to be involved in maintaining a near-neutral intracellular pH in acidic conditions. All cells have the ability to buffer the cytoplasm, either releasing or sequestering protons as appropriate using basic amino acids such as lysine, arginine and histidine. The buffering capacity of respiration inhibited cells of *Thiobacillus acidophilus* and *Acidithiobacillus facile* were measured and compared to the buffering capacity of growth medium containing Tris buffer. The buffering capacity of the microorganisms was shown to be approximately 100 nmol H^+ mg protein^{-1} pH

unit^{-1} (Matin, 1990), showing a much smaller decrease in cytoplasmic acidification than would be expected given the net H$^+$ influx into the cells compared to the buffered medium. Comparison of buffering capacity between *E. coli* and *Acidithiobacilus acidophilum* was carried out, with buffering capacities of 85 nmol H$^+$ mg protein $^{-1}$ and 97 nmol H+ mg protein $^{-1}$, respectively (Zychlinsky and Matin, 1983) (Figure 1-3).

Of particular concern to acidophiles is the uptake of weak acids (HA) as they function as uncouplers of the respiratory chain, which shuttle the net uniport of protons and 'uncouple' proton transport from cellular processes (Ciaramella *et al.,* 2005). These compounds can cross the cell wall in their undissociated form as they are uncharged at acidic pH (the external pH), but once they enter the cell, near-neutral pH, they become undissociated (H$^+$ and A-) and can no longer leave the cell in their charged form. Accumulation of weak acids by this process is problematic for cells, as protonation of the cytoplasm can occur quickly therefore some acidophiles have developed active mechanisms of weak acid degradation in order to overcome this problem (Ciaramella *et al.,* 2005) (Figure 1-3).

Other mechanisms involved in pH homeostasis include the use of DNA and protein repair chaperones and the use of iron to stabilise enzymes and enzyme complexes (Baker-Austin and Dopson, 2007). However, after comparisons of the genomes of several acidophilic microorganisms there appears to be no definitive individual adaptation or set of adaptations present in every acidophile which enables growth in low pH environments.

1.6   Molecular Biology Techniques and Microbial Identification

It has been recorded that less than 0.1% of the total microbial population of a natural habitat can be successfully isolated in pure culture. This is due to the bacteria being highly selective with their growth requirements. For this reason, a variety of media must be employed to obtain diverse microbial populations (Hill *et al.,* 2000). Therefore, every process which avoids the need to cultivate microorganisms before examining their characteristics and diversity will have the potential to become a powerful tool in microbial ecology (Marilley *et al.,* 1998).

Compared to traditional visual and microscopic diagnostic methods, molecular methods have a higher specificity and sensitivity and therefore may be useful to find infections pre-symptoms and identify the target diseases without time-consuming isolation and culturing of pathogens (Martin *et al.,* 2000). Therefore, traditional microbiology methods which have been used to classify organisms depending on phenotypic characters such as morphology, physiology, and metabolism are being supplemented by genotypic analysis (Scow *et al.,* 2001). Amann *et al.* (1997) suggested that fluorescently labeled, rRNA-targeted nucleic acid probes are very useful for many fields of microbial ecology and also, described stages in the molecular methods to identify microorganisms from environmental samples (Figure 1-4).
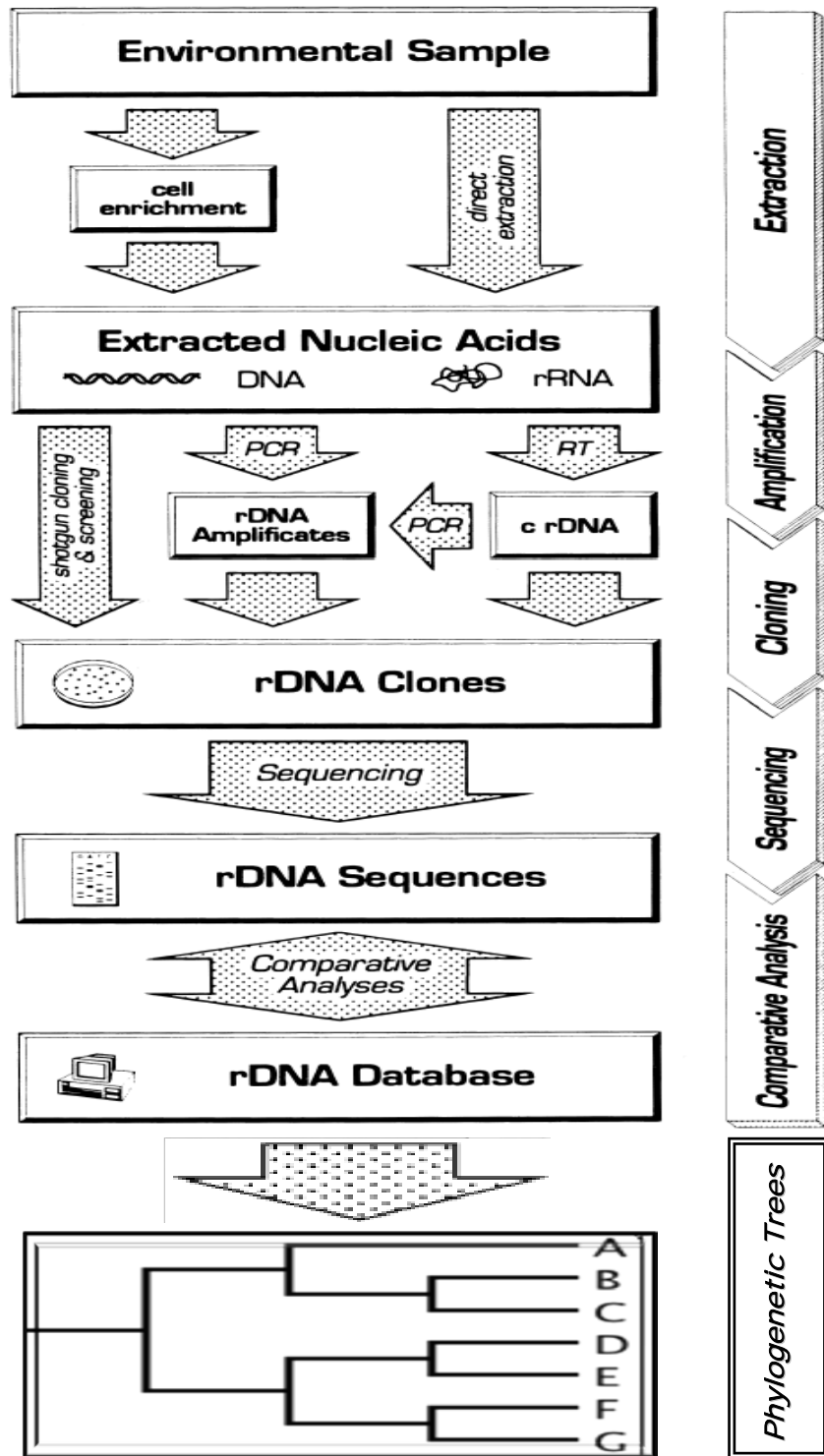
Figure 1-4: Flow diagram of the different stages in the molecular methods used to identify microorganisms from environmental samples, diagram modified (Amann *et al.,* 1997).

Nucleic acid sequence information (16S rRNA gene for prokaryotes or 18S rRNA gene for eukaryotes) is being used to identify and determine the degree of similarity between groups of organisms and relationships of microorganisms and all other life-forms. Therefore, the large genetic sequence database of known species can be used to identify sequences of rDNA from unknown species isolated from environmental samples.

In fact, there are many modern molecular applications involving the extraction and analysis of DNA and RNA, (PCR, gene clone libraries, amplified ribosomal DNA restriction analysis, restriction fragment length polymorphism and fluorescent *in situ* hybridization) now being widely used to identify microorganisms without previous cultivation within their natural habitats, such as in soil, drinking water, oligotrophic lakes and marine environments (Bockelmann *et al.,* 2000). Pernthaler and Amann (2005) showed that 16S rRNA gene is most suitable to define microbial diversity in novel environments. Therefore, extraction of complete or partial sequences of DNA isolated from natural environments or directly from cells concentrated on membrane filters could be amplified by PCR (Figure 1-5) with bacterial primers. PCR products are ligated into vectors and then transferred into *E. coli.* Crump *et al.* (1999) used a molecular approach (PCR-amplified 16S rRNA genes) to study particle-attached and free-living bacteria in the Columbia River and this study showed the large number of particle-attached bacterial clones (almost 75%) linked to members of the Proteobacteria. While, 48% of the free living bacterial clones correlated with cosmopolitan freshwater bacteria (Beta-Proteobacteria, Gram-positive bacteria, and *Verrucomicrobium spp*).
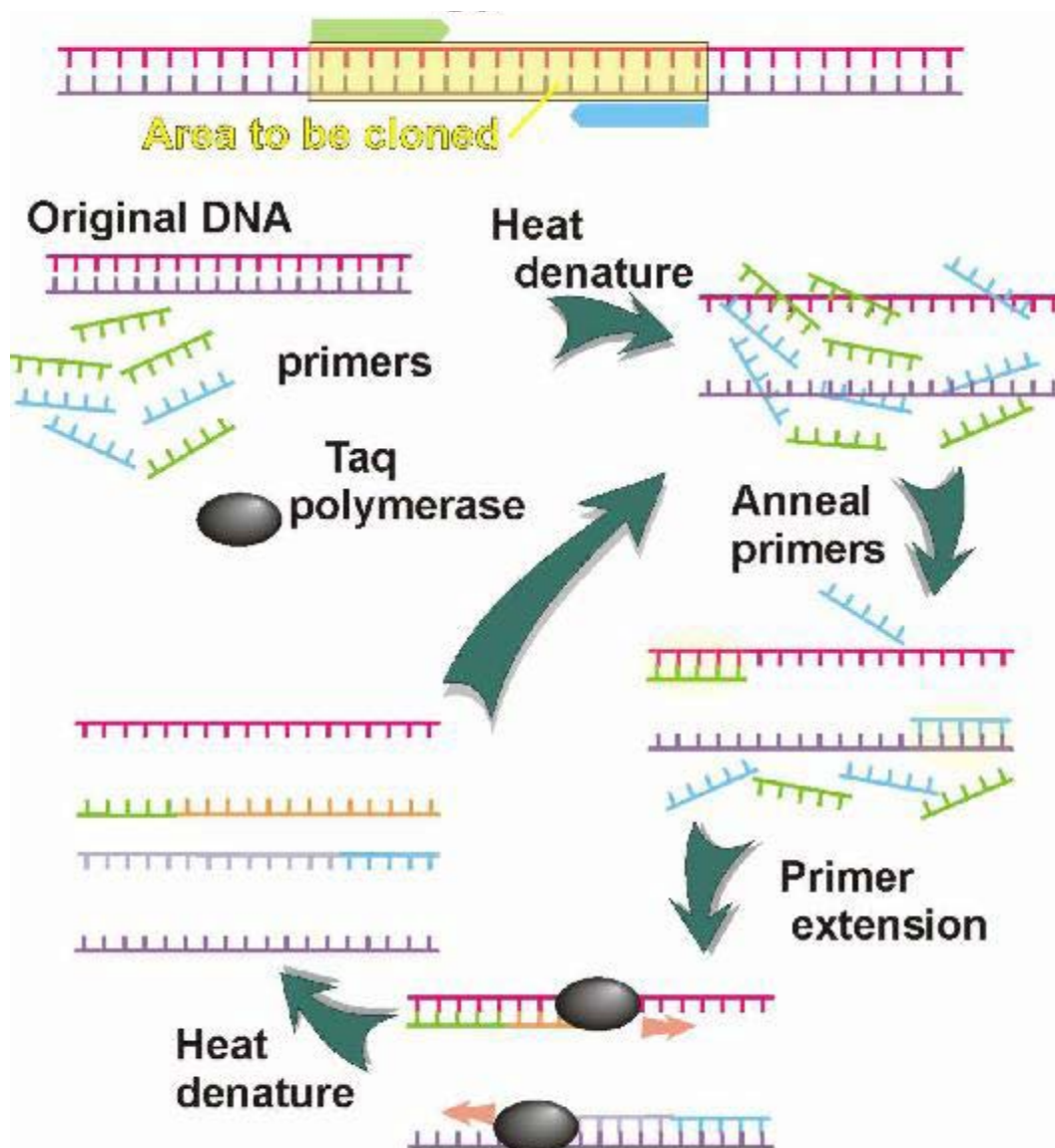
Figure 1-5: Schematic diagram illustrating the processing steps of (PCR), beginning with denaturing the double-stranded DNA into single strands, followed by primers annealing to their respective complementary DNA sequence in the template and finally the target DNA template replicated (extension) by *Taq* polymerase enzyme, these steps recycle for 20 to 35 times to produce approximately 68 billion copies (Vierstraete, 1999).

Diagram taken from http://universe-review.ca/

Johnson *et al.* (2001) isolated acidophilic bacteria from three samples of metal-rich waters with different pH values from a sub-arctic copper mine in Norway. The solid selective medium was used to isolate acidophiles and 16S rRNA sequence was used to identify the isolated microorganisms, the results indicated that *Acidithiobacillus ferrooxidans* was the dominant acidophile in all three waters. Yoon *et al.* (2004) 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, molecular phylogenetic analyses of RNA and PCR amplification of 16S rDNA were used.

Drancourt and Raoult (2005) showed that the sequence of the 16S rRNA gene might not be enough for characterization of a new bacterial species. Some essential phenotypic characters must be applied to describe unknown bacteria, for example Gram-stain, colony morphology, and motility, conditions for optimum growth such as temperature, spore formation and electron microscopic examination. Biochemical tests such as oxidase and catalase tests and the capability to metabolize major carbohydrates could also be helpful for initial description of unknown strains.

The polymerase chain reaction (PCR) (Figure 1-5) 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. The number of cycles of amplification is performed in the same vial so that enough copies of the desired sequence are reproduced for analysis (Figure 1-5).

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, originally 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 regions which often contain a high degree of species specificity. The gene is large enough, with statistically relevant sequence information (Figure 1-6).
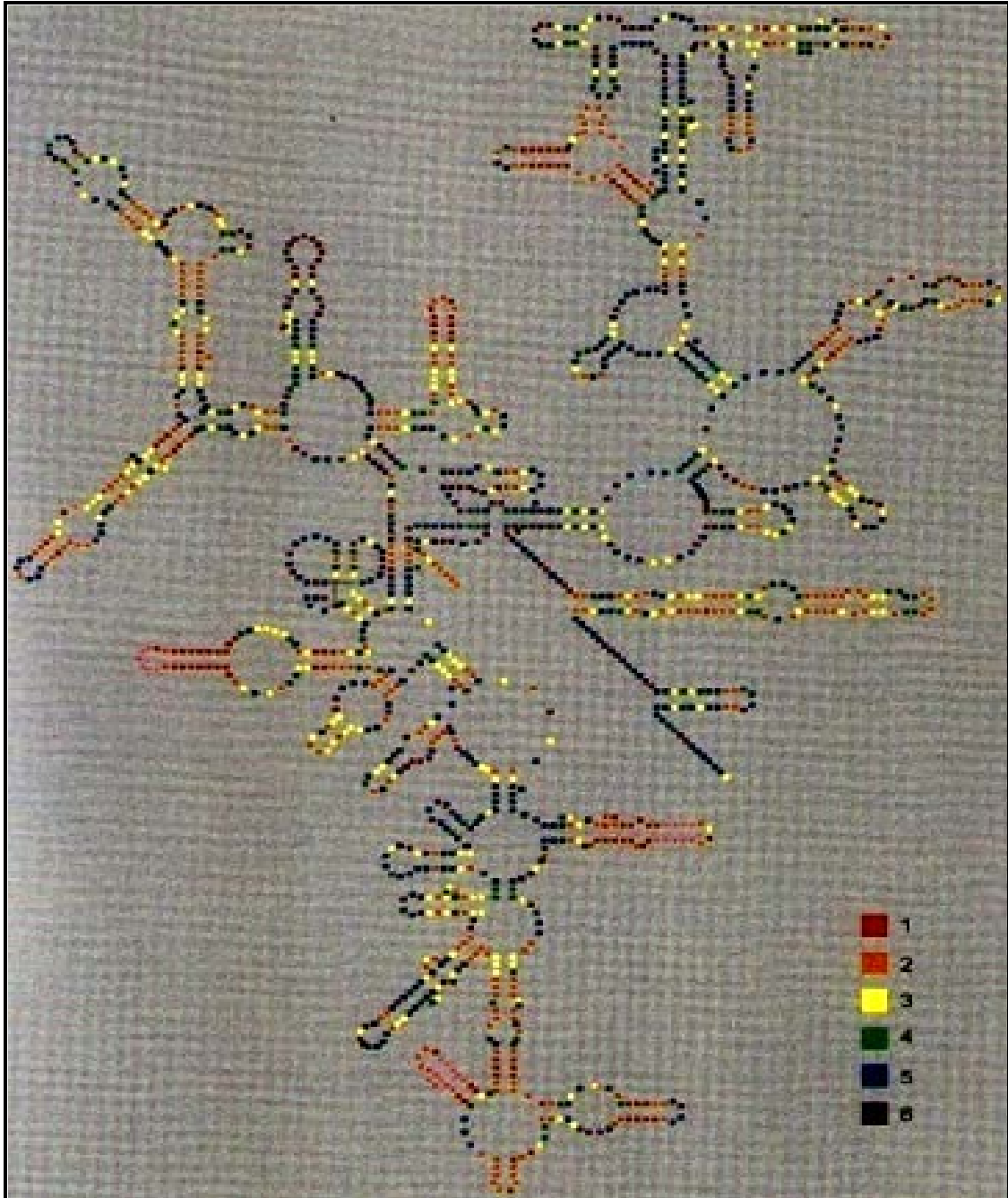
Figure 1-6: Color map superimposed on the 16S rRNA secondary structure model of *E. coli*. Nucleotides are subdivided into five groups of increasing variability. The most variable positions are in red, the most conserved in blue. Absolutely conserved positions are indicated in purple. Nucleotides present in *E. coli* but absent in >75% of the bacterial sequences considered are indicated in pink. Taken from, Van de Peer (1996).

1.7 Aims of the Project

The Rivelin Valley River and Limb Brook in Sheffield were used for industrial activities over a long period dating back to the industrial revolution. So it was assumed that both sites would still contain some polluted acidic environments. Therefore, various liquid and sediment samples were collected from different places from both valleys at different times in order to isolate extremophilic microorganisms from these habitats. Many different media such as FeTSB, M9 minimal and ½ LB medium were set up at pH 3 to select for acidophilic microorganisms.

The microbial strains isolated in pH 3 medium were further characterised to categorise them as acidophilic or acid tolerant (Chapter 3). Four microbial strains were identified using 16S rRNA and 18S rRNA sequencing (Chapter 4). The physiological characteristics such as growth rates, respiration rates and effects of high salinity on the growth rate of two species (*Debaryomyces hansenii* and *Micrococcus luteus*) were determined (Chapter 5). Also the accumulation of compatible solutes *by D.* hansenii *and M.* luteus was demonstrated using NMR spectroscopy (Chapter 5). In addition, scanning electron microscope (SEM) and transmission electron microscope (TEM) were used to examine the cell structure of the two species under different acidic conditions (Chapter 5). In the final result chapter the bioenergetics of acidophilic growth *of D. hansenii* were investigated using silicone oil technique (Chapter 6).

CHAPTER TWO

2 MATERIALS AND METHODS

2.1 Area of Study

2.1.1 Rivelin Valley

"The River Rivelin is a river in South Yorkshire, England (Figure 2-1). It rises in the Hallam moors, North West of Sheffield, and on the outskirts of Sheffield joins the River Loxley (at Malin Bridge). The Rivelin Valley, through which the river flows, is a three and a half mile long woodland valley which includes the popular Rivelin Valley Nature Trail which was created in 1967. The valley has farmland on its gentler upper slopes.

A relatively fast flowing river (it drops 80 metres between Rivelin Mill Bridge and Malin Bridge), the Rivelin is fed by a constant release of water from the nearby moorland peat. Its flow was exploited for centuries as a power source, driving the water wheels of up to twenty industries (forges, metal-working and flour mills) along its course" (Wikipedia web site).

2.1.2 Limb Valley

The Limb Brook is a stream in Sheffield, South Yorkshire, England. It rises at the village of Ringinglow, flowing east through Whirlow and Ecclesall Woods into Abbeydale in the Beauchief area, where it merges with the River Sheaf (Figure 2-1). Near this point part of the stream has been diverted to provide the goit (leat or millstream) for the Abbeydale Industrial Hamlet millpond, and this channel flows through what is now Beauchief Gardens.

Limb Brook lies entirely within the City of Sheffield boundaries, but used to form (with the connecting River Sheaf and Meers Brook) part of the border between Yorkshire and Derbyshire. This boundary dates back to the Anglo-Saxon kingdoms of Mercia and Northumbria (Addy, 1888), (Wikipedia web site).

2.2 Chemical Analysis of Water Samples

In order to identify the levels of cations and anions present, water samples from the River Rivelin and from Limb Brook were chemically analyzed. 10 ml of each raw water sample were sent to Chemistry Department in the University of Sheffield for the required analysis.

2.3 Sample Collection

Water samples were collected from Rivelin River and Limb Brook and evidence of iron deposition was found. However, the pH of the samples from the Rivelin River was only slightly acidic at around pH 6.5, while the pH of the samples from Limb Brook was pH 5.

Various samples were collected from the River Rivelin at two different times; the first collection was on 15th February 2009, and the second collection was on 6th April 2009. During the both collections, the samples were transferred into sterile 50 ml Falcon tubes. Each sample was labelled at the time and a photograph taken at each sampling location. The pH of samples was measured *in situ* using a portable pH meter. Samples were stored at 4°C until required. Same procedure of sample collecting was performed when various samples were collected from a ditch near Limb Brook on the 7th May 2010.

Figure 2-1: Map locations of Sheffield main rivers taken from (Addy, 1888).

2.4 Growth Media

2.4.1 FeTSB Liquid Medium

Ferrooxidans medium was prepared by using three solutions, solution A (Basal salt solution) was prepared by dissolving 2.4 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4.7H_2O$, 0.05g KH_2PO_4, 0.05 g KCl and 0.014 g $Ca(NO_3)_2$ in 900 ml distilled water. Solution B was prepared by dissolving 0.5 g NaCl and 0.25 g K_2HPO_4 in 100 ml distilled water. Solution C was prepared by dissolving 29.13 g $FeSO_4.7H_2O$ in 80 ml distilled water and sterilized by filtration. Then, 4 ml was added from solution B to the solution A and made up to 920 ml with distilled water and autoclaved.

To prepare 50 ml culture, 46 ml was added from solution A+B to 4 ml solution C (Johnson *et al.,* 1987).

2.4.2 FeTSB Solid Medium

Ferrooxidans solid medium was prepared by using four solutions, solution A (Basal salt solution) was prepared by dissolving 1.26 g $(NH_4)_2SO_4$, 0.49 g $MgSO_4.7H_2O$ in 600 ml distilled water. Solution B (tryptone soya broth (TSB)) was prepared by dissolving 1.7 g bactotryptone, 0.5 g NaCl and 0.25 g K_2HPO_4 in 100 ml distilled water. Solution C (Ferrous sulphate solution) was prepared by dissolving 18.21 g $FeSO_4.7H_2O$ in 50 ml distilled water, solution D (gelling solution) was prepared by dissolving 7 g agarose in 250 ml.

Next, 10 ml of solution B was added to the solution A, and made up to 700 ml using distilled water, then the 50 ml of solution C and the 250 ml of gelling solution were added to the previous solution to prepare one litre of FeTSB solid medium. Each

solution has been autoclaved separately except solution C which was sterilized by the filtration method. All solutions were allowed to cool to approximately 50°C, combined aseptically and gently inverted to mix before being poured into sterile plastic Petri dishes and left to set (Johnson *et al.,* 1987).

2.4.3 M9 Medium

M9 minimal medium was prepared by dissolving 11.3 g M9 minimal salts (Sigma M-6030) in 980 ml distilled water and adjusting the pH as necessary with 1 M H_2SO_4 or 1 M NaOH, and the following four solutions were autoclaved separately and added as follows; 1 ml $MgSO_4$ (1 M), 9 ml NH_4Cl (5 g in 45 ml distilled water), 9 ml glucose (15 g in 45 ml distilled water) and 1 ml $CaCl_2$ (100 mM). Solid M9 minimal medium was prepared by adding 15 g agar No 1 directly to the pH 7 medium, while pH 3 plates were made by preparing a separate gelling solution of 15 g agar No 1 (final concentration 1.5% w/v).

2.4.4 ½ LB Medium

Half Luria Bertani (1/2 LB) plates were prepared by adding 2.5 g yeast extract, 5 g tryptone and 2.5 g NaCl to 490 ml of distilled water, adjusting the required pH using 1 M H_2SO_4 or 1 M NaOH and autoclaving. A separate solidifying solution was prepared by adding 10 g of agar No.1 to 500 ml of distilled water, and then continues as described in section (2.4.2). The liquid medium consisted of 2.5 g yeast extract, 5 g tryptone and 2.5 g NaCl per litre.

2.4.5 LB Medium

Luria Bertani (LB) medium consisted of 5 g yeast extract, 10 g tryptone, 10 g NaCl in 1000 ml of distilled water to produce liquid medium and was adjusted for the required

pH using 1 M H_2SO_4 or 1 M NaOH. For solid medium 15 g of bacteriological agar No.1 were added per litre.

2.4.6 YPD Medium:

YPD medium was prepared by dissolving 20 g peptone, 10 g yeast extract and 20 g glucose in 990 ml distilled water and was adjusted to the required pH with 1 M H_2SO_4 or 1 M NaOH. 10 g agar No. 1 was added if solid YPD medium was required.

2.5 Isolation of Acidophilic or Acid tolerant Microorganisms

Isolation of acidophilic or acid tolerant microorganisms was carried out using FeTSB, M9 and ½ LB medium. 1 ml of each river water sample was inoculated into 50 ml of FeTSB, M9 and ½ LB. All flasks were incubated overnight at 25ºC with shaking (250 rpm).

In parallel, approximately 200 µl of raw environmental sample was spread plated onto FeTSB, ½ LB and M9 minimal media. Each plate was labelled and incubated at 25^oC. Growth of liquid and solid cultures was monitored daily for 3 days and any growth obtained in liquid culture or any colonies appearing on plates were streaked onto fresh plates in order to isolate pure cultures. After the third generation of plate growth, a single colony from each isolate was inoculated into approximately 10 ml of the appropriate liquid medium and sub-cultured into 250 ml flasks, containing 50 ml of medium, and shaken at 250 rpm.

2.6 Purity of Acidophilic Strains

The purity of strains was monitored by streaking a loopful of the culture on agar plates of pH 3 M9 and ½ LB medium. After incubation, the plates were first

examined for colony morphology and then single colonies were picked off and observed as a wet mount using a phase contrast microscope.

2.7   Maintenance of Acidophilic Strains

All isolated strains were maintained by adding 2 ml of overnight culture (active inoculum) to 2 ml of sterile glycerol (50% v/v) and kept in a freezer at – 20ºC. When required, the frozen cells were thawed at room temperature and inoculated into 250 ml conical flasks plugged with cotton wool containing 50 ml of M9 medium of the same pH, and incubated overnight at 25ºC on a rotary shaker at 250 rpm. In addition, once every two months each strain of acidophilic bacteria was maintained by streaking three pH 3 M9 or ½ LB medium plates.

The plates were incubated for 24 – 48 h at 25ºC. When pure colonies had developed on the plates, a piece of parafilm was stretched around each plate's edge and then one set of plates was stored at room temperature and the other put in the fridge until required. Sub-culturing of the strains was also performed routinely once every two weeks to minimize contamination over time by adding 1 ml of overnight culture into 250 ml conical flasks containing 50 ml of M9 or ½ LB medium of the same pH. The flasks were incubated at 25ºC on a rotary shaker at 250 rpm and then kept at room temperature until required.

2.8   Identification of Acidophilic Strains

2.8.1   Colony Morphology

The differences between colony shapes when growing on FeTSB, M9 or ½ LB media were noted.

2.8.2 Cell Morphology

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

2.8.3 Motility of Bacteria

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

2.9 Effect of Medium pH on Growth of Isolated Strains and Growth Curve Determination

The effect of different pH values on the growth of isolated strains was determined using overnight cultures in parallel half enrichment medium (½ LB) and minimal medium (M9). 1 ml of active inoculum from each strain was inoculated into ½ LB and M9 medium pH 7, pH 5 and pH 3 and the optical density (OD) at 600 nm was measured using the Unicam Helisα spectrophotometer against a distilled water blank

in 1 ml plastic cuvettes. To produce a growth curve, 4 x 250 ml conical flasks containing 50 ml of each pH value ½ LB and M9 medium were inoculated with 1 ml of the same pH adapted cells from an overnight culture. The OD_{660} was measured against a water blank immediately after inoculation then every two hours over an incubation period at 25ºC on a rotary shaker at 250 rpm. The growth curves were plotted against time of incubation.

2.10 Measurement of External pH of Overnight Cultures of Strains in Different Media

In order to investigate the behaviour of isolated strains with different pH values, the pH of overnight cultures was measured using a Mettler Toledo MP225 pH meter.

To test the external pH, four 250 ml flaks containing 50 ml of either M9 or ½ LB or LB or YPD media at pH 7, pH 5 and pH 3 were inoculated with 1 ml of active inoculum from each strain and then incubated overnight at 25ºC on a rotary shaker at 250 rpm. The overnight cultures were centrifuged for 10 min at 3000 g, then 10 ml of supernatant was taken and the pH was measured.

2.11 Measurement of Respiration Rate (Oxygen Uptake) of Isolated Strains

Respiration rate was measured using a modified Clarke oxygen electrode (Hansatech Scientific Instruments, Kings Lynn, UK) with a Servoscribe 1S potentiometric chart recorder as described by Delieu and Walker (1972). Schematic drawings of the oxygen electrode unit are shown in Figures 2.2 and 2.3. 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.

$$4H^+ + O_2 + 4e- \longleftrightarrow 2H_2O$$
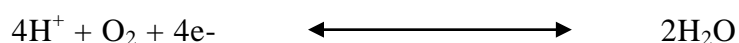
The flow of current in the circuit when the polarising volts were set between 0.5 and 0.8 V varied in linear relationship to the partial pressure of oxygen in solution. The current flowing was measured by connecting the electrode to a sensitive potentiometric chart recorder. The reaction chamber of the oxygen electrode was maintained at a constant temperature similar to the culture temperature (25°C), by circulating water from a temperature controlled water bath (Figure 2-2 and Figure 2-3).

A 12 V, 100 W tungsten halogen lamp was used to illuminate the chamber when required, approximate output intensity 1800 μmol m^{-2} s^{-1}.
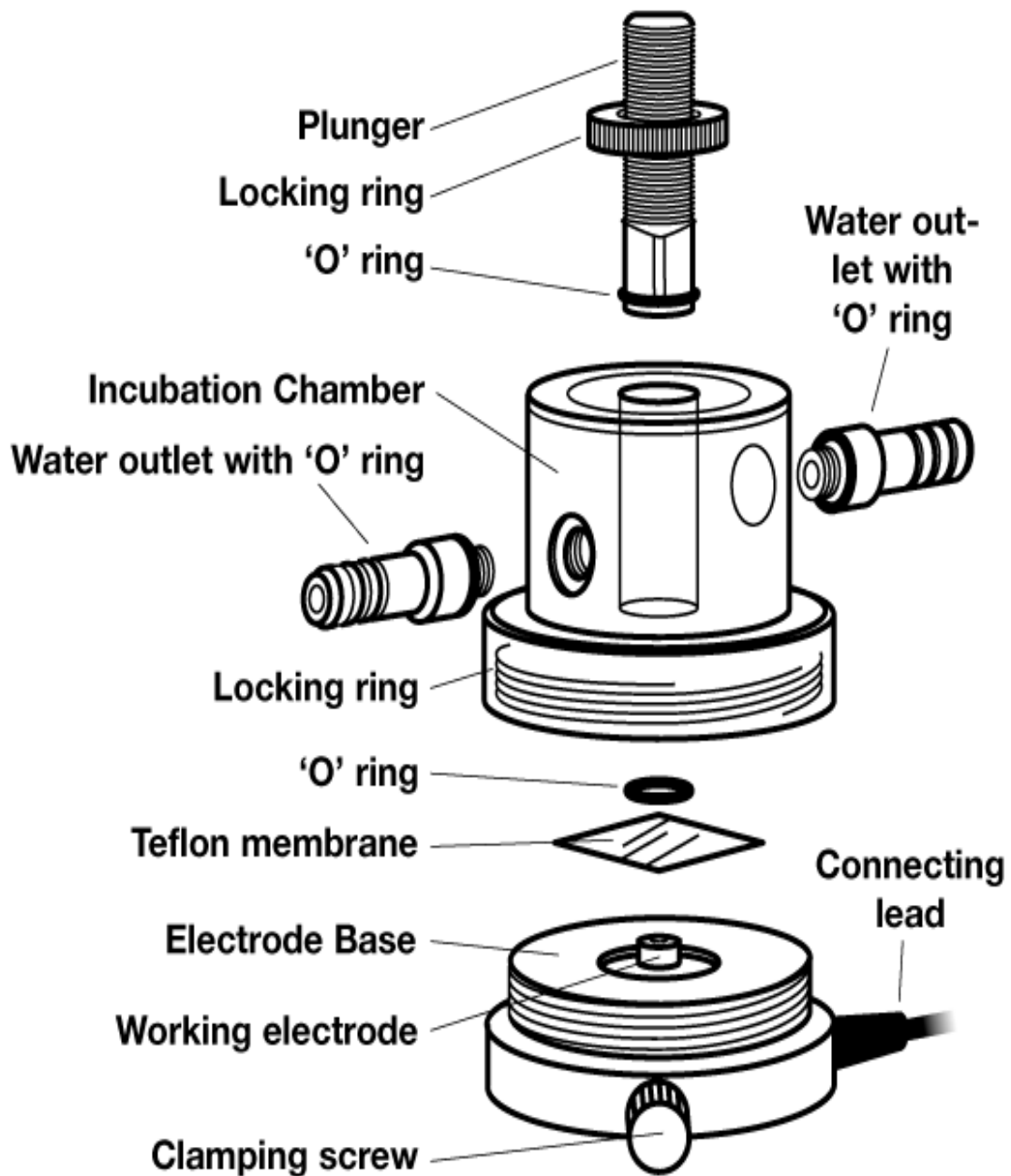
Figure 2-2: Schematic diagram illustrating the key components of an oxygen electrode. Figure from (http://www.rankbrothers.co.uk/prod1exp.htm).
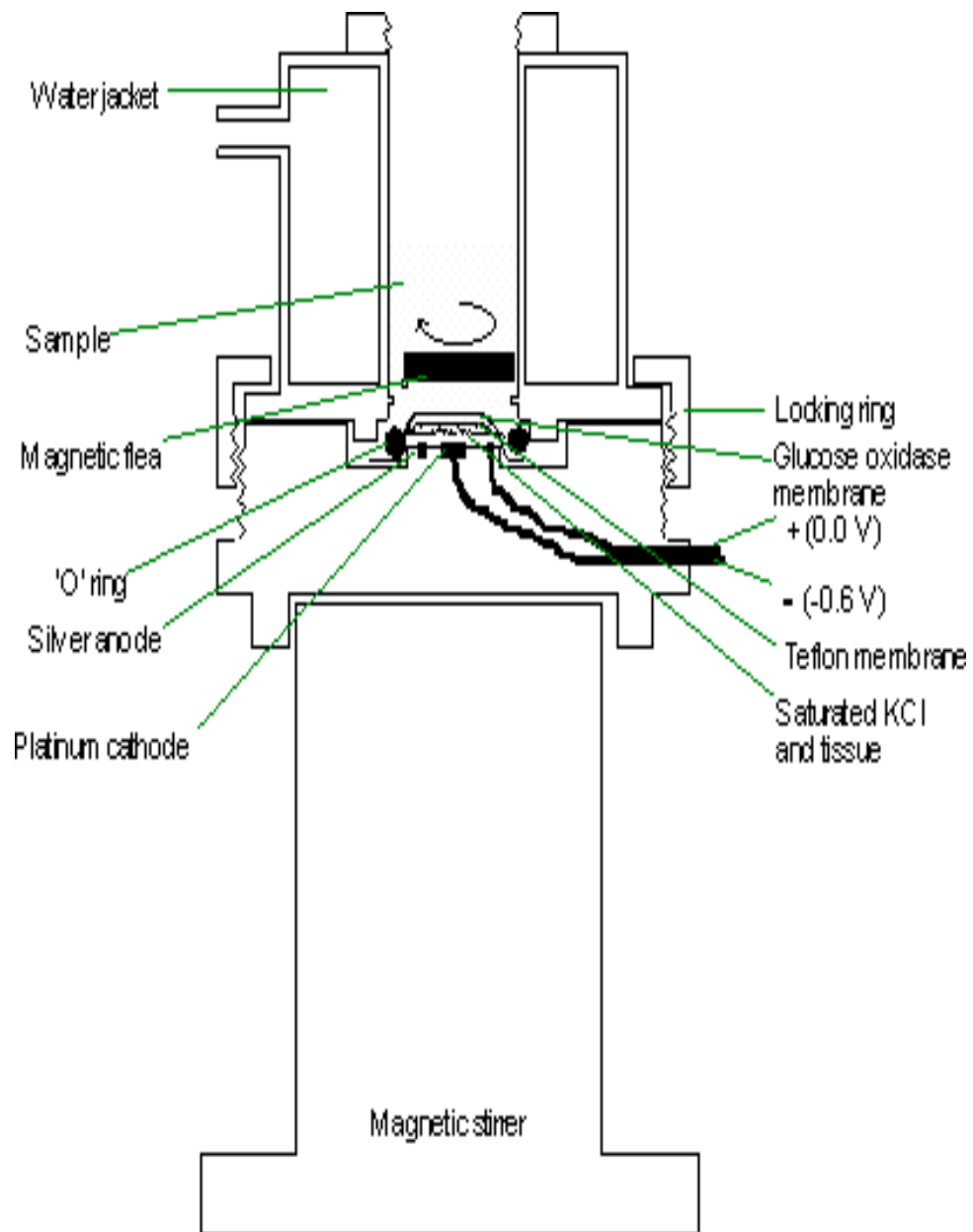
Figure 2-3: A diagram of the oxygen electrode showing more details of the electrode components. Only a Teflon membrane was used in the current work and the tissue was cigarette paper.

Figure from (www.lsbu.ac.uk/biology/enzyme/oxelectrode.html).

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

$$Na_2S_2O_2 + 2O_2 + H_2O \longleftrightarrow NaHSO_4 + NaHSO_3$$
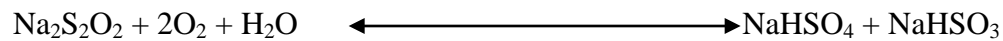
To calculate the respiration rate we need to know the concentration of O_2 in air saturated water at 25°C and the protein content of the bacterial suspension. Then the following equation is used:

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

$$\frac{standard}{range} \quad x \quad \frac{number\ of\ units}{time} \quad x \quad \frac{60}{\mu g\ of\ protein\ per\ sample}$$

- Standard: Oxygen amount in 2 ml medium (sample) = 0.660 μmoles at 30°C or 0.722 μmoles at 25°C

- Range: Units taken from calibration (0 – 100%)

- Number of units: Number of units was 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 (µg): this relates to amount of protein in a sample of 2 ml of cells from Bradford assay (see section 2.12).

To prepare cells for measurements in the oxygen electrode, the OD_{600} of all cultures was measured and 5 to 10 ml of cells from each sample (grown overnight at pH 7, pH 5, and pH 3 in ½ LB or LB or YPD medium) were transferred to a 50 ml Falcon tube and harvested by centrifugation at 3000 g for 10 minutes. Each pellet was then resuspended in a fresh 10 ml of same medium as they were grown and same pH. The cells were also normally concentrated twofold and the protein content was determined (section 2.12). The viability of the cultures grown overnight was measured by adding 0.5 ml of concentrated cells to 1.5 ml of fresh medium of the same pH in the chamber of oxygen electrode (keeping the overall volume at 2 ml to allow standardisation of the results) and the plunger placed on top and was left for 5 minutes without illumination to induce oxygen uptake. The plunger was removed and the sample unloaded using a Pasteur pipette. Distilled water was used to clean both the chamber and the plunger. When the effect of increasing external salinity on the respiration rate was studied the same procedure was employed except that the washing step was carried out with sodium free desired medium. Then the cells were resuspended in different concentrations of sodium chloride for the measurements in the oxygen electrode.

2.12 Determination of Protein Content

2.12.1 Determination of Standard Curve

A standard curve was produced using bovine serum albumin (BSA) in the range of 0 - 100 µg by dissolving 250 mg of BSA in 50 ml of distilled water. The final

concentration of protein in the stock solution was 5 mg ml^{-1} (5 µg µl^{-1}). The stock solution was used to make a range of protein concentrations by a series of dilutions as shown in Table 2-1.

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

2.12.2 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 2.12.1.

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 2.11) was placed into a clean test tube and then 0.9 ml of 1M NaOH was added. The sample was then thoroughly mixed and heated at 90°C for 10 minutes.

- The test tube was cooled on ice for 5 minutes and dried outside and then centrifuged for 10 minutes in bench centrifuge at full speed (3000 g).

- 0.3 ml of the resulting supernatant was added to 3 ml of Bradford's reagent in a fresh test tube and the contents were thoroughly mixed. After 5 minutes incubation at room temperature the optical density was measured in the Unicam Helisα spectrophotometer against 0.1 ml water plus 3 ml of Bradford's reagent (blank) at 595 nm.

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

Table 2-1: Components in test tubes which were needed to make a standard protein curve.

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

2.13   Effect of High Sodium Concentration on the Growth of the Cells of RV$_4$ and LV$_1$ Strains

To prepare sodium free M9 minimal medium, the pH of medium was adjusted to pH 7, pH 5 and pH 3 as necessary using either 1 M HCl or 1 M KOH (instead of NaOH) and 200 mM potassium carbonate buffer was used instead of sodium carbonate with different concentrations of sodium chloride (0, 0.175, 0.4, 1 and 1.5 M) being added. The same procedure was used to prepare YPD and LB medium except that a tiny sodium chloride in yeast extract (approximately 0.05 mM NaCl) cannot be removed. Then media containing different sodium chloride concentrations were prepared (0.4, 1, 1.5, 2, 2.5 M).

An overnight culture (50 ml) grown in M9, YPD or LB medium to mid-exponential phase at pH 10 was transferred into a 50 ml Falcon tube and then harvested by centrifugation in a bench top centrifuge (3000 g) for 15 minutes. The pellet was resuspended (washed) three times in 50 ml of sodium free medium of the same pH and then 1 ml of cells was added into a number of 250 ml conical flasks containing 50 ml of desired medium with different concentrations of sodium chloride (0 – 2.5 M). The optical density (OD) at 600 nm was measured in the Unicam Helisα spectrophotometer against sterilized medium blank, immediately after inoculation, and then all flasks were incubated at 25ºC on a rotary shaker at 250 rpm, the OD at 600 nm was measured every hour or every 3 hours or daily depending on the speed of growth and used as growth parameter.

2.14 Effect of High Sodium Concentration on the Respiration Rate of the Cells of RV$_4$ and LV$_1$ Strains

The effect of increasing external salinity in the media on the oxygen consumption of both RV$_4$ and LV$_1$ strains was determined using YPD and LB medium, respectively and a modified Clarke type oxygen electrode as described in section 2.11. The pH adjustment and media preparation were done as explained in section 2.13.

To prepare cells for measurements in the oxygen electrode, 40 ml of overnight culture grown to mid-exponential phase at pH 7, pH 5 and pH 3 in YPD medium for RV$_4$ strain and LB medium for LV strain were transferred into a 50 ml Falcon tube and harvested by centrifugation in a bench top centrifuge at 3000 g for 15 minutes and the pellet was resuspended (washed) three times in 20 ml of sodium free YPD or LB medium. The respiration rate of the cells was measured in the oxygen electrode by adding 0.1 ml of concentrated cells to 1.9 ml of fresh YPD or LB medium with different NaCl concentrations and 1.9 ml of normal YPD and LB were also used as controls (keeping the overall volume at 2 ml to allow standardization of the results). A Bradford assay (see section 2.12) was performed on the concentrated cell suspensions to determine the protein concentration for use in calculating the respiration rate of the cells.

2.15 Effect of Iron, Sulphur and Aluminium on the Growth of RV$_4$ and LV$_1$ Cells

To prepare media containing iron, sulphur and aluminium, M9 minimal medium has been used with both RV$_4$ and LV$_1$ strains. First of all, normal M9 minimal medium has been prepared as usual and pH adjusted using H_2SO_4 and NaOH. Then the desired

concentrations of iron, sulphur and aluminium dissolved with distilled water were prepared and autoclaved separately (the concentrations were adjusted to be the same concentrations in the rivers (see Chapter 3, section 3.2.2), left to cool down, and then mixed together with M9 medium.

To study the effects of iron, sulphur and aluminium, on the growth rates of strains RV_4 and LV_1, 4 x 250 ml conical flasks containing 50 ml of each amended medium against a control (M9 medium without addition of iron, sulphur or aluminium) were inoculated with 1 to 3 ml of pre-adapted cells from an overnight culture. The OD_{660} was measured against a medium blank immediately after inoculation, then every three hours over an incubation period at 25ºC on a rotary shaker at 250 rpm. The growth was plotted against time of incubation.

2.16   Anaerobic Growth of Bacteria

Anaerobic growth of bacteria was carried out using fresh overnight cultures in normal LB medium. Triplicate LB medium plates were inoculated with a single colony from each strain and then placed in an anaerobic jar and incubated overnight at 25ºC. The growth was monitored to check if anaerobic growth was possible.

2.17   Physiological Characteristics of Yeast Species

2.17.1   Cycloheximide Resistance

50 μl of Cycloheximide stock solution (100 mg ml^{-1} in DMSO) was added to 50 ml M9 medium and inoculated with 1 ml of Yeast strain cells, left overnight in $25^{o}C$ incubator with shaking, and then the optical density (OD) at 600 nm was measured using the Unicam Helisα spectrophotometer against sterilized medium as a blank in 1 ml plastic cuvettes.

2.17.2 **Assimilation of Glucose**

Normal M9 medium was used to check the ability of Yeast strain to use glucose as a carbon source. The growth was checked as described in section 2.17.1.

2.17.3 **Assimilation of α-Methyl glucoside**

M9 (α-Methyl glucoside) medium did not contain any carbon source except α-Methyl glucoside. To prepare 1 litre of M9 (α-Methyl glucoside) medium, 33.9 g disodium phosphate, 15 g monopotassium phosphate, 2.5 g sodium chloride and 20 ml (20mM) α-Methyl glucoside, were dissolved and then then made up to 1 litre using distilled water. Growth was detected as described in section 2.17.1.

2.17.4 **Assimilation of Nitrate**

A modified M9 (nitrate) medium was prepared with nitrate as the sole source of nitrogen. To prepare 1 litre of M9 (nitrate) medium, 33.9 g disodium phosphate, 15 g monopotassium phosphate, 2.5 g sodium chloride, 5 g nitrate and 20 ml 1 M glucose, were dissolved and then made up to 1 litre by adding distilled water. Growth was measured as described in section 2.17.1.

2.17.5 **Assimilation of Cadaverine**

M9 (cadaverine) medium was prepared as described for M9 (nitrate) medium in section 2.17.4 but 117 ml of 1 M cadaverine was added instead of nitrate, and then the growth was measured as described in section 2.17.1.

2.18 NMR Analysis of Compatible Solutes

2.18.1 Preparation of Samples for NMR Analysis

Samples (5ml) of RV_4 and LV_1 strains were put into 15 ml tubes, centrifuged at 3000 g for 10 minutes and the supernatant was discarded. The pellets were kept in -80°C freezer until the analysis was due.

The pellets were thawed, resuspended in 1 ml of distilled water and vortexed for 1 minute at room temperature. Then the samples were sonicated (2 x 20 seconds) and centrifuged at full speed in the microfuge for 10 minutes. The supernatants were transferred into two 1.5 ml Eppendorf tubes, transferred to $-80°C$ freezer for 2 hours and then freeze dried for two days. Freeze dried samples were prepared for Nuclear Magnetic Resonance (NMR) analysis by dissolving them in 500 µl of D_2O in an Eppendorf tube and then 5 µl of trimethyl syle propionate (TSP) were added. Next, the dissolved sample was transferred into an NMR tube and run in the NMR (Frings *et al.*, 1993).

2.18.2 Effect of Adaptation to Different Salinity, pH and Growth Media on Compatible Solutes Accumulated by RV_4 and LV_1 Strains

RV_4 and LV_1 strains were adapted to different concentrations of NaCl (0.4 and 1 M) in M9 minimal medium and (0.4, 1, 1.5, 2 and 2.5 M) in YPD medium for RV_4 and LB medium for LV_1 strain, with 3 different pH values (pH 7, pH 5 and pH 3). After incubation for 24 – 48 hours at 25^0C with shaking, 5 ml of each sample were centrifuged for 10 minutes, the supernatant was poured off and the pellet was kept in freezer until used. NMR analysis was carried out as described in section 2.18.1.

2.19 Electron Microscopy

Electron microscopy was carried out using a scanning electron microscope (SEM) and a transmission electron microscope (TEM). Both types were used to examine RV_4 and LV_1 cells under normal conditions (pH 7) and extreme condition (pH 3). The analysis was performed in the Biomedical Science Department.

2.19.1 Scanning Electron Microscope (SEM)

To produce very high-resolution images of a sample surface, SEM was used. First step was to prepare the sample for examination by pelleting 1.5 ml of overnight culture for 10 minutes at 3000 g and discarding the supernatant. The cells were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer for 4 hours at 4^0C, then the cells were washed in 0.1 M phosphate buffer, twice with 15 intervals at 4^0C.

Secondary fixation was carried out in 2% aqueous osmium tetroxide for 1 hour at room temperature. The wash step was repeated and dehydration was achieved through a graded series of ethanol solutions as follows:

75% ethanol for 15 minutes

95% ethanol for 15 minutes

100% ethanol for 15 minutes

100% ethanol for 15 minutes

100% ethanol dried over anhydrous copper sulphate for 15 minutes.

All the above steps were carried out at room temperature.

The cells were then air dried from hexamethyldisilazane. Initially they were placed in a 50/50 mixture of 100% ethanol and hexamethyldisiazane for 30 minutes followed

by 30 minutes in 100% hexamethyldisilazane. The cells were then allowed to air dry overnight before mounting.

Upon completion of drying, the cells were mounted on 12.5 mm diameter stubs, attached with Sticky Tabs and then coated in an Edwards S150B sputter coater with approximately 25 nm of gold.

The cells were examined in a Philips XL-20 Scanning Electron Microscope at an accelerating voltage of 20 Kv.

2.19.2 Transmission Electron Microscope (TEM)

TEM has been used to obtain thin, minimally deformed cells that allow for the observation of the internal structures of cells. After preparing cells as described in section 2.19.1 in an Eppendorf tube, fresh 3% glutaradehyde in 0.1 M phosphate buffer was added to the pellet (re-suspended to ensure optimal fixation) and left overnight at 40°C. The cells were then washed in 0.1 M phosphate buffer, twice with 30 min intervals at 40°C.

Secondary fixation was carried out in 2% aqueous osmium tetroxide for 2 hours at room temperature, washed in buffer as above. Followed by dehydration through a graded series of ethanol solutions at room temperature as described in section 2.19.1, except the last dehydration step was repeated twice.

The cells were then placed in an intermediate solvent, propylene oxide, for two changes of 15 minutes duration. Infiltration was accomplished by placing the cells in a 50/50 mixture of propylene oxide/Araldite resin. The cells were left in this 50/50 mixture overnight at room temperature.

The cells were left in full strength Araldite resin for 6-8 hours at room temperature (resin was changed after 3-4 hours) after which they were embedded in fresh Araldite resin for 48-72 hours at 60°C.

Araldite resin contained:

CY212 resin	10 ml
DDSA hardener	10 ml
BDMA accelerator	1 drop per 1 ml of resin mixture

Semi-thin sections approximately 0.5 μm thick were cut on a Leica ultramicrotome and stained with 1% Toluidine blue in 1% Borax.

Ultrathin sections, approximately 70-90 nm thick, were cut on a Leica ultramicrotome and stained for 25 minutes with saturated aqueous uranyl acetate followed by staining with Reynold's lead citrate for 5 minutes. The sections were examined using a FEI Tecnai TEM at an accelerating voltage of 80 Kv. Electron micrographs were taken using a Gatan digital camera.

2.20   Silicone Oil Technique

Centrifugation through silicone oil was used to completely separate cells from medium (Gimmler *et al*., 1978). A range of oils with different densities were produced by mixing individual silicone oils with different densities (Dow Corning 200/1 grade oil was mixed with Dow Corning 550 grade oil). Cell suspensions of the density to be used in experiments (with no isotopes added) were used to find the oil which is dense enough to restrict mixing with the medium, but which allows the cells to pass through the oil during centrifugation and produce a pellet (Figure 2-4).

2.20.1 Determination of Intracellular Volume (ICV) of RV₄

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

Two 1 ml samples of concentrated cells (10 ml of cells from an overnight culture grown in M9 minimal medium at pH 7 or pH 3 were transferred to a 50 ml Falcon tube and harvested by centrifugation at 3000 g for 10 minutes. The yeast cell pellet was resuspended in 8 ml of fresh medium of the same pH and placed in 1.5 ml Eppendorf tubes. 10 µl of $^{3}H_2O$ (1850 kBq ml^{-1}) were added to one sample of concentrated cells to give 18.5 kBq ml^{-1} and 20 µl of ^{14}C-dextran (MW = 70000, 1850 kBq ml^{-1}) were added to second sample of concentrated cells to give 14.8 kBq ml^{-1}. Both samples were vortexed thoroughly.

After 5 minutes incubation at room temperature the 1.5 ml Eppendorf tubes were vortexed again, triplicate samples (300 µl) were taken from each 1.5 ml Eppendorf tube and layered onto 300 µl of the silicone oil mixture (right density) in fresh 1.5 ml Eppendorf tubes. The samples were then centrifuged for 1 minute at 13000 rpm (11340 g) in a bench top micro centrifuge (MSE, Micro-Centaur). From the aqueous supernatant fraction, triplicate samples (50 µl) were taken and placed into separate scintillation vials containing 5 ml of FluoranSafe scintillation fluid (VWR) and labelled appropriately.

The tips of the 1.5 ml Eppendorf tubes, which contain the pellet of yeast, were carefully cut off using a razor blade within the oil layer and placed cut end down into 1.5 ml Eppendorf tubes containing 300 µl distilled water. They were then centrifuged

for approximately 15 seconds at 13000 rpm (11340 g) to remove the pellet from the tips which were then discarded.

Each pellet was resuspended in the water and then the whole sample was pipetted out and placed into separate scintillation vials containing 5 ml of FluoranSafe scintillation fluid (labelled appropriately) and then dispersed using a vortex agitator. All the vials were placed in racks and counted for 5 minutes per vial in a Beckman LS 1801 Liquid Scintillation Counter.

The $^{3}H_2O$ was evenly distributed throughout the pellet, whereas the ^{14}C-dextran was only found in the spaces between the cells and the pellet due to its high molecular weight (Figure 2-4). The pellet volume (PV) and the extracellular volume (ECV) were calculated from the ratio of $^{3}H_2O$ and ^{14}C-dextran in the pellet and supernatant fractions respectively using the following equations given in Hard and Gilmour (1996).

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

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

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

* dpm = Disintegrations per minute
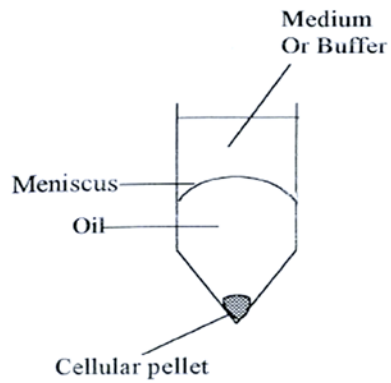
1. Oil is not dense enough

2. Oil is too dense

3. Oil is in the right density

Figure 2-4: Silicone oil density selection for intracellular volume, membrane potential and internal pH determinations. After centrifugation a pellet was formed and then medium remained on top of silicone oil of the correct density (3), silicone oil of the incorrect density (1 and 2).

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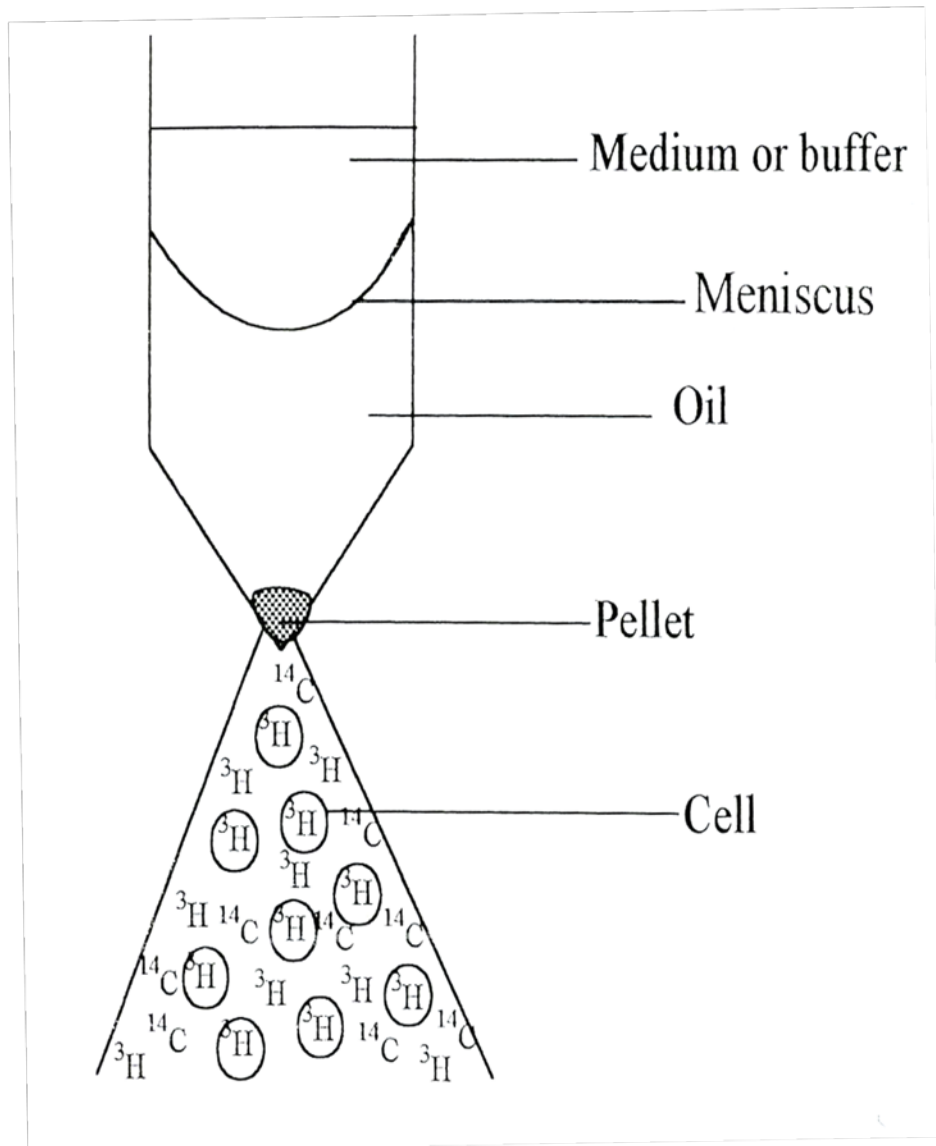
Figure 2-5: Intracellular volume (ICV) determination using ^3H_2O and ^{14}C-dextran. The ^3H_2O distributes throughout the pellet and ^{14}C- dextran is only found in the spaces between cells in the pellet.

2.20.2 The Measurement of Membrane Potential (ΔΨ) in RV₄ Cells

Estimation of membrane potential was carried out using the silicone oil technique as described by Rottenberg, (1979) and (1989). The experimental procedure was similar to the method used to determine intracellular volume (ICV) (section 2.20.2), with the exception that 5 µl of 9250 kBq ml^{-1} ^{14}C-TPP$^+$ (tetraphenylphosphonium) were added to 1 ml of concentrated cells to give a final concentration of 18.4 kBq ml^{-1} ^{14}C-TPP$^+$. The membrane potential was then calculated as follows:

1) Dpm ^{14}C-TPP$^+$ in 1 µl = Dpm ^{3}H-TPP$^+$ of supernatant divided by 50 $\Rightarrow$ A

2) ^{14}C-TPP$^+$ within the pellet which is outside the cells = multiply A by extracellular volume in µl (calculated from parallel samples treated with ^{3}H$_2$O and ^{14}C- dextran) $\Rightarrow$ B

3) Dpm µl^{-1} cell volume = Dpm ^{14}C-TPP$^+$ in pellet minus B and divided by intracellular volume in µl (calculated from parallel samples treated with ^{3}H$_2$O and ^{14}C-dextran) $\Rightarrow$ C

4) Ratio of $\dfrac{A}{C}$ = concentration of ^{14}C-TPP$^+$ inside the cells / concentration of ^{14}C-TPP$^+$ outside cells (a$_o$)

Using the Nernst equation:

$$\Delta\Psi \ (mV) = \ \text{--} \ \frac{RT}{ZF} \ \ln \frac{ai}{ao}$$

Where:-

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

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

At 25°C and converting from ln to $\log_{10}$ (x 2.303):

$$\Delta\Psi \text{ (mV)} = -58.8 \times \log \frac{ai}{ao}$$

2.20.3 Determination of Internal pH (pHi) of RV$_4$ Cells

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

The silicone oil method was used in an identical manner to that used for intracellular volume (ICV) calculation (section 2.20.2) and membrane potential determination (section 2.20.2), except that 5 µl of ^{14}C-benzoic acid were added to 1 ml of cell suspension (sample) to give a final concentration of 18.5 kBq ml^{-1}. The calculations were exactly the same as those used in section 2.20.2 to calculate the ratio $\frac{ai}{ao}$.

To determine the internal pH from the $\frac{ai}{ao}$ ratio

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

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

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

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

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

pK of benzoic acid $= 4.2$

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

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

See section 2.20.2 for definitions of R, T and F

2.20.4 Measurement of Isotope Uptake over Time

In order to determine uptake of each isotope by RV_4 strain a time course experiment was carried out. Essentially the silicone oil method (section 2.20) was carried out using only one isotope. An appropriate volume of isotope was added to five 1 ml samples of cells and the silicone oil procedure carried out at 1, 5, 10, 15 and 30 minute intervals. Thirty scintillation vials containing 5 ml of FluoranSafe scintillation fluid and samples were labelled appropriately and then dispersed using a vortex agitator. All the vials were placed in racks and counted for 5 minutes per vial in a Beckman LS 1801 Liquid Scintillation Counter. The mean disintegration per minute

(dpm) for the pellet and supernatant triplicates per time point were calculated and plotted against time.

2.21 Molecular Identification of Unknown Organisms

2.21.1 Genomic DNA Extraction

CTAB method as described by Chen *et al*., (2001) (Appendix B) was used as first attempt to extract genomic DNA from all four strains.

The second method used was QIAgen Genomic-Tip kit following manufacturers guidelines.

ANACHEM Key Prep kit was the third method used in this study to extract genomic DNA from all four strains using liquid cultures and then following manufacturer's protocol.

2.21.2 Polymerase Chain Reaction (PCR) Amplification of 16S rRNA

Following extraction of genomic DNA, polymerase chain reaction (PCR) was carried out in order to amplify the 16S rRNA gene, the primers used to amplify the 16S rRNA gene were two universal bacterial primers: Forward primer (f D1: 5'CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCA G 3') and Reverse primer (r D1: 5' CCC GGG ATC CAA GCT TAC GGC TAC CTT GTT ACG ACT T 3') designed to target the conserved regions of the 16S rRNA gene (Weisburg *et al.,* 1991). The reaction mixture contained the following reagents in a 0.2 ml thin walled PCR tube: 39 µl Distilled Water, 5 µl 10x Buffer, 2.5 µl 50 mM $MgCl_2$, 0.5 µl Forward Primer, 0.5 µl Reverse Primer, 1 µl 25 mM dNTPs, 1 µl genomic DNA and 0.5 µl Taq polymerase (Bioline).

Amplifications were carried out in a MyCycler thermocycler (BioRad) and began with an initial denaturation step consisting of 94°C for 3 min followed by 30 cycles consisting of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C followed by a final extension at 72°C for 5 minutes.

2.21.3 Purification of PCR Products

PCR reactions were cleaned up using QIAgen PCR purification kit and ANACHEM Key Prep Purification as per the manufacturer's protocols. This stage was used to remove any remaining primers and dNTPs. After the clean up, the PCR product can be sent for direct sequencing.

2.21.4 Agarose Gel Electrophoresis

After PCR and after purification process, gel electrophoresis was used to check and confirm the correct gene had been amplified (16S rRNA is 1.5 kbp). The gel was made by adding 2 ml of 50X TAE into a conical flask, add distilled water up to 100 ml and add 1 g of agarose to produce a 1% gel, This mixture was then heated in a microwave until the agarose had melted, after which it was allowed to cool whilst being stirred, and 5 µl of ethidium bromide (Biorad #161-0433) was added prior to pouring into a Biorad Subcell GT electrophoretic tank with a 30 well comb. Once the gel had set, it was covered with 1X TAE buffer and run at 90 − 100 V using a Biorad PowerPack 300.

PCR products were loaded on the gel as follows, 2 µl of the PCR reaction was added to 2 µl of Orange G loading dye and analysed on a 1 % agarose gel against 1 µl of 1

kb GeneRuler ladder (Fermentas) (Appendix B). Gels were visualised using the Uvitec "Uvidoc" mounted camera system.

2.21.5 TOPO Cloning Reaction

2.21.5.1 Ligation

The ligation stage is used to ligate 16S rRNA into a vector or plasmid which is a naturally occurring circular piece of DNA which most bacteria possess (Appendix B). Bacteria use them to spread antibiotic resistance genes between each other for example, which is useful to use as a selective marker.

2.21.5.2 Transformation & Miniprep Procedure

After ligation of 16S rRNA gene into the vector, competent *Escherichia coli* cells were transformed with the vector containing the 16S rRNA insert. Basically, transformation is the process of forcing the *E. coli* cells to take up the plasmid DNA. Competent *E. coli* cells are made especially so that they can take up plasmid. Ligation reaction and transformation were carried out by using TOPO 10 cloning reaction protocol.

2.21.5.3 Digestion

Digest step is usually used to confirm if the plasmid has the correct insert or not. This involves cutting the DNA in a specific place using a restriction enzyme. Restriction digests usually contain 1 µl of enzyme, 1 µl 10X buffer (appropriate to the enzyme), 2 µl plasmid DNA and 6 µl MilliQ water, and then the mixture was incubated at 37°C for 2 hours. The results are checked using gel electrophoresis and samples containing

the correct insert are sent to Medical School for sequencing. The sequences were then compared to other sequences using the NCBI Blast web site.

For high quality plasmid DNA, the PureLink™ HiPure Plasmid DNA MiniprepKit was used as described in the manufacturer's protocol.

2.21.6   Phylogenetic (DNA Sequence) Analysis

For the phylogenetic placement of RV_2 and RV_4, 16S rRNA gene sequences were processed by The National Collection of Industrial, Marine and Food Bacteria (NCIMB) using the MicroSeq database and the EMBL public database. RV_3 16S rRNA gene sequences were produced by Eurofins MWG while LV_1 16S rRNA gene sequences were processed by the Medical School in the University of Sheffield.

2.22   Statistics

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

CHAPTER THREE

3 Isolation and Initial Characterisation of Acidophiles from Rivelin and Limb Valleys

3.1 Introduction

Recent research has examined microbial life in extremely low pH environments either neutral or man-made environments. This research has focused on prokaryotic microorganisms due to the importance of these microorganisms (bacteria or archaea) in environmental pollution and biotechnology applications such as biodegradation. However, eukaryotic microorganisms (algae, fungi and yeasts) are also known to be obligate acidophiles which may form stable communities with prokaryotes (Norris *et al.,* 1992, Norris and Johnson, 1998, Johnson, 1998).

However, extremophiles have also been isolated from habitats where they are not expected to be actively growing (Echigo *et al.,* 2005). Therefore, it is possible that extremely tolerant or even extremophilic microorganisms can be isolated from a wide range of environments including fresh water (Kristjansson and Hreggvidsson, 1995, Schleper *et al.,* 1997, Purdy *et al.,* 2004, Oren, 2008).

As noted in Chapter 1, microorganisms that live at extreme pH values (acidophilic or alkaliphilic) must maintain a cytoplasmic pH that is compatible with optimal functional and structural integrity of the cytoplasmic proteins that support growth (Krulwich and Guffanti, 1983, Padan *et al.,* 2001). Although acidophiles tolerate pH gradients [pH gradient (ΔpH) = pH_{in} minus pH_{out}] several orders of magnitude greater than neutralophiles, acidophiles require a moderate intracellular pH. The ΔpH across the

cytoplasmic membrane is intrinsically linked to cellular bioenergetics because it is the major contributor to the proton motive force in acidophiles. However, the influx of protons through the F_0F_1 ATPase to produce ATP intensifies cellular protonation and, if left unchecked, will rapidly dissipate the ΔpH. The functions of proteins and nucleic acids are impaired by protonation, and interference caused by free intracellular protons can impair processes such as DNA transcription, protein synthesis and enzyme activities (Madshus, 1988, Baker-Austin and Dopson, 2007).

Nevertheless, internal pH regulation mechanisms in acidophilic microorganisms although poorly understood, are essential for the survival and growth of this ecologically and biotechnologically important group of microorganisms.

The Rivelin and Limb Valleys in Sheffield have long history of industrial activity dating back to the Industrial Revolution. Therefore, it was assumed that even today, the rivers would remain polluted acidic environments. For that reason, analysis of the microbial diversity present in water samples from Rivelin and Limb Valleys was carried out using classical microbiology techniques using batch culture to isolate microorganisms from Rivelin and Limb Valley water samples which can grow at low pH values such as pH 5 and pH 3.

In this chapter, initial characterisations were performed on all four isolated strains such as cell shape under light microscope, growth curves, their ability to grow in different pH values, respiration rates, the range of salinities that all isolated strains can tolerate and some salient physiological characteristics of both prokaryotic and eukaryotic microorganisms.

3.2 Results and Discussion

3.2.1 Sample collection

3.2.1.1 Rivelin Valley

The collections of samples from the River Rivelin were carried out at two different times.

3.2.1.1.1 First Collection

A total of three samples were collected from the Rivelin Valley on 15th February 2009 (Rivelin Valley was filled with snow which made it difficult to collect samples). Samples were numbered according to their type i.e. surface water, deep water and sediment). The pH of samples was measured upon returning to the laboratory and not on site, pH value for the three samples was 7.3, 6.68 and 7.15, respectively.

3.2.1.1.2 Second Collection

On 6th April 2009 a total of four samples were collected from Rivelin Valley. The pH of samples was measured in the field and the readings were 6.3 to 7 for the four samples. The samples were numbered and photographs taken during sampling in the Rivelin Valley (Figure 3-1).

3.2.1.2 Limb Valley:

A total of three samples were collected from water in the Limb Valley on the 7th May 2010. The pH of samples was measured in the field, the highest pH was 6.5 and the lowest was 5, this latter sample was taken from a ditch in the Limb Valley. The samples were numbered and photographs taken during sampling (Figure 3-2).

Figure 3-1: Photographs showing the sampling sites in the Rivelin Valley during the second collection on 6th April 2009.

Figure 3-2: Photographs showing the sampling sites in the Limb Valley. The collection was made on the 7th May 2010.

3.2.2 Chemical Analysis of Water Samples

Table 3-1 shows that both the Rivelin and Limb Valley water have similar concentrations of Ca^{2+}, Cu, K^+, Mn^{2+}, Ni^{2+}, Pb, and Zn^{2+}, while Table 3-2 shows the ions that are found at different concentrations in the two sets of samples. It is obvious from Table 3-2 that the concentrations of Al^{3+}, S, Si, Ti and Mg^{2+} were higher in the Limb Valley samples than in Rivelin River, while the concentrations of Fe^{2+} and Na^+ were higher in Rivelin Valley samples.

Table 3-1: Cations and anions of Rivelin and Limb Valley water samples which contain similar concentrations (mg/l).

Ions	Ca	Ni	Cu	K	Mn	Zn	Pb
Rivelin	29.5	0.005	0.04	4.4	0.34	0.16	0.04
Limb	25.6	0.053	0.014	3.7	1.01	0.098	0.13

Table 3-2: Cations and anions of Rivelin and Limb Valley water samples which contain different concentrations (mg/l).

Ions	Al	S	Si	Fe	Na	Mg	Ti
Rivelin	1.09	7.9	10.3	53.5	57.5	7.3	0.06
Limb	77.5	41.4	23.1	8.2	12.6	16.6	0.26

3.2.3   Isolation and Selection of Culturable Microorganisms

Initially in order to isolate acidophilic or acid tolerant microorganisms, FeTSB liquid and solid media (sections 2.4.1 and 2.4.2) were selected for isolation of strains (Johnson *et al.,* 1987). Unfortunately, after several attempts, there was no observation of any microbial growth in this medium.

Therefore, M9 minimal medium (section 2.4.3) was used for initial isolation of strains instead of FeTSB medium. M9 medium was adjusted to three different pH values 3, 5 and 7. Seven samples of water (four from the first collection and three from the second) were collected from different points on the Rivelin River (Figure 3-1), and three samples of water were collected from Limb River and the ditch beside the river. 1 ml of each river water sample was inoculated in to 50 ml of M9 medium. Flasks were incubated overnight at 25ºC with shaking (250 rpm). In parallel, a modified ½ LB medium which has half the normal concentration of LB medium components (section 2.4.4) was used as well as M9 in order to isolate a wider variety of acidophiles or acid tolerant microorganisms.

The biomass obtained from the flasks was spread on M9 and ½ LB agar plates and single colonies from these plates were used to purify three strains from Rivelin valley RV_2, RV_3, and RV_4, and one strain from Limb valley designed as LV_1. Three strains RV_2, RV_3 and RV_4 were able to grow well in both media at three different pH values 3, 5 and 7 while LV_1 strain took a longer time to adapt and grow in low pH, especially in M9 medium. Good growth was defined as reaching at least an OD_{600} of 1 after overnight growth at 25°C with shaking (250 rpm).

3.2.4 Initial Characterisation of Bacterial Strains RV$_3$ and LV$_1$

Strains RV$_3$ and LV$_1$ were identified as bacteria and their basic phenotypic and physiological characteristics are shown in Table 3-3. Gram staining overnight cultures showed that cells of both bacteria strains are Gram positive (Figure 3-3 and Figure 3-4). RV$_3$ cells are rod-shaped and motile, LV$_1$ cells are cocci and no motility was observed with LV$_1$ cells. RV$_3$ cells have ability to form internal spores, but in contrast no spores were observed with LV$_1$ cells. RV$_3$ cells grow as facultative aerobes, whereas LV$_1$ are obligately aerobic. The most obvious difference between RV$_3$ and LV$_1$ strains was the yellow pigment produced by LV$_1$ during growth.

3.2.5 Initial Characterisation of Yeast Strains RV$_2$ and RV$_4$

Table 3-4 illustrates some basic phenotypic characteristics of strains RV$_2$ and RV$_4$, which were identified as yeast strains. Both yeast strains are motile with oval cell shape (Figure 3-5 and Figure 3-6). The most noticeable difference between the two yeast strains was the pink colour of RV$_2$ colonies on M9 medium agar plats. Also it was noticed that RV$_2$ old colonies turned from pink to black colour.

Table 3-3: The basic characteristics of the isolated bacterial strains, cells were grown in ½ LB medium, pH 5 at 25℃.

Strains	RV_3	LV_1
Physical characterisation		
Gram stain	Positive	Positive
Cell morphology	Rod	Coccus
Cell arrangement	Chain, single	Staphylo, tetrad
Pigmentation	White	Yellow
Motility	Motile	Non motile
Oxygen requirement	Facultative aerobic	An obligate aerobic
Spore forming	Positive	Negative

Table 3-4: Basic physical characteristics of the isolated yeast strains, cells were grown in M9 medium, pH 5 at 25°C.

Strains	RV$_2$	RV$_4$
Physical characterisation		
Morphology	Oval	Oval
Motility	Motile	Motile
Oxygen requirement	Aerobic	Aerobic
Fresh colony colour	Pink	White
Old colony colour	Black	White
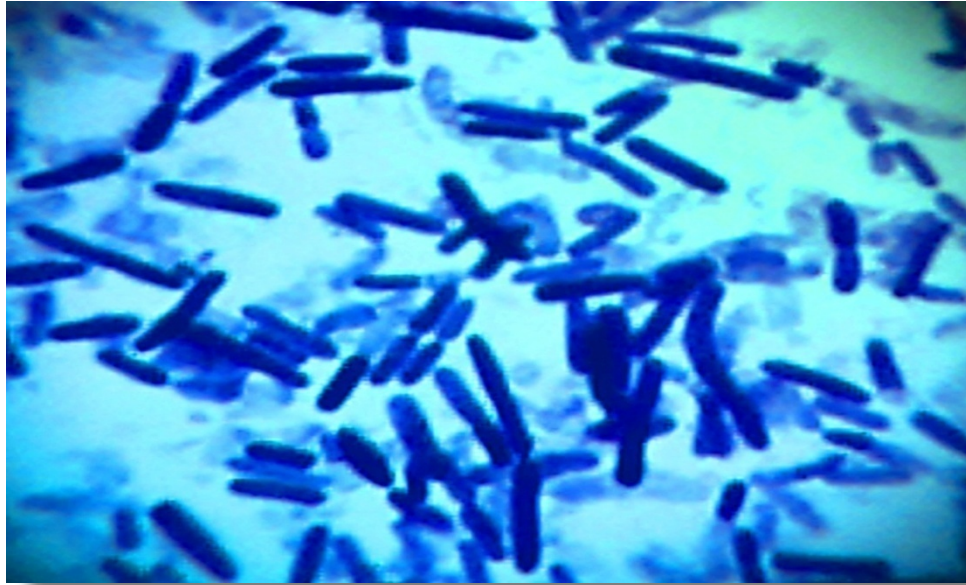
Figure 3-3: Phase contrast photomicrograph of RV_3 cells grown in ½ LB medium at pH 5 overnight at 25°C. Photomicrograph was taken using an Olympus Bx61 Upright microscope. Magnification was x 1000.
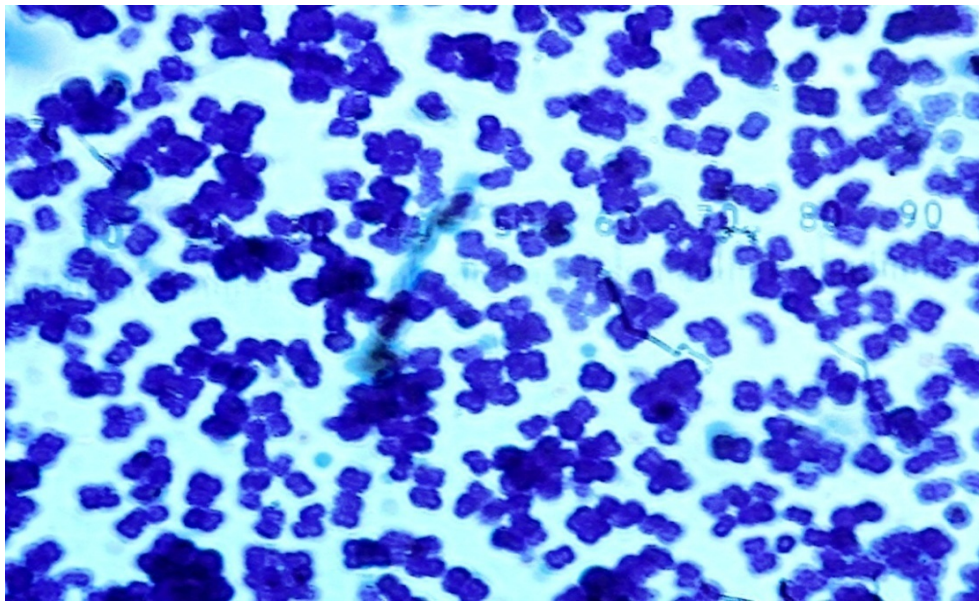
Figure 3-4: Phase contrast photomicrograph of LV_1 cells grown in ½ LB medium at pH 5 overnight at 25ºC. Photomicrograph was taken using an Olympus Bx61 Upright microscope. Magnification was x 1000.
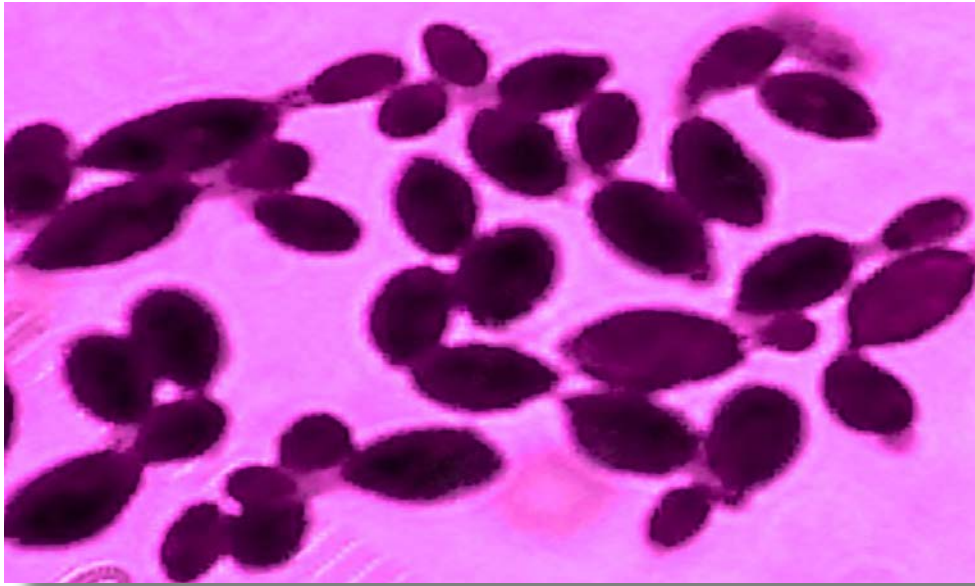
Figure 3-6: Phase contrast photomicrograph of RV_2 cells grown in M9 medium at pH 5 overnight at 25ºC. Photomicrograph was taken using the Olympus Bx61 Upright microscope. Magnification was x 1000.
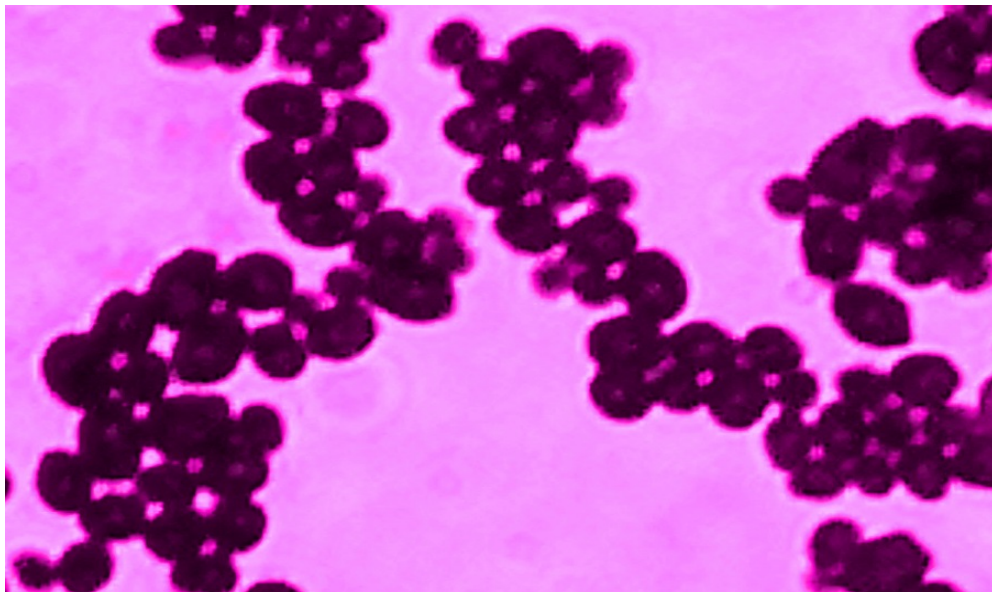
Figure 3-5: Phase contrast photomicrograph of RV_4 cells grown in M9 medium at pH 5 overnight at 25°C. Photomicrograph was taken using an Olympus Bx61 Upright microscope. Magnification was x 1000.

3.2.6 Further Characteristics of Isolated Yeast Strains

As described in section 2.17 some further physiological tests were performed to investigate and differentiate the isolated yeast strains RV_2 and RV_4.

Table 3-5 shows that both yeast strains RV2 and RV4 were unable to grow in the presence of 0.01 % (w/v) cycloheximide, while both strains fermented glucose. The characteristics of carbon and nitrogen assimilation by RV_2 and RV_4 strains were found to differ. RV_2 can assimilate nitrate, but RV_4 was negative for nitrate assimilation. In contrast, RV_4 strain was able to assimilate both unusual carbon sources tested α-Methyl glucoside and cadaverine, while RV_2 strain could not.

These salient physiological characteristic results will help to confirm the molecular based identification of the yeast species described in Chapter 4.

Table 3-5: Some of physiological characterisations of isolated yeast strains RV_2 and RV_4.

Strains	RV_2	RV_4
Cycloheximide resistance	Negative	Negative
Glucose fermentation	Positive	Positive
Assimilation of:		
Nitrate	Positive	Negative
α-Methyl glucoside	Negative	Positive
Cadaverine	Negative	Positive

3.2.7 Growth Characterisation of Isolated Strains RV$_2$, RV$_3$, RV$_4$ and LV$_1$ at Different pH Values in Different Culture Media

Immediately after isolation and at an early stage in the adaptation process, growth curves were produced for RV$_2$, RV$_3$, RV$_4$ and LV$_1$ to investigate the effect of external pH on the growth rates at pH 7, pH 5 and pH 3 in M9 minimal medium and ½ LB medium. Growth was quantified by direct optical density (OD) measurement at 600 nm using a Unicam Helisα spectrophotometer.

3.2.7.1 M9 Minimal Medium

Figure 3-7 shows that all isolated strains can grew well in M9 minimal medium at pH 7, although LV$_1$ strain grew slightly slower than the other strains. Similar results were found in pH 5 and pH 3 M9 medium but the gap between LV$_1$ and the other stains became slightly wider (Figure 3-8 and Figure 3-9). It was also clear that RV$_4$ strain was the best strain in terms of adapting and thriving in M9 minimal medium at low pH (Figure 3-8 and Figure 3-9). These interpretations of the growth curve results are supported by calculating the doubling times which are shown in Table 3-6.

3.2.7.2 Half Enrichment Medium (1/2 LB)

Growth curves shown in Figure 3-11 indicated that all isolated strains RV2, RV3, RV$_4$ and LV$_1$ can grow very well at pH 7 in ½ LB medium, but when the pH value goes down to pH 5 the growth of LV$_1$ strain was decreased while the rest of strains still maintained very good growth (Figure 3-12). At pH 3 (Figure 3-13), RV4 strain again seems to be the best strain that successfully thrives and achieves a high growth level at pH 3 in ½ LB medium. Table 3-7 and Figure 3-14 show the doubling time calculations of the growth rates for all strains on ½ LB at different pH values. It is

clear that all strains at all pH values grow faster on the richer ½ LB medium than on the minimal M9 medium.

To conclude the RV_4 strain is the most acid tolerant of the four strains tested, showing very little change in growth rate over the pH range pH 3 to pH 7 (Table 3-6 and Table 3-7). In contrast, LV_1 strain grew more slowly at low pH in both media (Table 3-6 and Table 3-7), and LV_1 cells needed a longer time to adapt and grow particularly in M9 medium (data not shown). On the other hand, RV_2 and RV_3 strains seem to prefer neutral pH rather than growing in low pH but they showed good resistance to the acidity which means their cells have been successfully adapted to tolerate pH 3 in both media (Table 3-6 and Table 3-7).
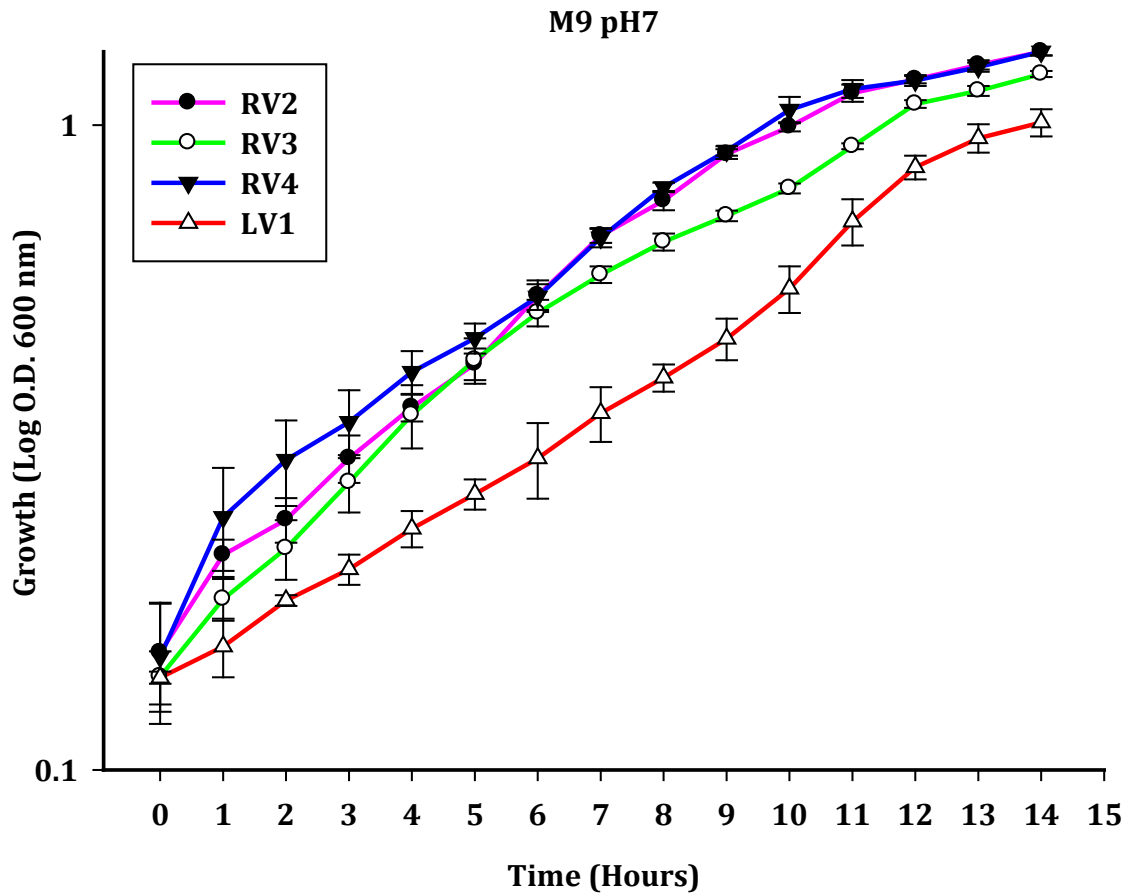
Figure 3-7: Growth curves for RV_2, RV_3, RV_4 and LV_1 cells growing in M9 minimal medium at pH 7, incubated in a 25ºC constant temperature room on an orbital shaker at 250 rpm overnight. The OD for each strain was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.
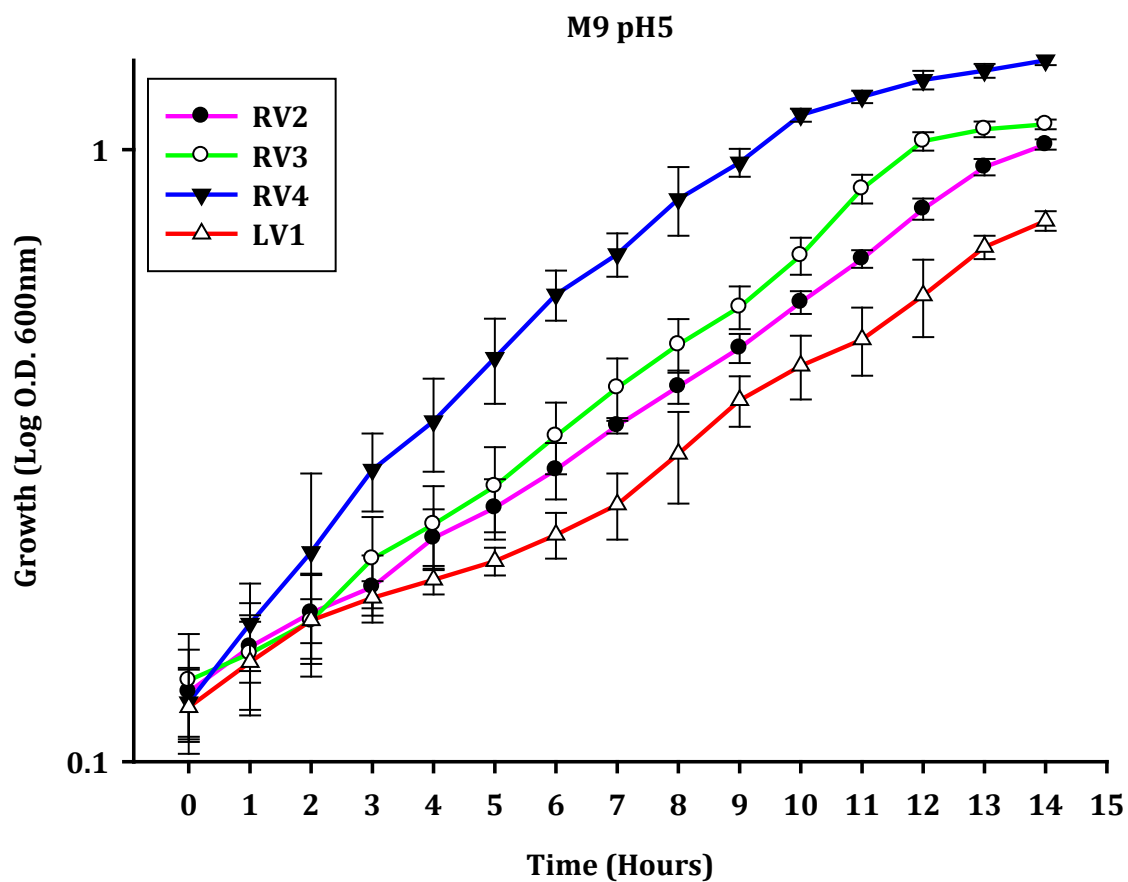
Figure 3-8: Growth curves for RV_2, RV_3, RV_4 and LV_1 cells growing in M9 minimal medium at pH 5, incubated in a 25ºC constant temperature room on an orbital shaker at 250 rpm overnight. The OD for each strain was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.
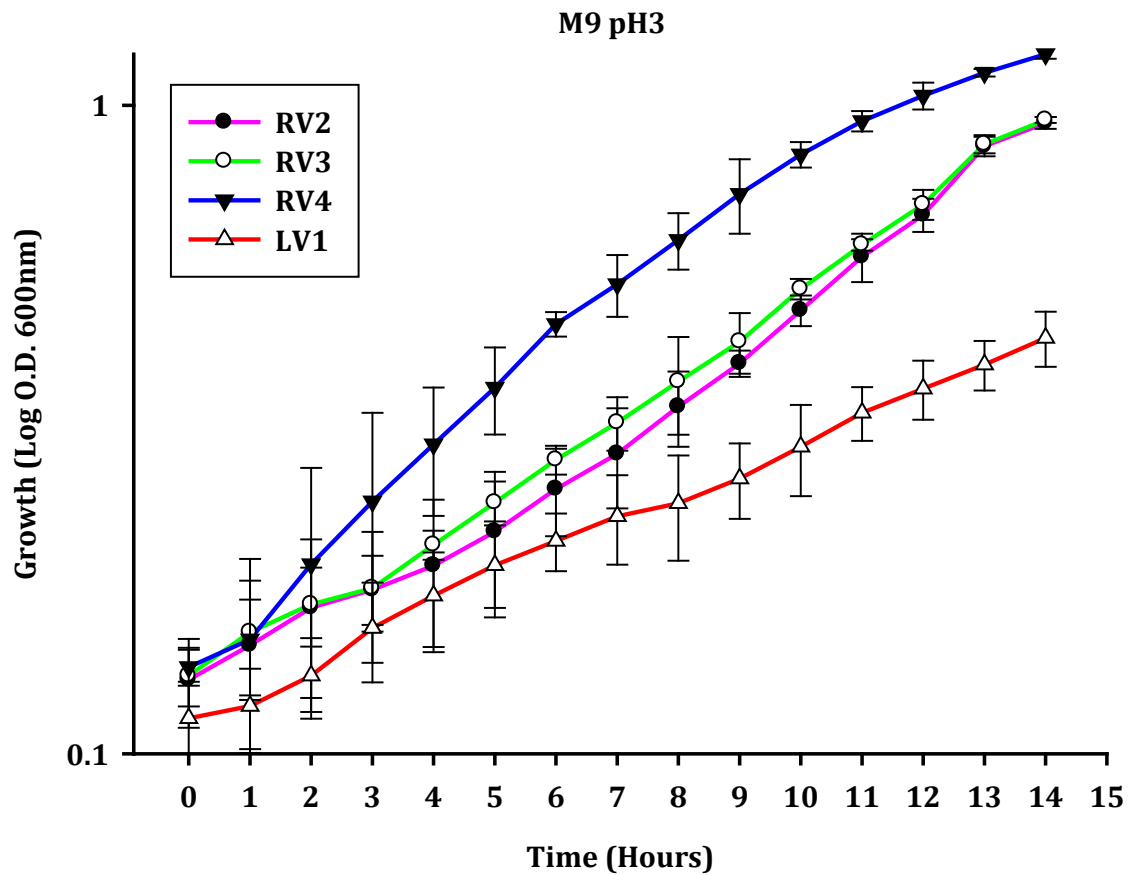
Figure 3-9: Growth curves for RV2, RV3, RV4 and LV1 cells growing in M9 minimal medium at pH 3, incubated in a 25ºC constant temperature room on an orbital shaker at 250 rpm overnight. The OD for each strain was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.

Table 3-6: Effect of external pH on the doubling times of strains RV_2, RV_3, RV_4 and LV_1 in M9 minimal medium. Each point represents the mean from four replicate samples.

	Doubling Time of Strains in M9 medium (Minutes)			
	RV2	RV3	RV4	LV1
pH7	200	198	189	225
pH5	269	258	180	293
pH3	292	284	182	408
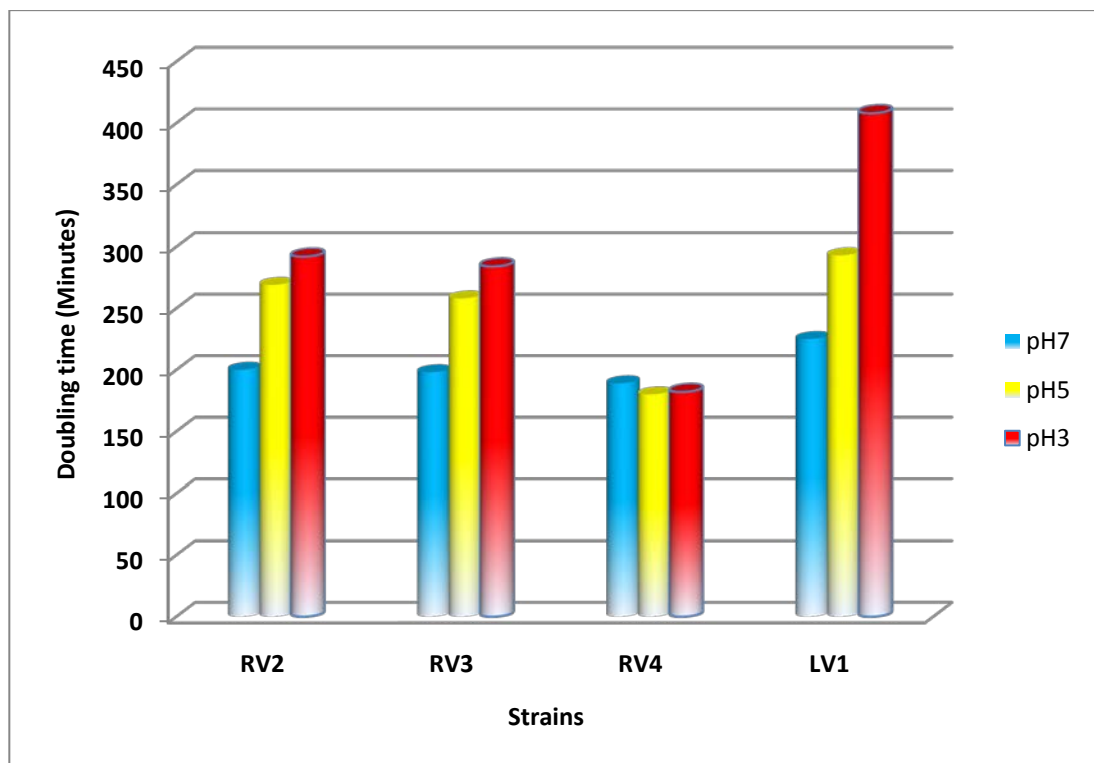
Figure 3-10: Effect of external pH on the doubling times of strains RV_2, RV_3, RV_4 and LV_1 in M9 minimal medium. Each point represents the mean from four replicate samples.

1/2 LB pH7
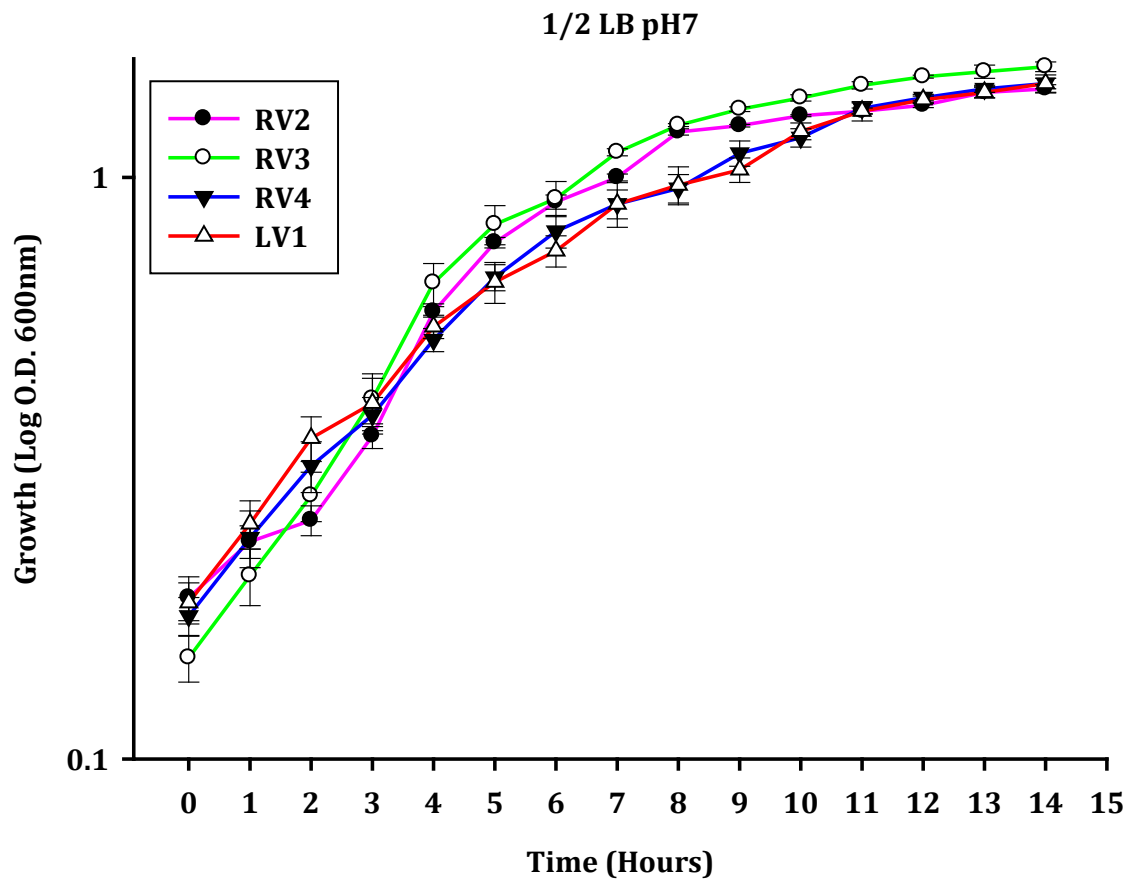
Figure 3-11: Growth curves for RV_2, RV_3, RV_4 and LV_1 cells growing in ½ LB medium at pH 7, incubated in a 25ºC constant temperature room on an orbital shaker at 250 rpm overnight. The OD for each strain was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.
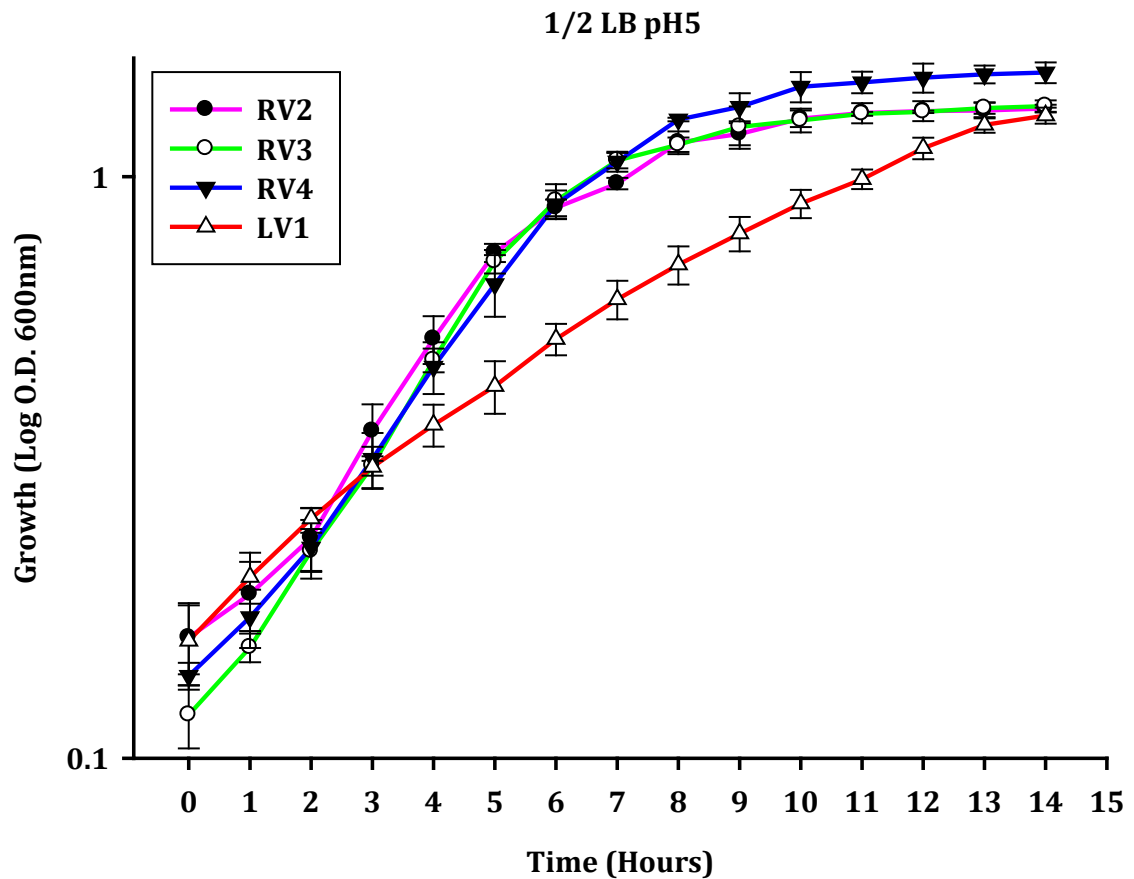
Figure 3-12: Growth curves for RV2, RV3, RV4 and LV1 cells growing in ½ LB medium at pH 5, incubated in a 25ºC constant temperature room on an orbital shaker at 250 rpm overnight. The OD for each strain was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.
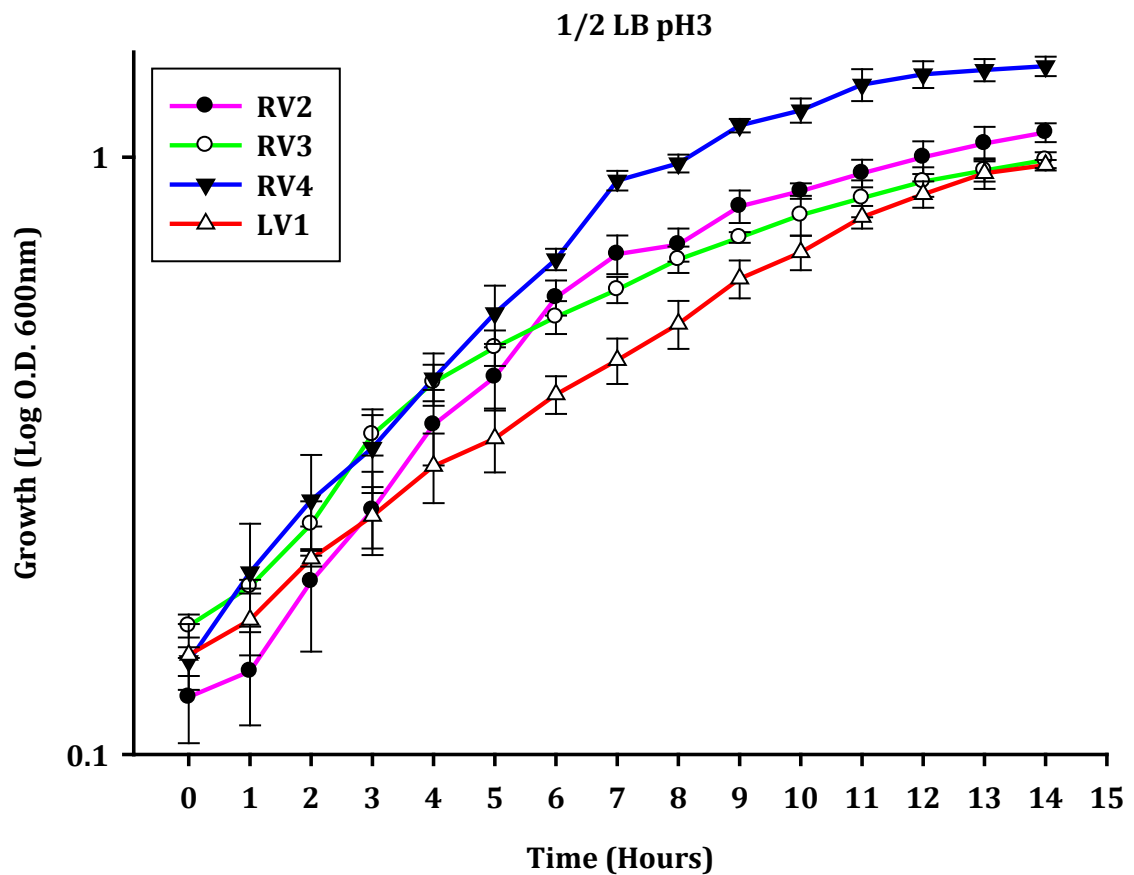
Figure 3-13: Growth curves for RV2, RV3, RV4 and LV1 cells growing in ½ LB medium at pH3, incubated in a 25ºC constant temperature room on an orbital shaker at 250 rpm overnight. The OD for each strain was measured at 600 nm. Data points are the means of three replicates plus or minus standard deviation.

Table 3-7: Effect of external pH on the doubling times of strains RV_2, RV_3, RV_4 and LV_1 in ½ LB medium. Each point represents the mean from four replicate samples.

Doubling Time of Strains in 1/2 LB medium (Minutes)				
	RV2	**RV3**	**RV4**	**LV1**
pH7	110	102	156	138
pH5	145	134	125	246
pH3	192	186	162	276
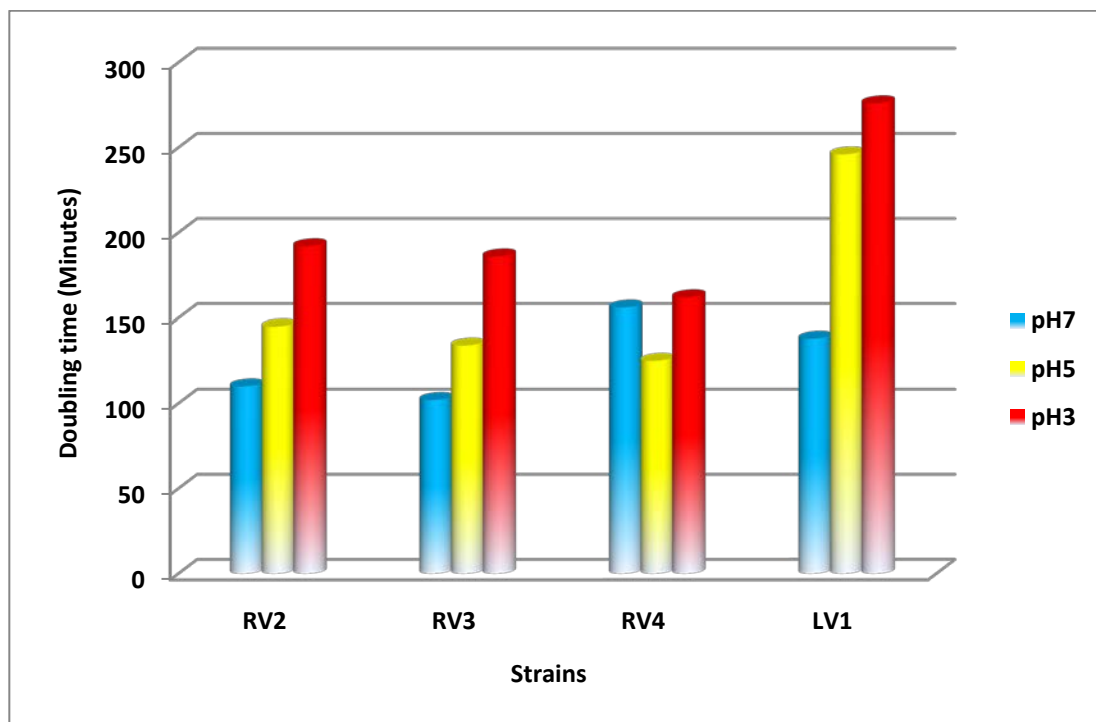
Figure 3-14: Effect of external pH on the doubling times of strains RV_2, RV_3, RV_4 and LV_1 in ½ LB medium. Each point represents the mean from four replicate samples.

3.2.8 Effect of External pH on Respiration Rate of RV_2, RV_3, RV_4 and LV_1 Strains

As described in section 2.10, the respiration rate of strains RV_2, RV_3, RV_4 and LV_1, was measured using a Clarke-type oxygen electrode in order to observe the effect of external pH on the rate of uptake of oxygen over a pH range of pH 7 to 3 in ½ LB medium.

Cells were grown in ½ LB medium at pH 3, 5 and 7, and then respiration rate was measured in the growth pH (control) plus the other two pH values. The red values in Tables 2 to 5 are the control values,

Table 3-8 shows that the respiration rate of RV_2 cells was highest at neutral pH and the respiration decreased with decreasing pH. There was a significant fall in the rate of O_2 uptake from 4.2 µmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 7 to 2 µmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 3. This result was confirmed with the cells grown at pH 7 and shocked at pH 5 and pH 3 where the respiration rate decreased from 4.2 µmoles O_2 taken up mg^{-1} cell protein h^{-1} to 2.8 and 2.6 µmoles O_2 taken up mg^{-1} cell protein h^{-1}, respectively.

Similar results occurred with RV_3 strain, the respiration rate goes down with the increasing of acidity (Table 3-9). Respiration rate was 3.9 µmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 7 and decreased to 3.3 and 3.2 µmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 5 and pH 3, respectively. The respiration rate of RV_3 cells grown at pH 7 and resuspended in pH 5 and pH 3 drop from 3.9 µmoles O_2 taken up mg^{-1} cell

protein h^{-1} to 3.3 and 2.8 μmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 5 and pH 3, respectively.

In contrast, RV$_4$ cells adapted to pH 3 have the highest rate of respiration at all three pH values tested (Table 3-10). The O_2 uptake decreased from 3 μmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 3 to 2.2 and 1.5 at pH 5 and pH 7, respectively. This finding was confirmed when for the cells grown at pH 7 and shocked at pH 5 and pH 7 the respiration rate rose from 1.5 μmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 7 to 1.6 and 1.9 at pH 5 and pH 3, respectively.

Table 3-11 shows that the O2 uptake rate of LV1 strain was decreased with the increasing of external pH. The respiration rate drops from 5.2 μmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 7 to 4.2 and 3.7 μmoles O_2 taken up mg^{-1} cell protein h^{-1} at pH 5 and pH 3, respectively. The shock experiments gave same results, when cells were grown at neutral pH and resuspended at low pH the respiration rate fell, while when the cells were grown at low pH and resuspended at higher pH the respiration rate increased.

In general, these results supported and agreed with the growth curve results (Figure 3-11 and Figure 3-13), which suggested that RV$_4$ strain is the most acidophilic microorganism of the four strains while LV$_1$ strain was the weakest one in terms of adapting to grow at low pH.

Table 3-8: Respiration rate for strain RV_2 subjected to pH stress in ½ LB medium (µmoles mg^{-1} protein h^{-1}). 5 ml of cells from each strain grown at pH 7, 5 and 3 in ½ LB medium overnight at 25°C on an orbital shaker at 250 rpm were harvested by centrifugation and resuspended in either 10 ml of fresh ½ LB medium of the same pH value or shocked by resuspension in a different pH value. The O_2 uptake was measured as described in section 2.11 immediately after resuspension in the fresh medium. Data points are the means of three replicates plus or minus standard deviations.

	Fresh 1/2 LB Shock Medium		
	pH3	**pH5**	**pH7**
Overnight cultures	↓	↓	↓
pH3	2.0 ±0.1	1.8 ±0.1	2.0 ±0.1
pH5	3.3 ±0.2	3.6 ±0.2	3.6 ±0.3
pH7	2.6 ±0.03	2.8 ±0.03	4.2 ±0.03

Table 3-9: Respiration rate for strain RV$_3$ subjected to pH stress in ½ LB medium (μmoles mg^{-1} protein h^{-1}). 5 ml of cells from each strain grown at pH 7, 5 and 3 in ½ LB medium overnight at 25°C on an orbital shaker at 250 rpm were harvested by centrifugation and resuspended in either 10 ml of fresh ½ LB medium of the same pH value or shocked by resuspension in a different pH value. The O$_2$ uptake was measured as described in section 2.11 immediately after resuspension in the fresh medium. Data points are the means of three replicates plus or minus standard deviations.

	Fresh 1/2 LB Shock Medium		
	pH3	pH5	pH7
Overnight cultures	↓	↓	↓
pH3	3.2 ±0.3	3.7 ±0.2	3.7 ±0.03
pH5	3.0 ±0.3	3.3 ±0.2	3.4 ±0.2
pH7	2.8 ±0.2	3.2 ±0.3	3.9 ±0.3

Table 3-10: Respiration rate for strain RV_4 subjected to pH stress in ½ LB medium (μmoles mg^{-1} protein h^{-1}). 5 ml of cells from each strain grown at pH 7, 5 and 3 in ½ LB medium overnight at 25°C on an orbital shaker at 250 rpm were harvested by centrifugation and resuspended in either 10 ml of fresh ½ LB medium of the same pH value or shocked by resuspension in a different pH value. The O_2 uptake was measured as described in section 2.11 immediately after resuspension in the fresh medium. Data points are the means of three replicates plus or minus standard deviations.

	Fresh 1/2 LB shock Medium		
	pH3	pH5	pH7
Overnight cultures	↓	↓	↓
pH3	3.0 ±0.1	3.0 ±0.2	2.9 ±0.0
pH5	2.0 ±0.2	2.2 ±0.1	2.0 ±0.1
pH7	1.9 ±0.05	1.6 ±0.1	1.5 ±0.1

Table 3-11: Respiration rate for strain LV_1 subjected to pH stress in ½ LB medium (µmoles mg^{-1} protein h^{-1}). 5 ml of cells from each strain grown at pH 7, 5 and 3 in ½ LB medium overnight at 25°C on an orbital shaker at 250 rpm were harvested by centrifugation and resuspended in either 10 ml of fresh ½ LB medium of the same pH value or shocked by resuspension in a different pH value. The O_2 uptake was measured as described in section 2.11 immediately after resuspension in the fresh medium. Data points are the means of three replicates plus or minus standard deviations.

	Fresh 1/2 LB shock Medium		
	pH3	pH5	pH7
Overnight cultures	↓	↓	↓
pH3	3.7 ±0.3	4.0 ±0.2	4.3 ±0.3
pH5	4.1 ±0.2	4.2 ±0.1	4.4 ±0.06
pH7	4.0 ±0.1	4.4 ±0.2	5.2 ±0.3

3.2.9 Effect of High Salinity on the Overnight Growth of RV$_2$, RV$_3$, RV$_4$ and LV$_1$ Strains in ½ LB Medium at Different pH Values

In order to investigate the range of salinities that the isolated strains can tolerate, cells of each strain were repeatedly sub-cultured in ½ LB medium containing higher levels of NaCl at different pH values 7, 5 and 3on an incremental basis. Initially, all strains were adapted to grow in 0.4, 1 and 1.5 M NaCl, then 2, 2.5 and 3 M NaCl over a period of several weeks.

Figure 3-15 shows the effect of different concentrations of sodium chloride on the growth of all isolated strains in ½ LB medium at pH 7. The results indicate that RV$_4$ and LV$_1$ strains seem to be halophiles or at least halotolerant microorganisms, both strains were able to grow with optimally at 0.4 to 1.0 M NaCl respectively, but when the NaCl concentration was increasing up to 1.5 M there was an inhibitory effect and the growth of both strains was significantly slower at 2 M NaCl. Similar results were found for RV$_4$ and LV$_1$ at pH 5 and pH 3 as shown in Figure 3-16 and Figure 3-17 showing clearly that both strains were capable of tolerating high salinity concentrations.

On the other hand, Figure 3-15 shows that RV$_2$ and RV$_3$ strains were able to grow optimally at 0.1 M NaCl. Growth was similar to growth in control ½ LB medium at pH 7 which contains 50 mM NaCl. Increasing the concentration of NaCl to more than 0.4 M NaCl significantly reduced the growth rate of both strains and there was no growth above 1 M NaCl. Furthermore, when pH was decreased to pH 5 and pH 3 both strains barely grew when salinity reached 0.4 M NaCl and there was no growth at salinities above this level as shown in Figure 3-16 and Figure 3-17. So, these results suggested that RV2 and RV3 strains cells could not tolerate more than 0.4 M NaCl.

1/2 LB pH7
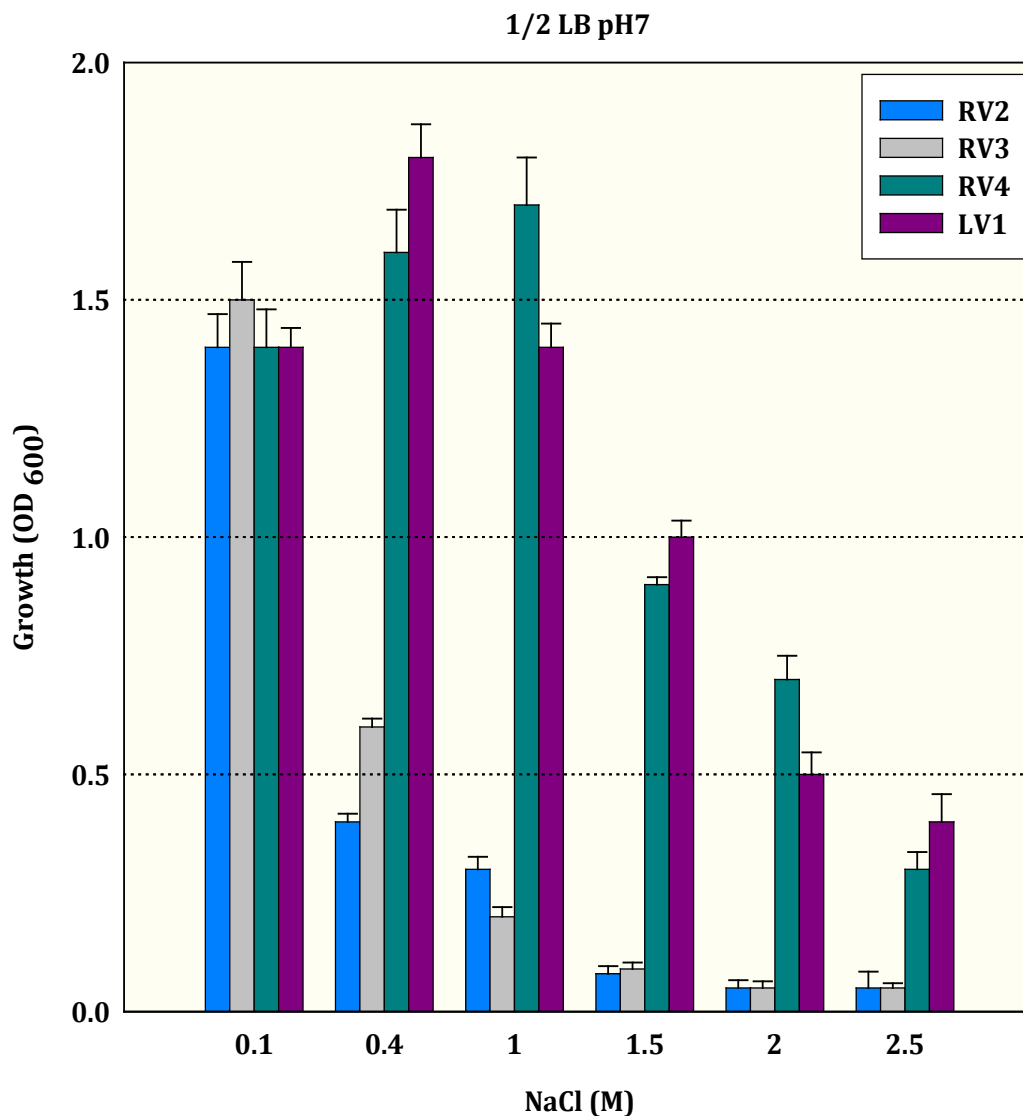
Figure 3-15: Effect of different NaCl concentrations on the overnight growth of RV_2, RV_3, RV_4 and LV_1 strains. Cells were grown in ½ LB medium at pH 7. The OD was measured at 600 nm after overnight incubation at 25°C with shaking at 250 rpm. Data points are the means of three replicates plus or minus standard deviations.
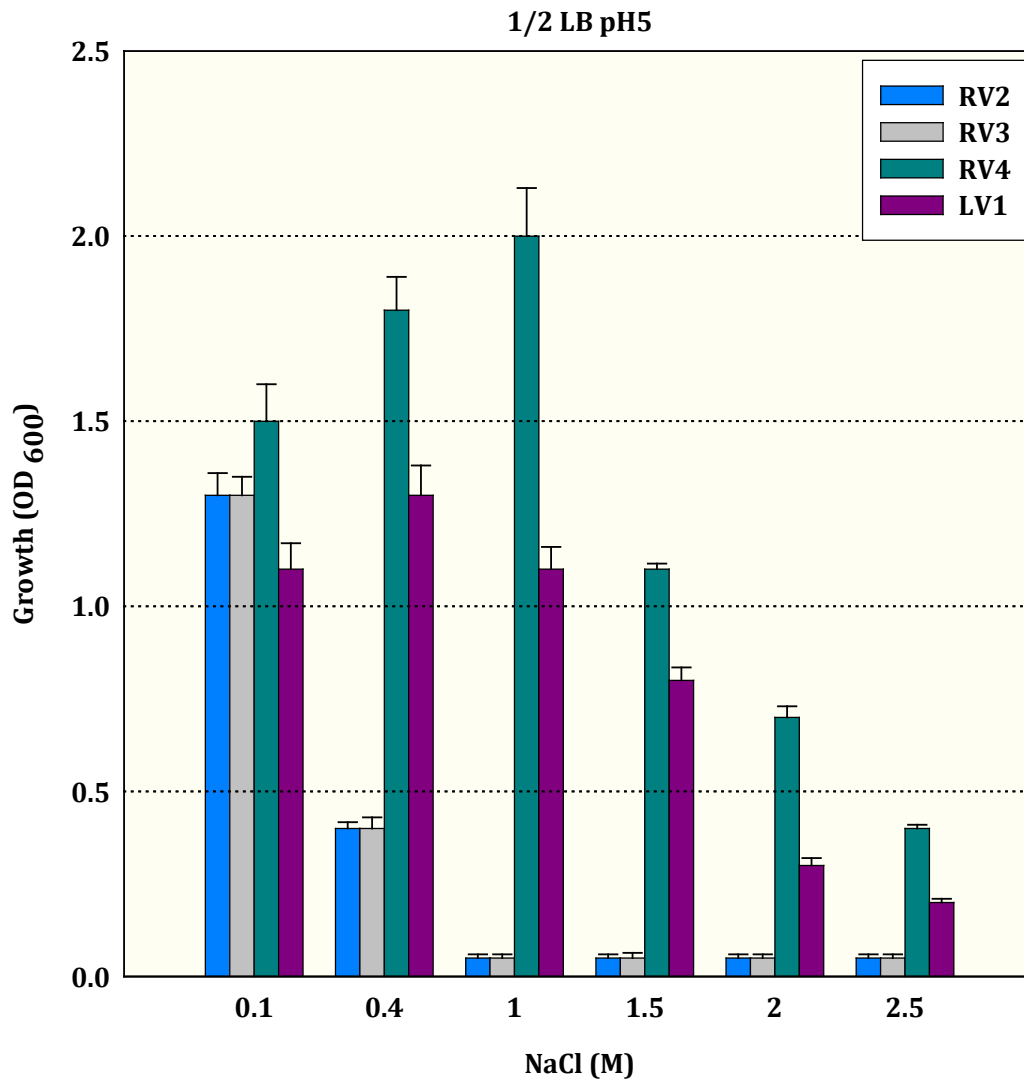
Figure 3-16: Effect of different NaCl concentrations on the overnight growth of RV_2, RV_3, RV_4 and LV_1 strains. Cells were grown in ½ LB medium at pH 5. The OD was measured at 600 nm after overnight incubation at 25°C with shaking at 250 rpm. Data points are the means of three replicates plus or minus standard deviations.
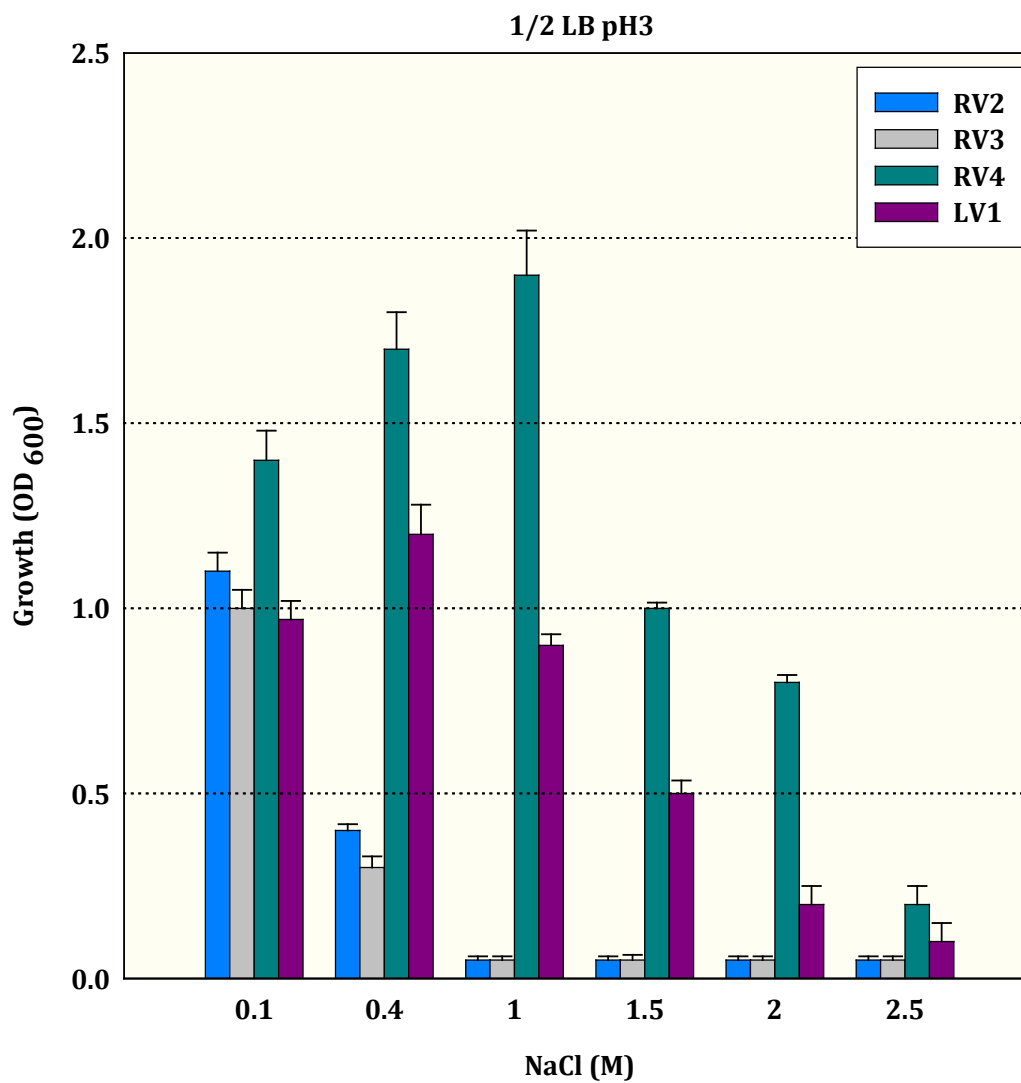
Figure 3-17: Effect of different NaCl concentrations on the overnight growth of RV_2, RV_3, RV_4 and LV_1 strains. Cells were grown in ½ LB medium at pH 3. The OD was measured at 600 nm after overnight incubation at 25°C with shaking at 250 rpm. Data points are the means of three replicates plus or minus standard deviations.

3.2.10 Measurement of External pH of Overnight Cultures of RV_2, RV_3, RV_4 and LV_1 strains

As described in section 2.12 the external pH of overnight cultures of all strains was measured in order to investigate the ability of the strains to alter the external pH of M9 and ½ LB media set to pH values 7, 5 and 3.

Table 3-12 illustrated that all four strains did not made any significant changes to the external pH in M9 medium, except RV_4 strain where the pH was reduced from 5 to 3.65. In contrast when RV_2, RV_3, and RV_4 were grown in ½ LB medium at pH 3 their cells increased the pH to 7.7, 6.5, and 6.8 respectively, while only a slight increase from pH 3 to pH 4.3 happened with LV_1 strain possibly due to the weaker growth of LV_1 at pH 3 (Table 3-13).

Large changes of external pH also occurred when strains were cultured in ½ LB medium at pH 5 and pH 7, all strains increased the pH values as shown in Table 3-13.

Table 3-12: Overnight cultures external pH of RV_2, RV_3, RV_4 and LV_1 strains at different pH values in M9 minimal medium.

	Overnight external pH			
	RV_2	**RV_3**	**RV_4**	**LV_1**
Initial external pH				
pH3	3.4 ±0.02	3.3 ±0.03	3.1 ±0.01	3.2 ±0.02
pH5	5.5 ±0.04	5.6 ±0.04	3.65 ±0.03	5.9± 0.05
pH7	7.2 ±0.05	7.3 ±0.04	7.0 ±0.06	7.3 ±0.03

Table 3-13: Overnight cultures external pH of RV_2, RV_3, RV_4 and LV_1 strains at different pH values in ½ LB medium.

	Overnight external pH			
	RV_2	RV_3	RV_4	LV_1
Initial external pH				
pH3	7.7 ±0.09	6.5 ±0.08	6.8 ±0.04	4.3 ±0.06
pH5	8.5 ±0.1	8.9 ±0.09	8.8 ±0.08	8.2 ±0.09
pH7	9.0 ±0.07	8.9 ±0.09	9.2 ±0.1	8.6 ±0.08

3.3   Conclusions

Various samples of sediment and liquid were collected from Rivelin and Limb Valleys in Sheffield and minimal and half enrichment cultures were set up at pH 3, pH 5 and pH 7 in M9 and ½ LB medium at 25ºC to grow and isolate acidophilic or acid tolerant microorganisms. Four strains (RV_2, RV_3, RV_4 and LV_1) were chosen for further studies due to their good growth at different pH values of pH 7, 5 and 3, and also RV_4 and LV_1 were chosen for further studies due to their ability to grow at high salinities.

Two prokaryotic strains RV_3 and LV_1 were successfully isolated and shown to be Gram-positive, aerobic microorganisms. RV_3 strain was motile while LV_1 strain was non-motile. Moreover, the other two strains isolated from the Rivelin Valley samples (RV_2 and RV_4) were eukaryotic microorganisms and characterised as yeast strains. Both strains were able to ferment glucose and could not grow in the presence of cycloheximide. RV_2 strain was found to assimilate nitrate while RV_4 could not. On the other hand, RV_4 strain was able to assimilate α-methyl glucoside and cadaverine while RV_2 strain could not.

At an early stage in the adaptation process, LV_1 strain did not grow at pH 3 in M9 medium, while RV_2, RV_3 and RV_4 showed a good growth at all pH values in M9 minimal medium. However, RV_4 strain was the best strain in terms of producing the best growth at pH 5 and pH 3 in both media. LV1 strain showed the lowest growth at low pH, particularly in M9 medium, and it should be noted that repeated sub-

culturing for several weeks was required to allow LV_1 strain to adapt and grow at pH 3 in both media.

The impact of different external pH values on the respiration rates of the four strains was also measured. RV_2, RV_3 and LV_1 strains showed their highest respiration rates at pH 7, while RV_4 showed its highest respiration rate at pH 3. The respiration results showed broad agreement with growth curve results. So, on the basis of these characteristics, the RV_4 strain was classified as aerobic acidophilic yeast and was selected for further study.

The tolerance of the strains to different salinities was examined, and the results indicated that RV_4 and LV_1 strains were able to grow at high salinities up to 2.5 M NaCl in M9 minimal and ½ LB medium.

To conclude, it was decided to further investigate the bioenergetics of the growth of RV_4 strain at pH 3 including the determination of membrane potential, and internal pH (Chapter 6). Furthermore, it was decided that further physiological work was required to investigate the metabolic processes that allow RV_4 and LV_1 strains to adapt and grow at high salinity (Chapter 5).

However, first of all in the next chapter, the four strains will be identified using molecular identification techniques, including DNA extraction, PCR amplification and purification of 16S rRNA gene for bacteria strains and 18S rRNA gene for yeast strains.

CHAPTER FOUR

4 Molecular Identification of RV$_2$, RV$_3$, RV$_4$ and LV$_1$ Strains

4.1 Introduction

Using phenotypic information as the only method to identify species in microbiology is no longer recommended, but this information is helpful for initial description of unknown strains. Therefore, traditional microbiology methods which have been used use to classify organisms depending on phenotypic characters such as morphology, physiology, and metabolism are being supplemented by genotypic analysis (Scow *et al.,* 2001).

Since the discovery of the polymerase chain reaction (PCR), molecular methods are much more reliable for relating and classifying organisms than phenotypic information, because they are more informative with regard to evolutionary relationships and they are also now more readily available (Woese, 1987, Lexa *et al.,* 2001, Fenollar *et al.,* 2006, Yeung *et al.,* 2009)

Ribosomal RNA (rRNA) is the most commonly used molecular marker for several reasons. Firstly and most importantly, it is present in all organisms and has been for a very long time (Olsen *et al.,* 1986). The overall structure of rRNA is well conserved between all organisms which means that it is easy to identify rRNA by size alone (Olsen *et al.,* 1986, Woo *et al.,* 2000). rRNA molecules are a necessary element of one of the most basic cellular functions, protein synthesis, and thus, form a significant proportion of cellular mass, therefore rRNA is readily recoverable from all cell types (Olsen *et al.,* 1986). In addition, it is possible to

sequence rRNA directly and rapidly using reverse transcriptase (Woese, 1987, Hugenholtz and Pace, 1996). 16S rRNA gene sequence is also chosen because it contains regions of conserved, variable and hyper variable sequence. For example, archaeal 16S rRNA molecules are easily identified from bacterial 16S rRNA or eukaryal 18S rRNA by their unique structure between positions 180 and 197 (Woese *et al.,* 1990, Johnson *et al.,* 2001, Ma *et al.,* 2004). In addition, 16S rRNA molecules are large (approximately 13 times larger than 5S rRNA) and consist of many domains which also facilitate their use as molecular markers and contain sufficient information for identification and phylogenetic analyses of organisms (Woese *et al.,* 1990, Drancourt *et al.,* 2000, Patel, 2001).

In this chapter, RV_3 and LV_1 bacterial strains, isolated from Rivelin and Limb valleys respectively, were identified to the genus level by the use of 16S rRNA gene sequencing. The 16S rRNA gene was amplified using bacterial primers by the polymerase chain reaction (PCR) technique. PCR products were ligated into the cloning vector pCR2.1 and then transferred into *E. coli* competent cells. The plasmid was then isolated and the 16S rRNA gene sequenced as described in section 2.21.5. The sequences from RV_3 and LV_1 were compared with known 16S rRNA gene sequences using the NCBI Blast function. Phylogenetic trees were also constructed for both strains to predict their genetic relatedness to known strains in the database.

The yeast strains RV_2 and RV_4, after many unsuccessful attempts to extract their genomic DNA using many different methods such as chemical CTAB protocol, QIAgen Genomic Kit and Anachem Key Prep kits, were sent to The National

Collection of Industrial, Marine and Food Bacteria (NCIMB) for 16S rDNA sequencing and their identification was achieved by using the MicroSeq database and the EMBL public database.

4.2   Results and Discussion

4.2.1   Bacterial Strains

4.2.1.1 Extraction of Genomic DNA

High molecular weight genomic DNA was extracted from the bacterial strains as described in section 2.12. RV_3 and LV_1 cultures were grown overnight on ½ LB medium at pH 3 to produce the biomass required for DNA extraction. The DNA extraction process normally involves cell breakage by digesting cell wall, centrifugation to remove the cell fragments and debris and then nucleic acid precipitation and purification.

Figure 4-1 shows that genomic DNA was successfully extracted from RV3 strain (lanes 2 and 3) and LV_1 strain (lanes 6 and 7) using Anachem Key Prep Genomic DNA kit. In addition, the figure shows the purity of DNA and the efficiency of the Anachem Key Prep kit in removing polysaccharides, proteins and any other contaminating molecules. Also from this result it can be noted the high molecular weight DNA is free from RNA contamination to allow the amplification of 16S rRNA gene using PCR technique.
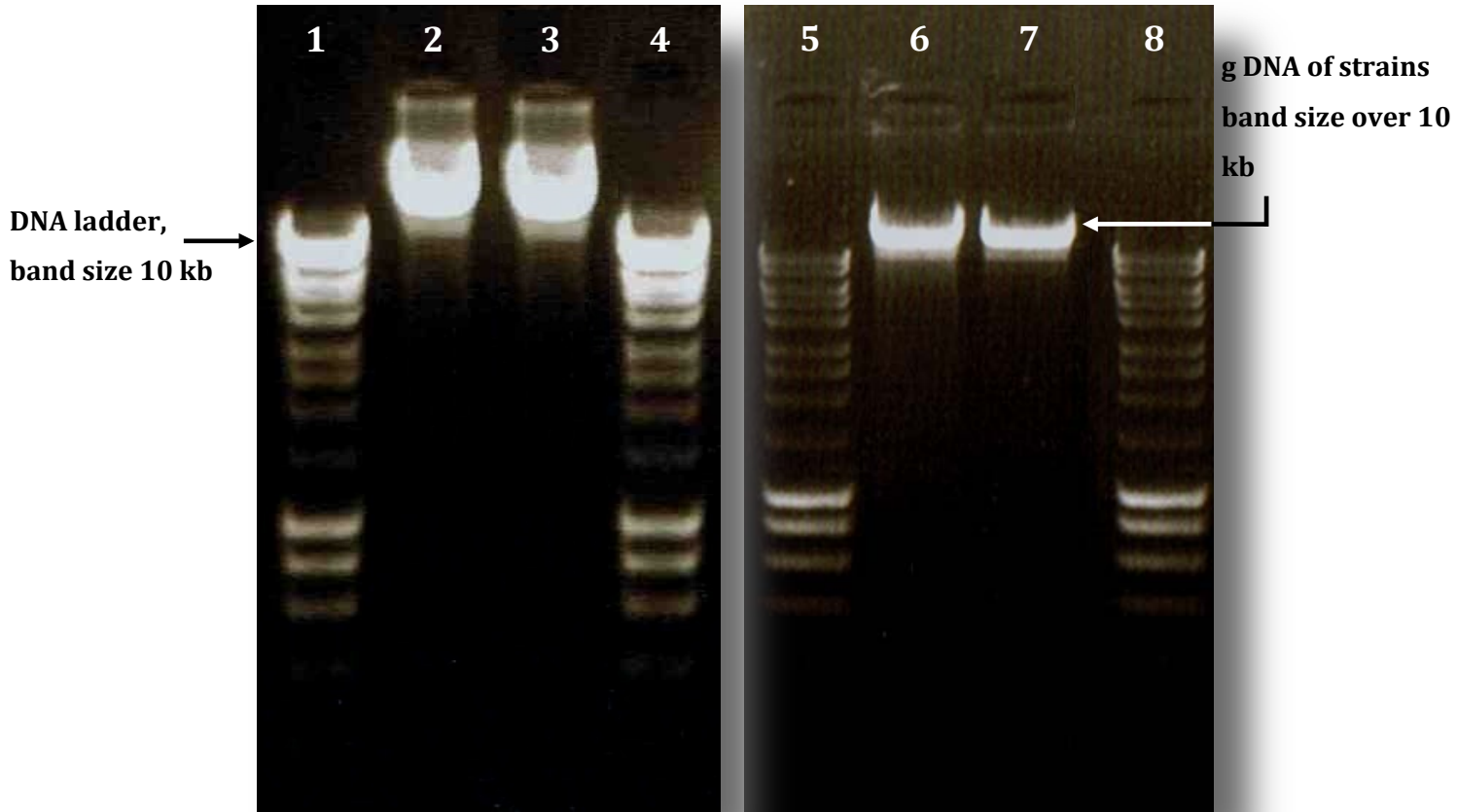
Figure 4-1: Agarose gel 1% electrophoresis with ethidium bromide showing 1 kb DNA ladder (lanes 1, 4 ,5 and 8) and genomic DNA extraction with a size over 10000 base pairs from both strains RV_3 (lanes 2 and 3) and LV_1 (lanes 6 and 7). Genomic DNA was extracted using Anachem Key Prep Genomic DNA kit.

4.2.1.2   PCR Amplification of 16S rRNA Gene of Bacterial Isolates

PCR amplification protocol was carried out in order to amplify the 16S rRNA gene from the genomic DNA extracted from RV_3 and LV_1 strains (see section 2.21.2). Two universal bacterial primers were used to amplify the 16S rRNA gene (Chen *et al.,* 2001). Universal primers: Forward primer 5`-AGAGTTTGATCCTGGCTCAG-3` and reverse primer 5`-GGTTACCTTGTTACGACTT-3` designed to target the conserved regions of the 16S rRNA gene (Lane, 1991).

Figure 4-2 shows the amplification product of 16S rRNA gene for RV3 (lanes 2 and 3) and LV_1 (lanes 4 and 5). The results demonstrated the success of the amplification process of 16S rRNA gene from genomic DNA for both strains with correct fragment band size for 16S rRNA gene of approximately 1500 base pairs. The PCR product (16S rRNA gene) was cleaned up using Anachem Key Prep PCR Purification Kit protocol (see section 2.21.3). The results shown in Figure 4-3 indicated that the purification of PCR product was successful and this step removes any possibility of the presence of compounds that could affect the purity of the 16S rRNA gene such as proteins, extra primers, salts and dNTPs.
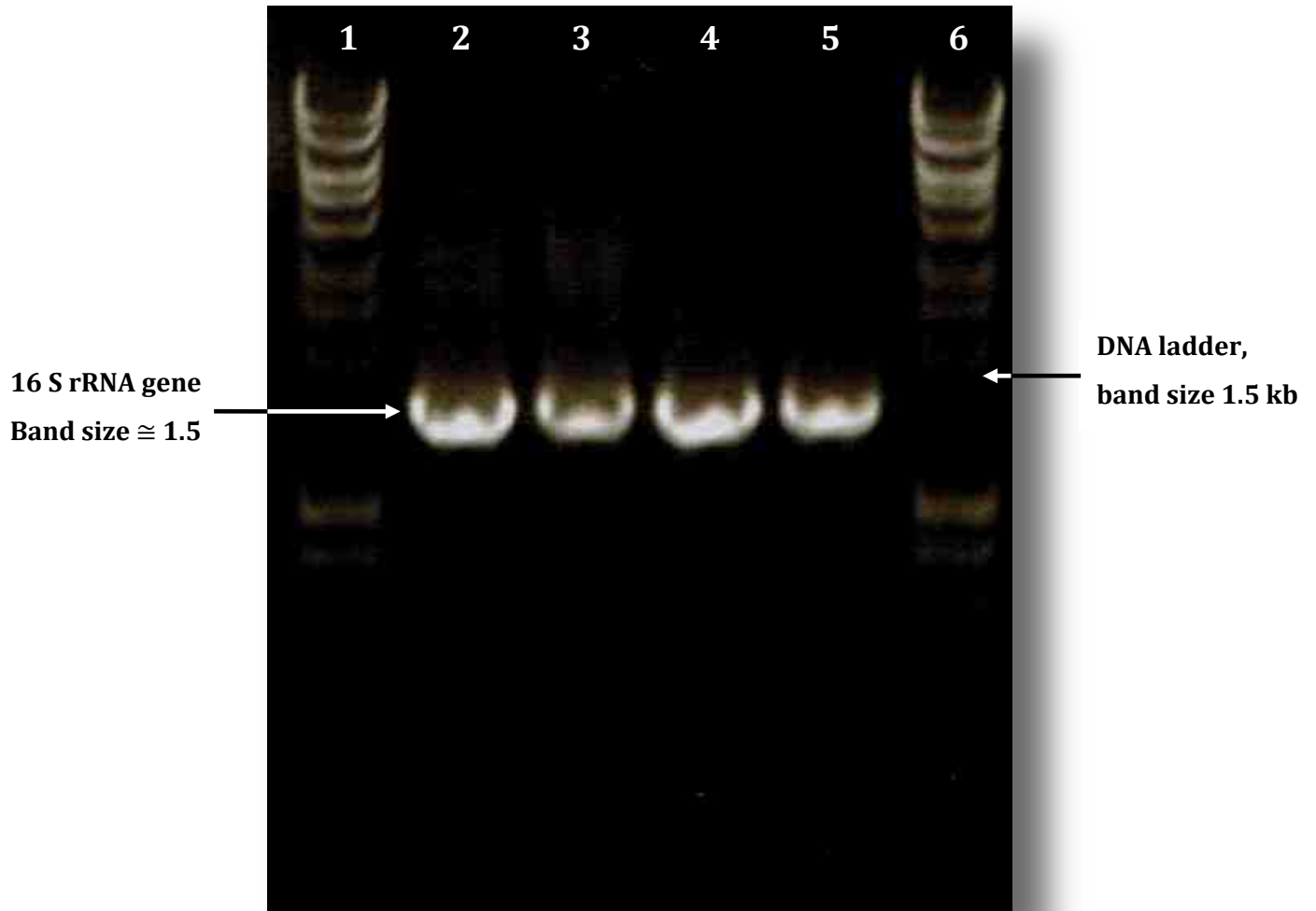
Figure 4-2: Agarose gel 1% electrophoresis with ethidium bromide showing the resolution of an approximate 1500 base pair amplification product (16S rRNA gene) from the PCR involving the universal bacterial primers and genomic DNA from strains RV_3 (lanes 2 and 3) and LV_1 (lanes 4 and 5). Lanes 1 and 6 show the 10 kb DNA ladder.
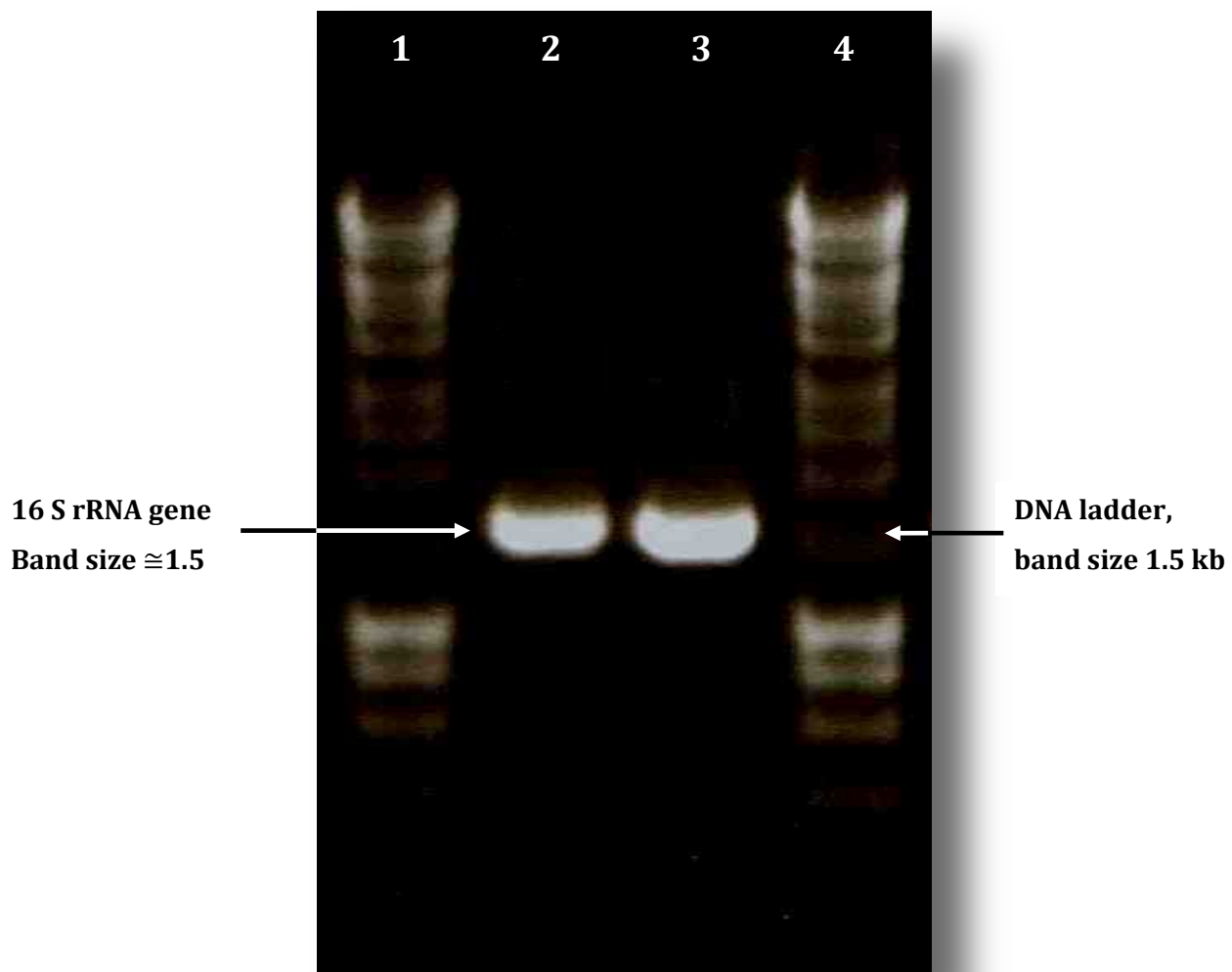
Figure 4-3: Agarose gel 1% electrophoresis with ethidium bromide showing the resolution of an approximate 1500 base pair purification products (16S rRNA gene) from the PCR after purification using Anachem Key Prep PCR Purification Kit. RV$_3$ strain (lane 2) and LV$_1$ strain (lane 3). Lanes 1 and 4 show the 10 kb DNA ladder.

4.2.1.3   Cloning of PCR Products and Transformation of E. coli

Bacterial 16S rRNA gene sequences can be obtained directly from PCR products. However, the sequence returned from direct sequencing of PCR products is often only 800 - 1000 bp, significantly shorter than the full 1500 bp. Therefore, in this study the amplified 16S rRNA gene of both strains RV_3 and LV_1 was ligated into pCR2.1 vector (3.9 kb) (see appendix B) in order to obtain a longer read of the sequences (1.5 kb) for better comparison with other sequences in the genomic DNA databases.

Transformation (the process of the *E. coli* DH5α competent cells taking up the plasmid by heat shock) with the vector containing the 16S rRNA gene insert was performed using ampicillin as the selectable marker with blue-white screening using X-gal as described in section 2.21.4. The successful ligation of the 16S rRNA gene into the vector is confirmed by the production of white colonies on the plates (Figure 4-4). Blue colonies confirm that there was no insertion, the lacZ gene was transcribed and the X-gal substrate was used by the *E. coli* cells.

Immediately after overnight growth on plates, single white colonies were picked (each colony was added to a small volume of liquid media containing the antibiotic) and grown in order to generate a large number of copies of the plasmid. Plasmid was isolated using QIAgen Mini-Prep kit following the manufacturer's protocol. Plasmid DNA was cut using *Eco*R1 to confirm that the plasmid has the correct insert. pCR2.1 is 3.9 kbp in length and has *Eco*R1 sites where the 16S rRNA sequence (1.5 kbp) should have inserted. Each digest produced two products (vector pCR 2.1 and 16S rRNA gene) at 3.9 kb and 1.5 kb band, respectively (Figure 4-5).
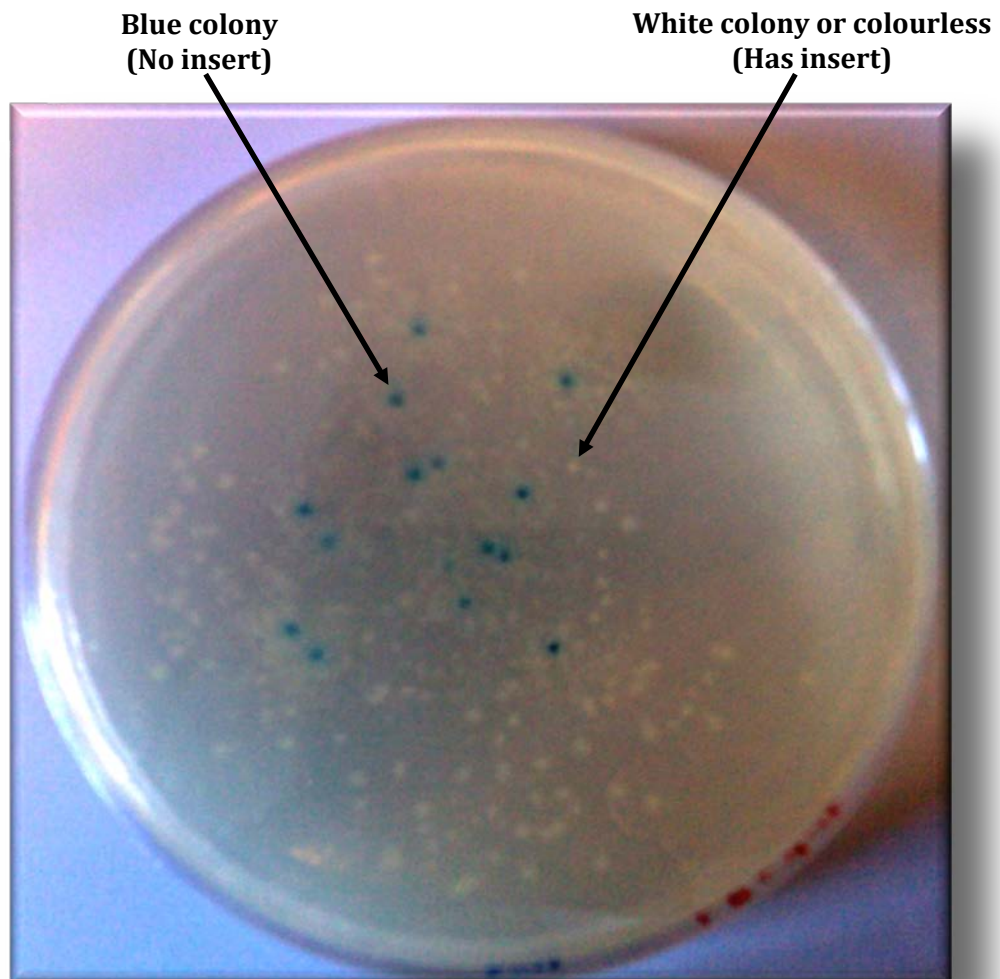
Figure 4-4: Selective LB agar plate containing 50 μl ml^{-1} ampicillin and 40 μl 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) showing the results of blue-white screen for the detection of successful ligation. When β-glactosidase hydrolyses X-gal, it releases a relatively insoluble blue dye. Many competent *E. coli* DH5α colonies on this plate are blue, indicating the presence of vector without cloned DNA. However, many of the colonies are colourless, indicating that amplified 16S rRNA gene has been inserted into the vector and the *lacZ* gene has been disrupted.
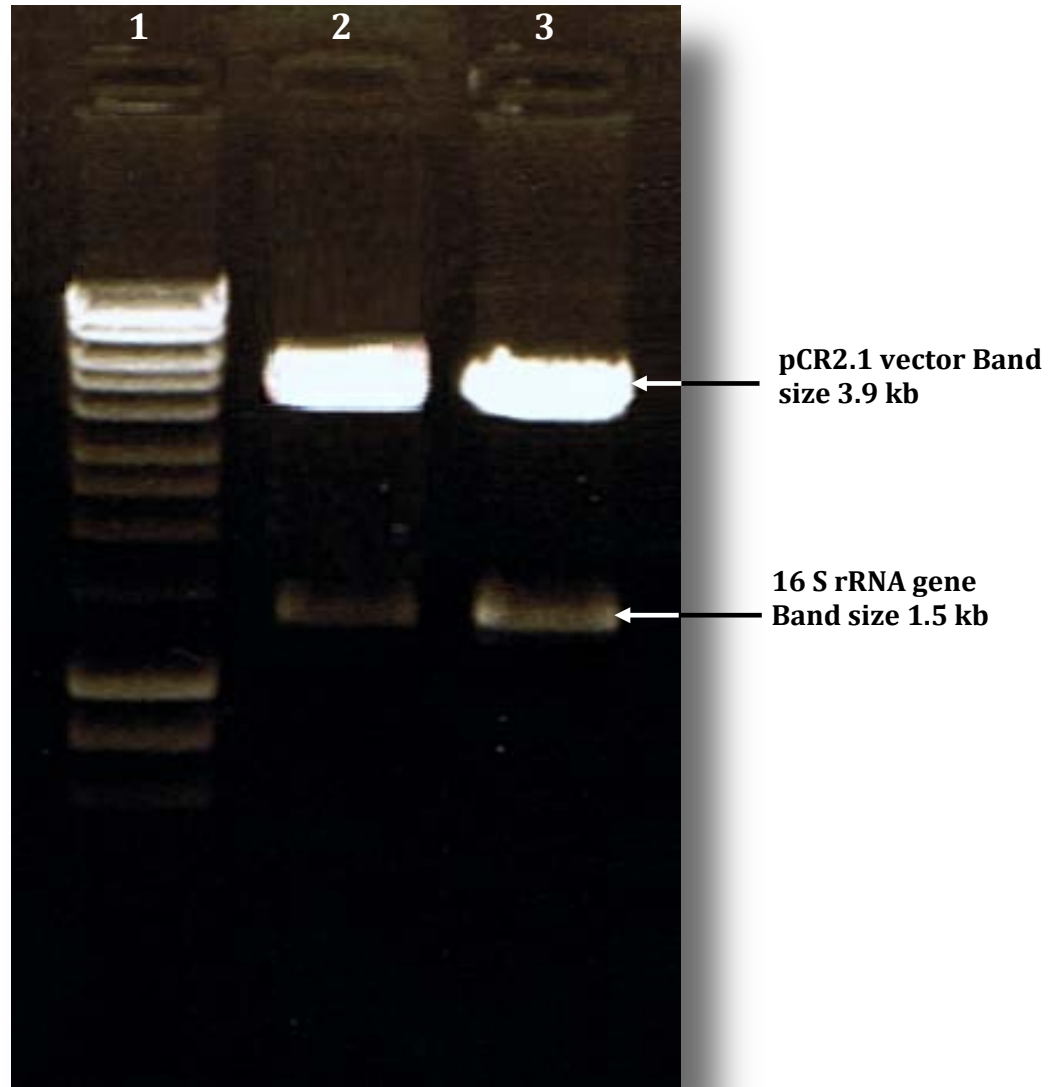
Figure 4-5: Agarose gel 1% electrophoresis with ethidium bromide showing the restriction endonuclease analysis of vector pCR2.1 digested with *Eco*R1 for both strains RV$_3$ (lane 2) and LV$_1$ (lane 3). Lane 1 shows the 10 kb DNA ladder. The upper band indicates the pCR2.1 vector (plasmid), with size 3.9 kb and the lower band indicates the 16S rRNA gene with size 1.5 kb.

4.2.1.4 Sequencing of 16S rDNA Gene of Bacterial Isolates

RV$_3$ vector which containing the correct sized insert was sent to Eurofins MWG in London while LV$_1$ vector was sent to the Medical School (University of Sheffield) for sequencing. Figure 4-6 and Figure 4-7 show good length sequences for RV$_3$ and LV$_1$ strains respectively (see Appendix C). The sequences were compared to other sequences using the NCBI Genbank.

```
CTATAATTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCC
CATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGC
ATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATT
AGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGT
GATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGA
ATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTC
GGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTT
GACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA
GGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGT
CTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGT
GCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAA
CACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGG
GAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTT
AGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTA
CGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG
TGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCC
TAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGC
TCGTGTCGTGAGATGTT
```

Figure 4-6: 16S rRNA gene sequence of facultative aerobic strain RV$_3$. Nucleotide sequence = 1020 letters.

```
GGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGG
GATAAGCCTGGGAAACTGGGTCTAATACCGGATAGGAGCGCCTACCGCATGGTGGGT
GTTGGAAAGATTTATCGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGG
TAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAA
TGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAA
ACCTCTTTCAGTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCACCGGCT
AACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTATCCGGAATTATT
GGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGTCGTGAAAGTCCGGGGCTTAA
CCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGCAGTAGGGGAGACTGGAATTC
CTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGT
CTCTGGGCTGTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGAT
ACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGACCATTCCACGGT
TTCCGCGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCT
AAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTC
GATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTTCTCGATCGCCGTAGAGATA
CGGTTTCCCCTTTGGGGCGGGTTCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
G
```

Figure 4-7: 16S rRNA gene sequence of obligate aerobic strain LV$_1$. Nucleotide sequence = 970 letters.

4.2.1.5 *Phylogenetic Analysis*

The 16S rRNA gene sequences of both bacterial isolates were compared with the highly similar sequences available from NCBI GenBank library by using the BLAST program. The results show that RV_3 was most similar to the species *Bacillus cereus* and LV_1 was most similar to *Micrococcus luteus* with sequence identity 100% for both strains (Table 4-1 and Table 4-2, respectively). Phylogenetic trees (neighbour joining) were constructed on the basis of the 16S rRNA gene sequences which show the relationship of the Rivelin and Limb valley isolates with the set of organisms that are the nearest matches (Figure 4-8 and Figure 4-10). Sequence comparisons are shown in Figure 4-9 and Figure 4-11.

On the basis of this analysis, RV_3 is most closely related to *Bacillus cereus* (Accession No: HQ596560.1) and LV_1 is most closely related to *Micrococcus luteus* (Accession No: JN545040.1).

Table 4-1: Similarity between 16S rRNA gene sequence of the facultative aerobic strain RV$_3$ and other related species/strains based on MegaBlast.

RV$_3$ Top Hits NCBI BLAST$^{®}$

Sequence Name	% Match
Bacillus cereus strain NMRL PED1	100
Bacillus cereus strain BPH33	100
Bacillus cereus strain PPB131	100
Bacillus cereus strain 57	100
Bacillus sp. Strain GZT	100
Bacillus sp. 5129	100
Bacillus cereus strain DZ4	100
Bacillus cereus strain M6	100
Bacillus cereus strain HT21	100
Bacillus cereus strain AcdSP4	100
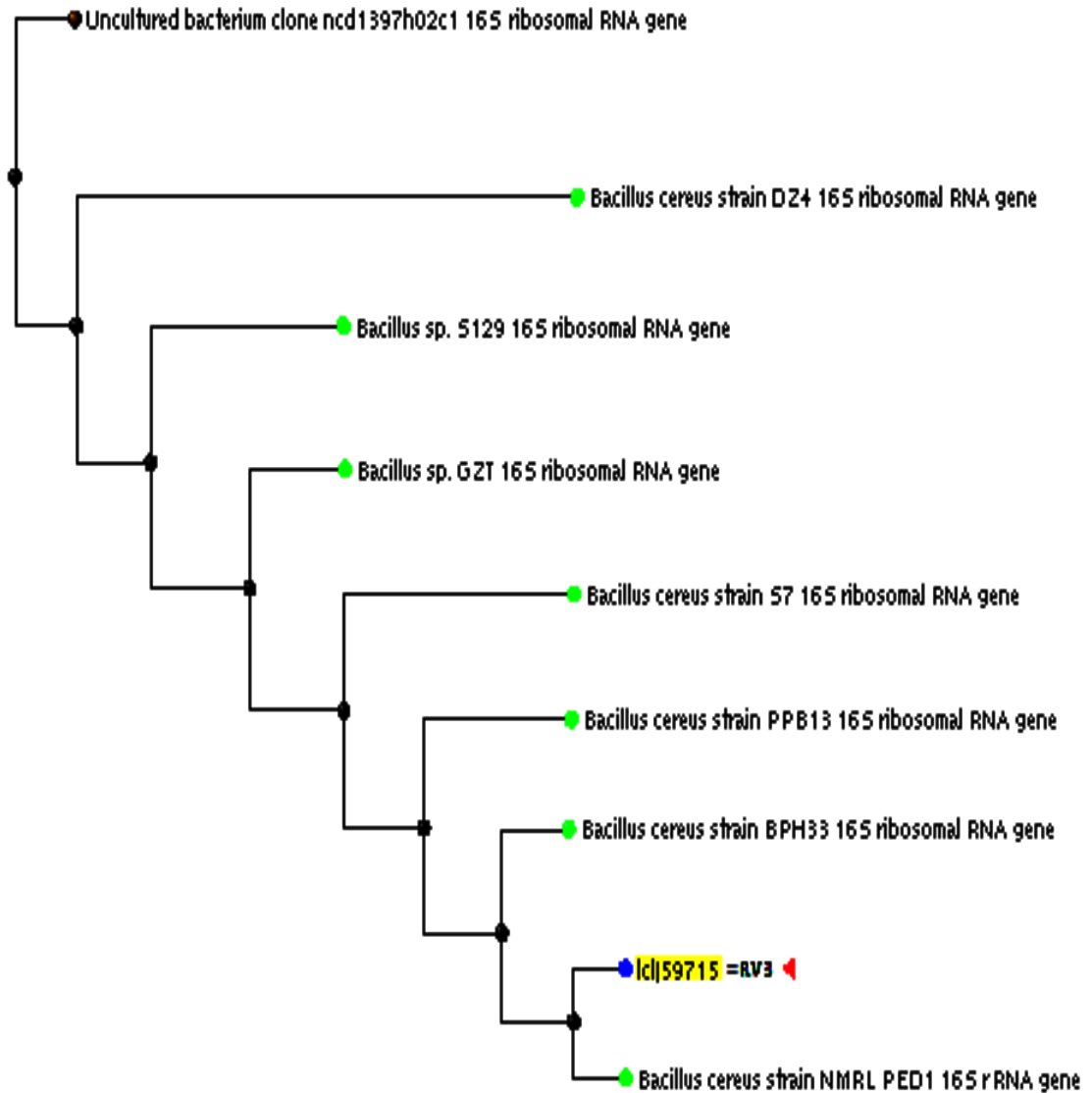
Figure 4-8: Neighbour joining phylogenetic tree for RV$_3$ strain associated with other members of the Protobacteria based on 16S rRNA gene sequences. The 16S rDNA sequence of RV$_3$ strain was determined and compared with those of related strains.

> gb|HQ596560.1| *Bacillus cereus* strain NMRL PED1 16S ribosomal RNA gene, partial sequence Length=1500 Score = 1884 bits (1020), Expect = 0.0 Identities = 1020/1020 (100%), Gaps = 0/1020 (0%) Strand=Plus/Plus

```
Query  241   GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTC   300
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  309   GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTC   368

Query  301   CGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGT   360
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  369   CGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGT   428

Query  361   AAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTAC   420
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  429   AAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTAC   488

Query  421   CTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG   480
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  489   CTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG   548

Query  481   CGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAA   540
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  549   CGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAA   608

Query  541   AGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGA   600
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  609   AGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGA   668

Query  601   AAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGA   660
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  669   AAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGA   728

Query  661   AGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGAT   720
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  729   AGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGAT   788

Query  721   TAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCC   780
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  789   TAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCC   848

Query  781   CTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGA   840
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  849   CTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGA   908

Query  841   AACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGC   900
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  909   AACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGC   968

Query  901   AACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCTC   960
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  969   AACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCTC   1028

Query  961   CTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTG   1020
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1029  CTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTG   1088
```

Figure 4-9: Sequence alignment of 16S rRNA gene sequence of a facultative aerobic environmental bacteria strain *Bacillus cereus* (Subject) versus *Bacillus cereus* strain NMRL PED1 (Query), which is the nearest matching species (See Table 4-2).

Table 4-2: Similarity between 16S rRNA gene sequence of the obligate aerobic strain LV_1 and other related species/strains based on MegaBlast.

LV_1 Top Hits NCBI BLAST$^{®}$

Sequence Name	% Match
Micrococcus luteus strain TS1	100
Micrococcus sp. Cp21	100
Micrococcus sp. 98TH11319	100
Micrococcus sp. 98TH11322	100
Micrococcus luteus strain CV39	100
Micrococcus luteus strain CJ-GTSA7	100
Micrococcus luteus strain C-SNA1	100
Micrococcus luteus strain VKRKHg9	100
Micrococcus luteus strain G3-6-08	100
Micrococcus luteus strain NSN12	100
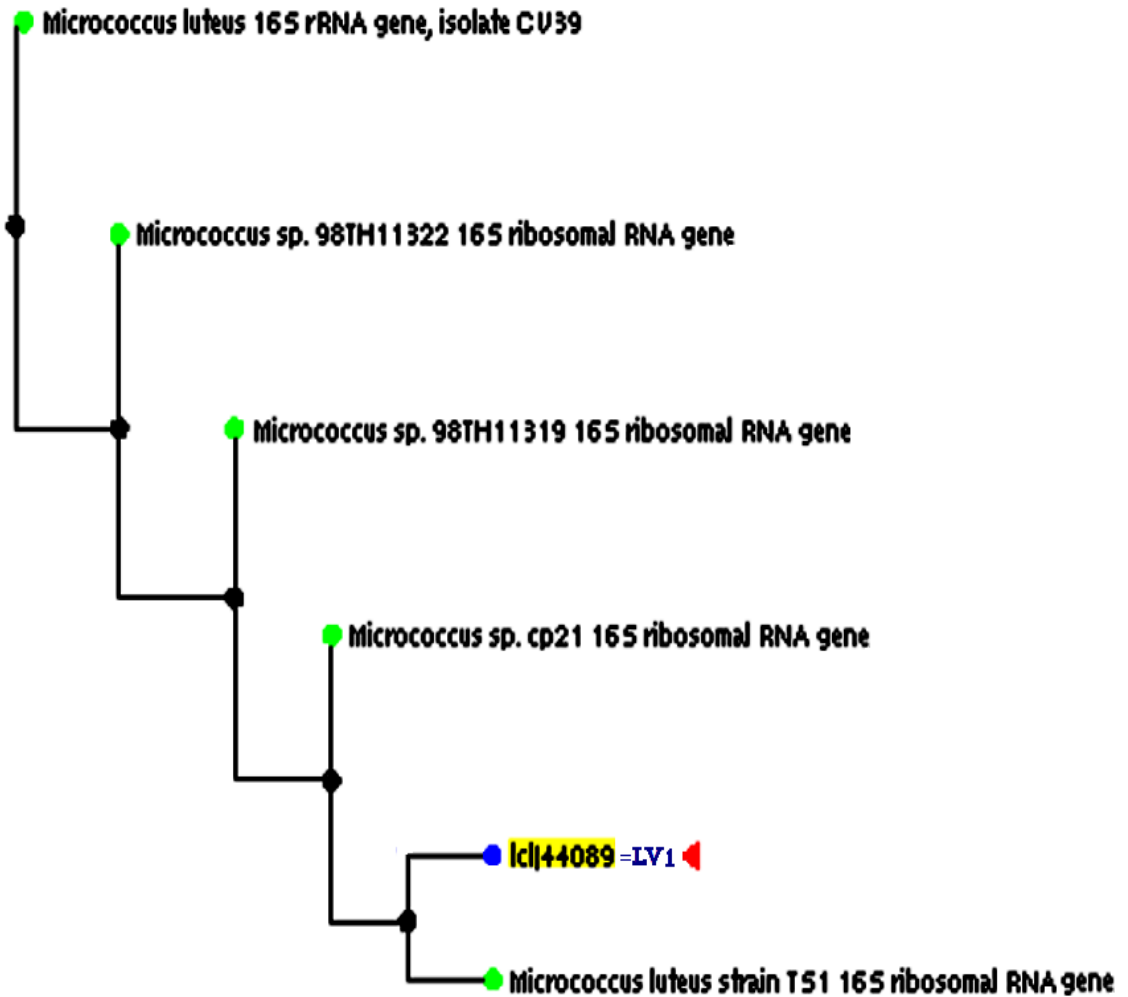
Figure 4-10: Neighbour joining phylogenetic tree for LV_1 strain associated with other members of the Protobacteria based on 16S rRNA gene sequences. The 16S rDNA sequence of LV_1 strain was determined and compared with those of related strains.

> [gb|JN545040.1|](#) *Micrococcus luteus* strain TS1 16S ribosomal RNA gene, partial sequence Length=1046 Score = 1788 bits (968), Expect = 0.0 Identities = 968/968 (100%), Gaps = 0/968 (0%) Strand=Plus/Plus

```
Query   241   CGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGA   300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   309   CGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGA   368

Query   301   TGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAA   360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   369   TGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAA   428

Query   361   GAAGCGAAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCG   420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   429   GAAGCGAAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCG   488

Query   421   GTAATACGTAGGGTGCGAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGT   480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   489   GTAATACGTAGGGTGCGAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGT   548

Query   481   TTGTCGCGTCTGTCGTGAAAGTCCGGGGCTTAACCCCGGATCTGCGGTGGGTACGGGCAG   540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   549   TTGTCGCGTCTGTCGTGAAAGTCCGGGGCTTAACCCCGGATCTGCGGTGGGTACGGGCAG   608

Query   541   ACTAGAGTGCAGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCA   600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   609   ACTAGAGTGCAGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCA   668

Query   601   GGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCTGTAACTGACGCTGAGGAGCGAAAG   660
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   669   GGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCTGTAACTGACGCTGAGGAGCGAAAG   728

Query   661   CATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAG   720
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   729   CATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAG   788

Query   721   GTGTGGGGACCATTCCACGGTTTCCGCGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGG   780
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   789   GTGTGGGGACCATTCCACGGTTTCCGCGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGG   848

Query   781   GAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAG   840
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   849   GAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAG   908

Query   841   CATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTTCTCGATC   900
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   909   CATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTTCTCGATC   968

Query   901   GCCGTAGAGATACGGTTTCCCCTTTGGGGCGGGTTCACAGGTGGTGCATGGTTGTCGTCA   960
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   969   GCCGTAGAGATACGGTTTCCCCTTTGGGGCGGGTTCACAGGTGGTGCATGGTTGTCGTCA   1028

Query   961   GCTCGTGT   968
              ||||||||
Sbjct   1029  GCTCGTGT   1036
```

Figure 4-11: Sequence alignment of 16S rRNA gene sequence of an obligate aerobic environmental bacteria strain *Micrococcus luteus* (Subject) versus *Micrococcus luteus* strain TS1 (Query), which is the closest matching species (See Table 4-2).

4.2.2   Yeast Strains

4.2.2.1   The Identification of Yeast Strains

Initially, the CTAB method with sonication was used in order to extract genomic DNA from both yeast strains, but no DNA has been obtained. So, QIAgen DNA extraction kits were used, but after many trials no genomic DNA was extracted (Figure 4-12). Another extraction kit (ANACHEM Key Prep) was used, and again even with optimised extraction protocol and sonication a very low quality genomic DNA was extracted from both strains which did not support the amplification process of 18S rRNA using PCR protocol (Figure 4-13). So, both isolates were sent as fresh colonies on plates to NCIMB in Aberdeen in order to identify them.

After two weeks, the sequencing of both strains was received (Figure 4-14 and Figure 4-15) and confirmed as eukaryotes The results show that both strains were yeast and most similar to the species *Aureobasidium pullulans* (RV2) and *Debaryomyces hansenii* (RV4) with sequence identities 100% and 99.72% respectively (Table 4-3 and Table 40-4). Phylogenetic trees (neighbour joining) constructed on the basis of the 18S rRNA gene sequences show the relationship of the Rivelin and Limb valleys isolates with the set of organisms that are the nearest matches (Figure 4-16 and Figure 4-18). Sequence comparisons are shown in Figure 4-17 and Figure 4-19.
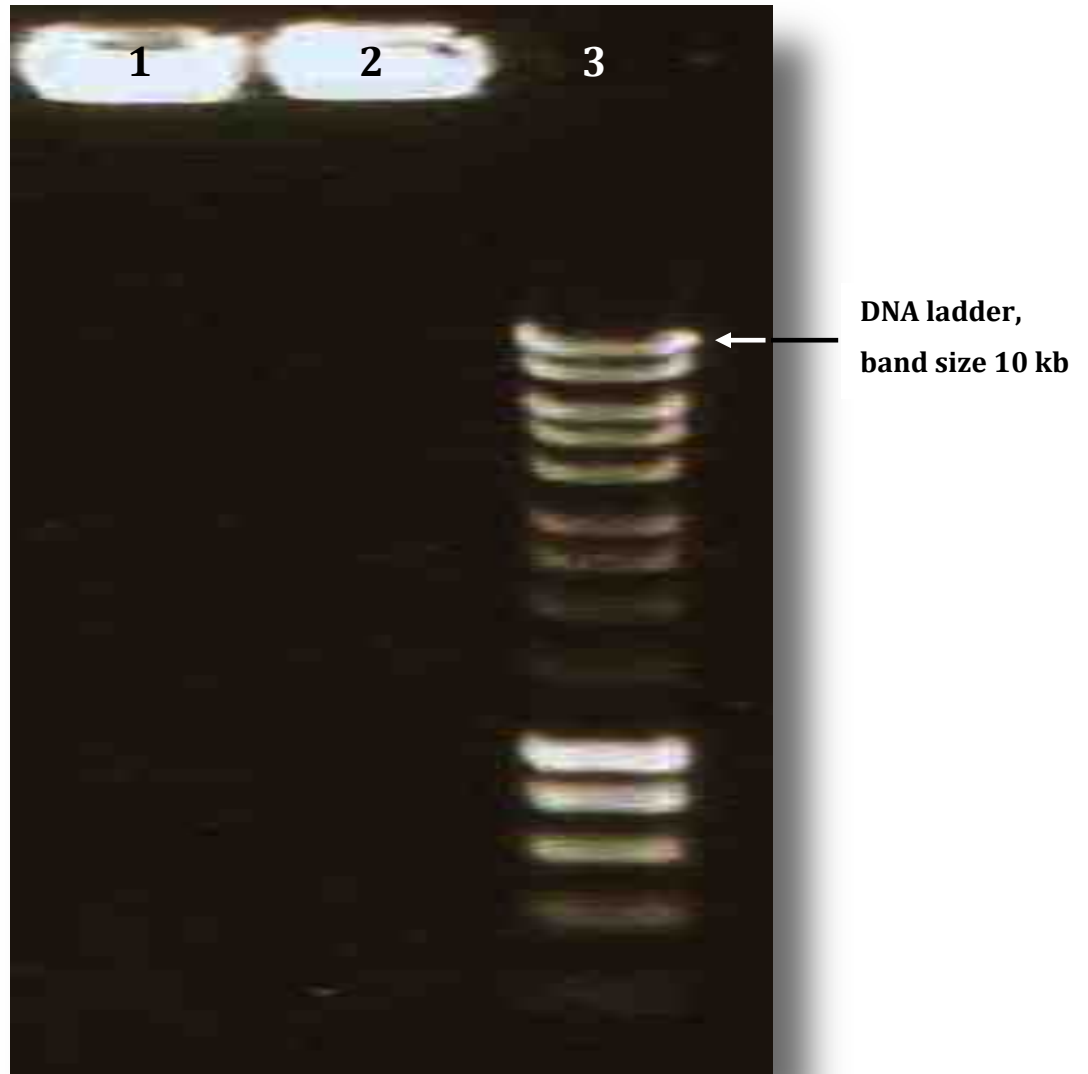
Figure 4-12 : Agarose gel 1% electrophoresis with ethidium bromide showing 10 regularly spaced bands of 1 kb DNA ladder (lane 3). The bands for both strains RV_3 (lane 2) and LV_1 (lane 1) did not migrate on the gel, which means that no genomic DNA was extracted. QIAgen Genomic DNA kit was used.

DNA ladder, band size 10 kb
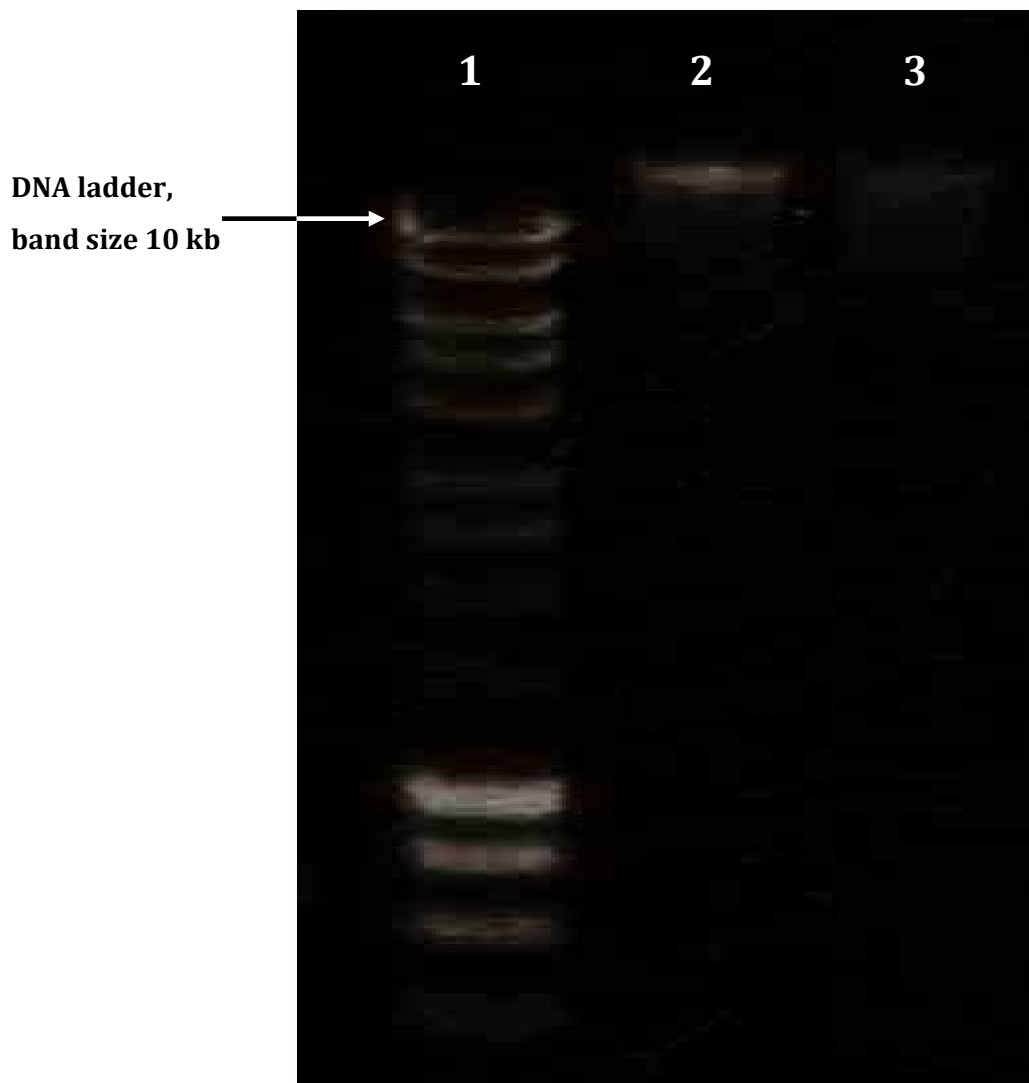
Figure 4-13: Agarose gel 1% electrophoresis with ethidium bromide showing 1 kb DNA ladder (lane 1). Very low quality genomic DNA was extracted for both strains RV_3 (lane 2) and LV_1 (lane 3). Anachem Key Prep Genomic DNA kit was used.

GAGTGATCGAAAGATGAAAAGCACTTTGGAAAGAGAG

TTAAAAAGCACGTGAAATTGTTGAAAGGGAAGCGCTT

GCAATCAGACTTGTTTAAACTGTTCGGCCGGTCTTCT

GACCGGTTTACTCAGTTTGGACAGGCCAGCATCAGTT

TCGGCGGCCGGATAAAGGCTCTGGGAATGTGGCCTCC

ACTTCGGTGGAGGTGTTATAGCCCAGGGTGTAATACG

GCCAGCCGGGACTGAGGTCCGCGCTTCGGCTAGGATG

CTGGCGTAATGGTTGTAAGCGAC

Figure 4-14: 18S rRNA gene sequence of RV$_2$ strain as received from NCIMB in Aberdeen. Nucleotide Sequence (283 letters).

```
CAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGA

AAAAGTACGTGAAATTGTTGAAAGGGAAGGGCTTGAGATC

AGACTTGGTATTTTGCGATCCTTTCCTTCTTGGTTGGGTT

CCTCGCAGCTTACTGGGCCAGCATCGGTTTGGATGGTAGG

ATAATGACTAAGGAATGTGGCTCTACTTCGGTGGAGTGTT

ATAGCCTTGGTTGATACTGCCTGTCTAGACCGAGGACTGC

GTCTTTGACTAGGATGTTGGCATAATGATCTTAAGCCAY
```

Figure 4-15: 18S rRNA gene sequence of RV$_4$ strain as received from NCIMB in Aberdeen. Nucleotide Sequence (279 letters).

Table 4-3: Similarity between 18S rRNA gene sequence of RV$_2$ strain and other related species/strains as received from NCIMB in Aberdeen.

RV$_2$ Top Hits NCBI BLAST$^®$

Sequence Name	% Match
Aureobasidium Pullulans	100
Kabatiella lini	99.72
Septoria pisticiae	91.0
Sydowia polyspora	90.32
Arthrographis cuboidae	86.16
Neotesudina rosatii	84.97
Dendrostibella mycophila	84.72
Stromatinia narcissi	84.3
Meria laricis	84.27
Oosporidium magaritiferum	83.99

Phylogenetic Tree

Specimen : NC000366_RV2
N.Join: 8.0%
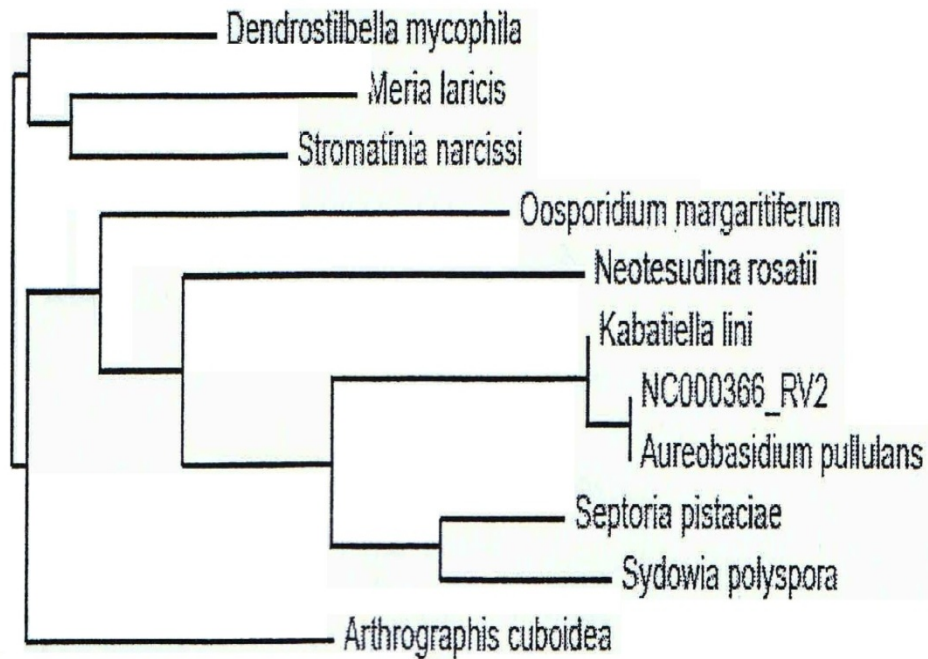
Figure 4-16: Neighbour joining phylogenetic tree for RV$_2$ strain associated with other members of the Eukarya as received from NCIMB in Aberdeen.

> ☑ *Aureobasidium pullulans* Sequence Length=548 Score =521 bits (282) Identities = 282/282 (100%) Gaps = 0/282(0%)


```
Query  1    GAGTGATCGAAAGATGAAAAGCACTTTGGAAAGAGAGTTAAAAAGCACGTGAAATTGTTG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  257  GAGTGATCGAAAGATGAAAAGCACTTTGGAAAGAGAGTTAAAAAGCACGTGAAATTGTTG  316

Query  61   AAAGGGAAGCGCTTGCAATCAGACTTGTTTAAACTGTTCGGCCGGTCTTCTGACCGGTTT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  317  AAAGGGAAGCGCTTGCAATCAGACTTGTTTAAACTGTTCGGCCGGTCTTCTGACCGGTTT  376

Query  121  ACTCAGTTTGGACAGGCCAGCATCAGTTTCGGCGGCCGGATAAAGGCTCTGGGAATGTGG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  377  ACTCAGTTTGGACAGGCCAGCATCAGTTTCGGCGGCCGGATAAAGGCTCTGGGAATGTGG  436

Query  181  CCTCCACTTCGGTGGAGGTGTTATAGCCCAGGGTGTAATACGGCCAGCCGGGACTGAGGT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  437  CCTCCACTTCGGTGGAGGTGTTATAGCCCAGGGTGTAATACGGCCAGCCGGGACTGAGGT  496

Query  241  CCGCGCTTCGGCTAGGATGCTGGCGTAATGGTTGTAAGCGAC     282
            ||||||||||||||||||||||||||||||||||||||||||
Sbjct  497  CCGCGCTTCGGCTAGGATGCTGGCGTAATGGTTGTAAGCGAC     538
```


Figure 4-17: Sequence alignment of 18S rRNA gene sequence of yeast strain RV$_2$ (Subject) with the closest matching species *Aureobasidium pullulans* (Query) using NCBI BLAST database (See Table 4-3).

Table 4-4: Similarity between 18S rRNA gene sequence of RV$_4$ strain and other related species/strains as received from NCIMB in Aberdeen.

RV$_4$ Top Hits NCBI BLAST$^®$

Sequence Name	% Match
Debaryomyces hansenii	99.72
Wingea robertsii	98.57
Debaryomyces udenii	98.22
Candida multigemmis	95.38
Candida krissi	94.3
Candida zeylanoides	94.3
Pitchia etchellsii	93.46
Schwanniomyces occidentalis	92.96
Candida railenensis	92.67
Debaryomyces polymorphus	92.6
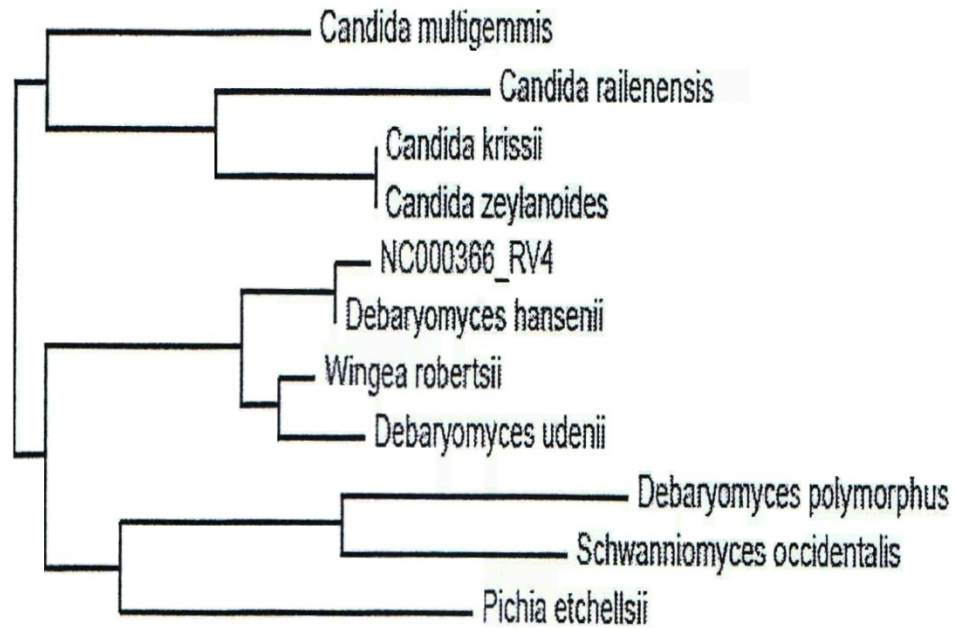
Figure 4-18: Neighbour joining phylogenetic tree for RV$_4$ strain associated with other members of the Eukarya as received from NCIMB in Aberdeen.

```
>☑  Debaryomyces hansenii Sequence Length= 553 Score =514
bits (278) Identities = 278/278 (100%) Gaps = 0/278 (0%)


Query  1    CAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAGTACGTGAAATTGTTG  60
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  268  CAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAGTACGTGAAATTGTTG  327

Query  61   AAAGGGAAGGGCTTGAGATCAGACTTGGTATTTTGCGATCCTTTCCTTCTTGGTTGGGTT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  328  AAAGGGAAGGGCTTGAGATCAGACTTGGTATTTTGCGATCCTTTCCTTCTTGGTTGGGTT  387

Query  121  CCTCGCAGCTTACTGGGCCAGCATCGGTTTGGATGGTAGGATAATGACTAAGGAATGTGG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  388  CCTCGCAGCTTACTGGGCCAGCATCGGTTTGGATGGTAGGATAATGACTAAGGAATGTGG  447

Query  181  CTCTACTTCGGTGGAGTGTTATAGCCTTGGTTGATACTGCCTGTCTAGACCGAGGACTGC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  448  CTCTACTTCGGTGGAGTGTTATAGCCTTGGTTGATACTGCCTGTCTAGACCGAGGACTGC  507

Query  241  GTCTTTGACTAGGATGTTGGCATAATGATCTTAAGCCA    278
            ||||||||||||||||||||||||||||||||||||||
Sbjct  508  GTCTTTGACTAGGATGTTGGCATAATGATCTTAAGCCA    545
```

Figure 4-19: Sequence alignment of 18S rRNA gene sequence of yeast strain RV$_4$ (Subject) versus *Debaryomyces hansenii* (Query) which is the nearest matching species (See Table 4-4).

4.3 Conclusions

Four microbial species have been isolated from Rivelin and Limb valleys, two bacterial strains (RV$_3$ and LV$_1$) and two yeast strains (RV$_2$ and RV$_4$). The bacterial species have been identified in the lab according to molecular identification techniques, starting with the extraction of genomic DNA, followed by PCR amplification and purification of 16S rRNA gene, and the transformation of competent *E.coli* cells. The sequences have been subjected to computer analysis and compared with other sequences using the NCBI GenBank which confirmed the identification of these bacteria as *Bacillus cereus* (RV$_3$) and *Micrococcus luteus* (LV$_1$) with sequence identities of 100%.

On the other hand, both yeast strains RV$_2$ and RV$_4$ were identified by The National Collection of Industrial, Marine and Food Bacteria (NCIMB) using the MicroSeq database and the EMBL public database. The identifications as received were *Aureobasidium pullulans* (RV$_2$) and *Debaryomyces hansenii* (RV$_4$) with sequence identities 100% and 99.72%, respectively.

Bacillus cereus is a Gram-positive, rod-shaped, facultative aerobe, which produces protective endospores and is beta hemolytic. It can be isolated from different environments, but usually from soil environments (Luksiene *et al.,* 2009). *B. cereus* commonly grows on plants and is adapted for growth in the intestinal tract of insects and mammals (Arnesen *et al.,* 2008). From these environments it is easily spread to foods, therefore, it can be isolated from a wide range of foods and also food ingredients, including rice, spices, dried foods, dairy products (milk powder, milk substitute, and dairy desserts), fresh vegetables and meat products (Reyes *et al.,* 2007, Arnesen *et al.,*

2008). The bacterium is responsible for gastrointestinal disease and non-gastrointestinal disease (wound and eye infections plus systemic infections) (Ehling-Schulz *et al.,* 2004). It is a major cause of two different forms of food poisoning (*B. cereus* foodborne disease), the emetic and the diarrhoeal syndromes (Finlay *et al.,* 2002a, Finlay *et al.,* 2002b, Ehling-Schulz *et al.,* 2004, Clavel *et al.,* 2007). The emetic type of food poisoning is probably the most dangerous as it has been associated with life-threatening acute conditions such as fulminant liver failure. It is caused by ingestion of a preformed toxin in the food, while the diarrhoeal poisoning is caused by a variety of toxins that can be formed in the food but also in the small intestine (Reyes *et al.,* 2007). The emetic toxin that causes vomiting is an extremely stable (heat and acid), small ring-form dodecadepsipeptide (peptide) which is resistant to proteolytic degradation, pH extremes and high temperature (Finlay *et al.,* 2002a, Finlay *et al.,* 2002b, Ehling-Schulz *et al.,* 2004, Arnesen *et al.,* 2008). Whereas the diarrhoeal disease is caused by one or more heat-labile protein enterotoxins produced during vegetative growth of *B. cereus* in the small intestine (Ehling-Schulz *et al.,* 2004, Arnesen *et al.,* 2008).

Micrococcus luteus is a Gram-positive bacterium, spherical or cocci shape, the cells arranged in tetrads producing yellow pigmented creamy colonies, obligate aerobe, which produce acid from glucose (Madigan *et al.,* 2009, Bergey and Boone, 2009). It can be found in a wide range of environments such as water, soil, air and dust (Baird-Parker, 1965, Rosypal *et al.,* 1966, Madigan *et al.,* 2009). It can also be isolated from the skin of mammals (Kloos *et al.,* 1974). *M. luteus* is commonly resistance to reduced water potential, can tolerate high salt concentrations and drying (Madigan *et al.,* 2009). In 1994, Moriguchi *et al.* have reported two salt-tolerant glutaminases which play major

roles in supporting growth of *M. luteus* cells at high salt concentrations (Moriguchi *et al.,* 1994). Despite *M. luteus* being a non-spore forming bacterium, Greenblatt and others recorded the ability of *M. luteus* to survive in oligotrophic environments. They investigated the presence of *M. luteus* in a 120 million year-old block of amber (Greenblatt *et al.,* 2004).

Aureobasidium pullulans is a yeast like fungus which can be found ubiquitously, it survives mainly in soil, water, air, limestone, rocks and commonly occurs on fruits (Domsch *et al.,* 1980, Deshpande *et al.,* 1992, Urzi *et al.,* 1999). Its colonies are white to pink at first but after a while they turned to black due to chlamydospore production and for this reason it is called the black yeast (Cooke, 1959, Domsch *et al.,* 1980, Hoog, 1993, Kurtzman and Fell, 1998). *A. pullulans* causes plant tissue softening so it demonstrated due to its osmotolerant properties (Hoog, 1993). It can be considered as a secondary saprophyte after the colonisation of bacterial mats or other fungi due to the low of its competitiveness in poor environments (Hoog, 1993). *A. pullulans* can divided to three varities, *A. pullulans* var *melanogenum*, *A. pullulans* var *pullulans* and *A. pullulans* var *aubasedani* Yurlova (Yurlova and De Hoog, 1997). A recent study carried out by Chi *et al.* demonstrated that *A. pullulans* can produce enzymes such as pullulan, amylase, mannanase, cellulose, proteinase, and siderophores which can used in many different biotechnological applications in different fields (Chi *et al.,* 2009).

A. pullulans has the ability to ferment glucose, assimilate nitrate, but α-methyl glucoside and cadaverine are not assimilated. Also *A. pullulans* cannot grow in the presence of cycloheximide (Kurtzman and Fell, 2000, Barnett *et al.,* 2000, Senses-Ergul *et al.,* 2006). In the previous chapter (section 3.2.6), exactly these characteristics were

found for RV_2 strongly supporting the molecular identification made in the current chapter i.e. that RV_2 is *Aureobasidium pullulans*.

Debaryomyces hansenii is classified as an ascomycetous yeast and associated with food spoilage especially salted and low-water-activity foods. It can also be found in salty water (Tokuoka, 1993, Kurtzman and Fell, 1998, Kurtzman and Robnett, 1998). It usually uses traditional sausages and cheeses as a habitat and can contribute to the production of special flavours (Seiler and Busse, 1990, Saldanha-da-Gama *et al.,* 1997). Therefore, the unusual ability of *D. hansenii* to sustain and thrive in high salinity has led researchers to investigate its ability to accumulate lipids, which could be very important for biotechnology applications such as oil production and degradation (Ratledge and Tan, 1990). Furthermore, it has been recorded that *D. hansenii* can produce thermophilic ß-glucosidases in wine production which are important in the production of alcohol-based fuels (Saha and Bothast, 1996). In 2006, Breuer and Harms used *D. hansenii* in the manufacture of many different foods such as dairy products and meat fermentation (Breuer and Harms, 2006). Recent taxonomy divided *D. hansenii* into two varieties, *D. hansenii* var. *hansenii* and *D. hansenii* var. *fabryi*, with different maximum growth temperatures (Breuer and Harms, 2006). *D. hansenii* can ferment glucose, and has the ability to assimilate α-methyl glucoside and cadaverine, whereas it cannot assimilate nitrate (Kurtzman and Fell, 2000, Barnett *et al.,* 2000, Senses-Ergul *et al.,* 2006). In previous chapter (section 3.2.6), these exact characteristics were found for RV_4, strongly supporting the molecular identification in this chapter (i.e. that RV_4 is *Debaryomyces hansenii*).

In the next chapter, the physiological adaptation of *Debaryomyces hansenii* (RV$_4$) and *Micrococcus luteus* (LV$_1$) to different pH values and different high salinity concentrations in M9 minimal and LB medium will be investigated.

CHAPTER FIVE

5 Physiological Growth of *Debaryomyces hansenii* (RV$_4$) and *Micrococcus luteus* (LV$_1$) Under Acidity and Salinity

5.1 Introduction

Extremophilic microorganisms have the ability to grow and survive under extreme environmental conditions that would be uncomfortable for the normal functioning of humans (Gomes and Steiner, 2004). Several of these extremophiles are able to withstand multiple extremes such as extreme acidity, high salinity and high temperature (Irwin and Baird, 2004).

Recently, extremophiles have been isolated from environments where they are not expected to be actively growing. Therefore, it is possible that acid tolerant or halotolerant prokaryotes or eukaryotes can be isolated from a wide range of environments including freshwater (Echigo *et al.,* 2005). However, most organisms have a relatively limited set of pH values and range of salt concentrations that enable growth and also, the minimum, optimum and maximum pH values or salt concentrations are often found to be dependent on the medium composition and growth temperature (Ventosa *et al.,* 1998, Oren, 2008). In 1990, Gilmour 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) (Gilmour, 1990).

However, microorganisms have different mechanisms to cope with acidity or salinity stresses depending on the stress encountered. For example, at high acidity stress (low pH) there are passive and active pH regulation mechanisms. In passive regulation the microorganisms produce biofilms to slow down the diffusion of molecules into the cell, or change their cell membrane to incorporate substances such as fatty acids that shield the cell against acidity, or they may secrete buffer molecules which help to raise the external pH (Raven and Smith, 1973, Ahmed and Booth, 1983, Booth, 1985). In addition, some microbes have the ability to pump hydrogen ions out of their cells (active mechanisms) (Mitchell, 1973, Baronofsky *et al.,* 1984, Gonzalez-Toril *et al.,* 2003). On the other hand, at high salinity concentrations there are two mechanisms that help microorganisms to maintain the osmotic equilibrium. Some microorganisms such as anaerobic bacteria (Rengpipat *et al.,* 1988) and halophilic archaea (Lanyi, 1974) are able to accumulate inorganic ions like Na^+ or K^+ , while some methanogenic archaea (Robertson *et al.,* 1990), most halophilic bacteria (Severin *et al.,* 1992) and halotolerant eukaryotes (Gunde-Cimerman *et al.,* 2009, Ben-Amotz and Avron, 1983) are able to accumulate or synthesize organic osmolytes (compatible solutes) (Brown, 1976).

Thereby, the importance of these extremophiles or extreme-tolerant microorganisms which adapt to grow and survive at both high acidity and high salinity is due to their special unique characteristics that enable them to produce extremozymes like amylase, cellulose, protease, catalase and lipase (Demirjian *et al.,* 2001, van den

Burg, 2003) and compatible solutes including glycine betaine, proline, glycerol, ectoine, alanine and sorbitol (Da Costa *et al.,* 1998, Bremer and KrÃmer, 2000, Demirjian *et al.,* 2001, Gomes and Steiner, 2004).

In fact, compatible solutes allow organisms to adapt to a wide range of salinity concentrations by adjusting the cytoplasm to the osmolarity of their environments, and they also protect proteins, membranes, and even whole cells against denaturation, inactivation, and inhibition by hyperosmotic stress (Yancey, 2005, Schubert *et al.,* 2007, Oren, 2008, Konrad and Bar-Zvi, 2008, Rajan *et al.,* 2010, Ma *et al.,* 2010, Fallet *et al.,* 2010). Moreover, the other reasons for the importance of compatible solutes and extremozymes are due to the applications which can be found in industrial biotechnology, medical biotechnology and other fields (Ratledge and Tan, 1990, Hough and Danson, 1999, Demirjian *et al.,* 2001, Irwin and Baird, 2004).

Nuclear magnetic resonance (NMR) spectroscopy is a suitable method for determination of intracellular potential compatible solutes in cells after exposure to high salinities (Landfald and Strom, 1986, Mendum and Smith, 2002). NMR has been used to determine the compatible solutes in various *Bacillus* species (Bursy *et al.,* 2007); *Halobacillus dabanensis* (Gu *et al.,* 2008); *Salinivibrio costicola* (Zhiu *et al.,* 2008), and *Halomonas* (Cummings *et al.,* 1993), also NMR was used to detect intact yeast cells and their compatible solutes (Salhany *et al.,* 1975, Jovall *et al.,* 1990, Block *et al.,* 2004).

Strain RV_4 was identified in Chapter 4 as a strain of *Debaryomyces hansenii* and in Chapter 3 *D. hansenii* (RV_4) was shown to thrive in extreme low pH and flourish in high salinity (Norkrans, 1966, Kreger-van Rij and W., 1984, Tokuoka, 1993, Almagro *et al.,* 2000). Strain LV_1 was identified as a strain of *Micrococcus luteus* in Chapter 4 and *M. luteus* (LV_1) was shown to survive in pH 5 and thrive in high salinity (Moriguchi *et al.,* 1994, Madigan *et al.,* 2009).

In the current chapter further studies are described that investigate the ability of the two strains to grow under many extreme stresses simultaneously such as low nutrients, low pH and high salinity. Furthermore, compatible solutes in both strains were determined using NMR spectroscopy and the effect of sulphur, aluminium and iron on the growth rate in M9 minimal medium was measured. In addition, the electron microscope was used to analyze *D. hansenii* and *M. luteus* cells grown under different conditions such as low nutrients, neutral pH and low pH in order to investigate any changes to the cell structure in response to different stresses.

5.2   Results and Discussion

5.2.1   Electron Microscope Analysis of *Debaryomyces hansenii* (RV_4) and *Micrococcus luteus* (LV_1) Cells Grown at pH 3 and pH 7

Scanning and transmission electron microscopes were used to observe any changes in the external structures of the cells of both *D. hansenii* and *M. luteus* under moderate and low pH values in low nutrient medium (M9 minimal medium). The analysis was carried out in the Biomedical Science Department (The University of Sheffield) as described in section 2.19.

Figure 5-1 shows scanning electron microscope images of *D. hansenii* grown at pH 3 (A and B) and at pH 7 (B and C), the main change observed was that the cells grown at pH 7 were larger than the cells grown at pH 3. Moreover, a transmission electron microscope was used to examine ultra-thin sections of *D. hansenii* cells grown under both conditions (Figure 5-2), the high resolution micrographs show that no significant changes were observed between cells at pH 3 (Figure 5-2; A1, A2) or pH 7 (Figure 5-3; B1, B2).

The same procedure was performed to investigate any changes that may accrue to *M. luteus* cells grown under acid stress in M9 minimal medium. However, despite previous results showing poor growth of *M. luteus* at low pH (Chapter three), there were no visible changes in the external structure of *M. luteus* cells observed when using the scanning electron microscope (Figure 5-3; pH 3; A, B. pH 7; C, D). However, high resolution TEM images of ultra-thin sections of overnight *M. luteus* cells grown in M9 medium at pH 3 (Figure 5-4; A1, A2) and pH 7 (Figure 5-4; B1, B2) show that the cell walls of cells grown at pH 3 were thicker than cell walls of cells grown at pH 7. Also it was noted that some large vacuoles were found in *M. luteus* cells when they were grown at pH 3 while no vacuoles were observed in cells when they were grown at pH 7. In addition, *M. luteus* cells appeared to form clumps more readily when grown at low pH which may help them to cope with the acidity and sustain life (Figure 5-4). The good quality SEM and TEM images of both microorganisms confirmed the phenotypic and phylogenic identifications arrived at in Chapters 3 and 4.
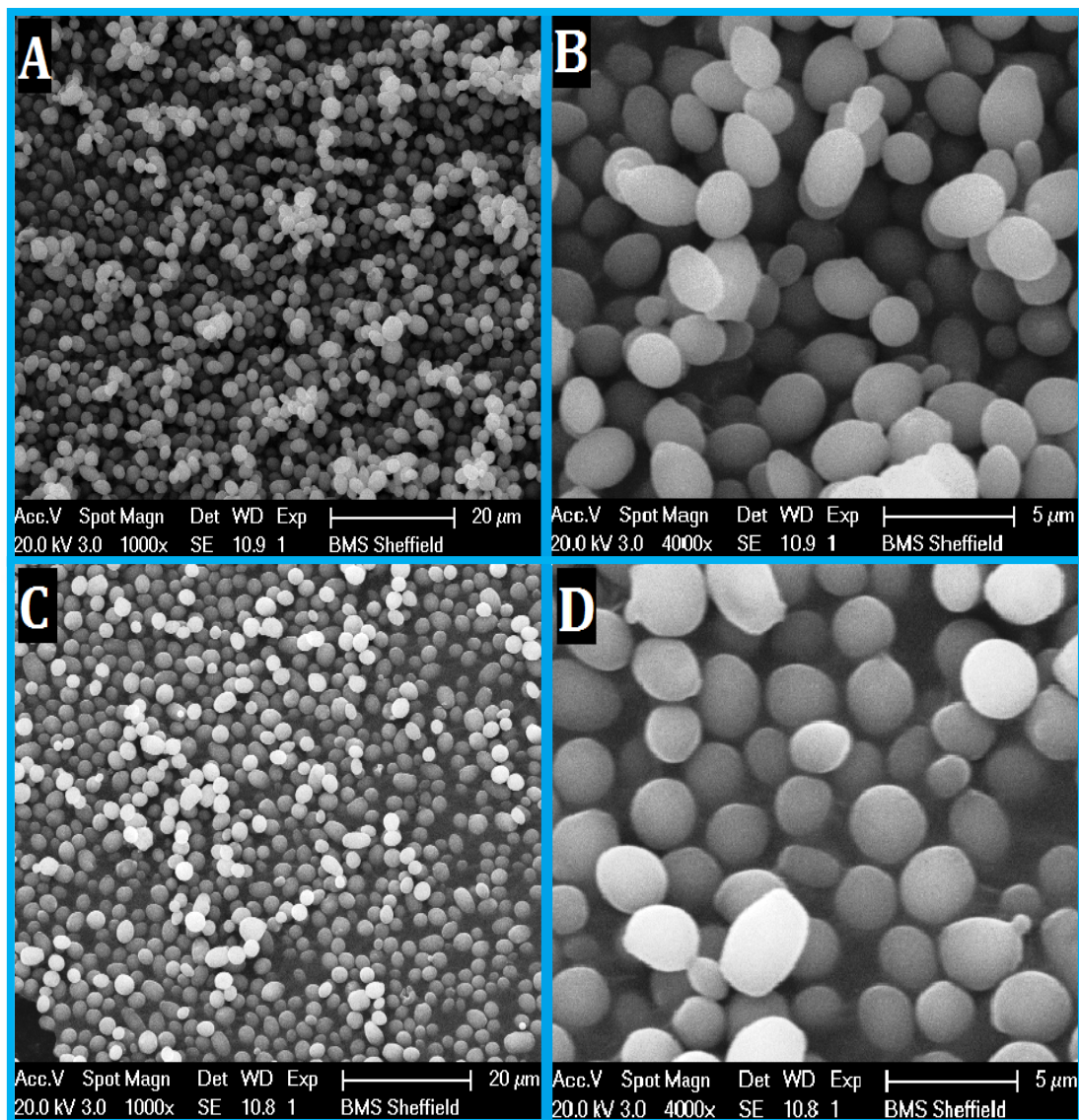
Figure 5-1: Electron micrographs of *D. hansenii* cells grown overnight in M9 minimal medium in a 25°C constant temperature room on an orbital shaker at 250 rpm at pH 3 (A and B) and pH 7 (C and D). Note the difference in cell size when grown at pH 7 (larger) (C and D) or when grown at pH 3 (smaller) (A and B). The cells viewed by SEM (1000x A, C), (4000x B, D).
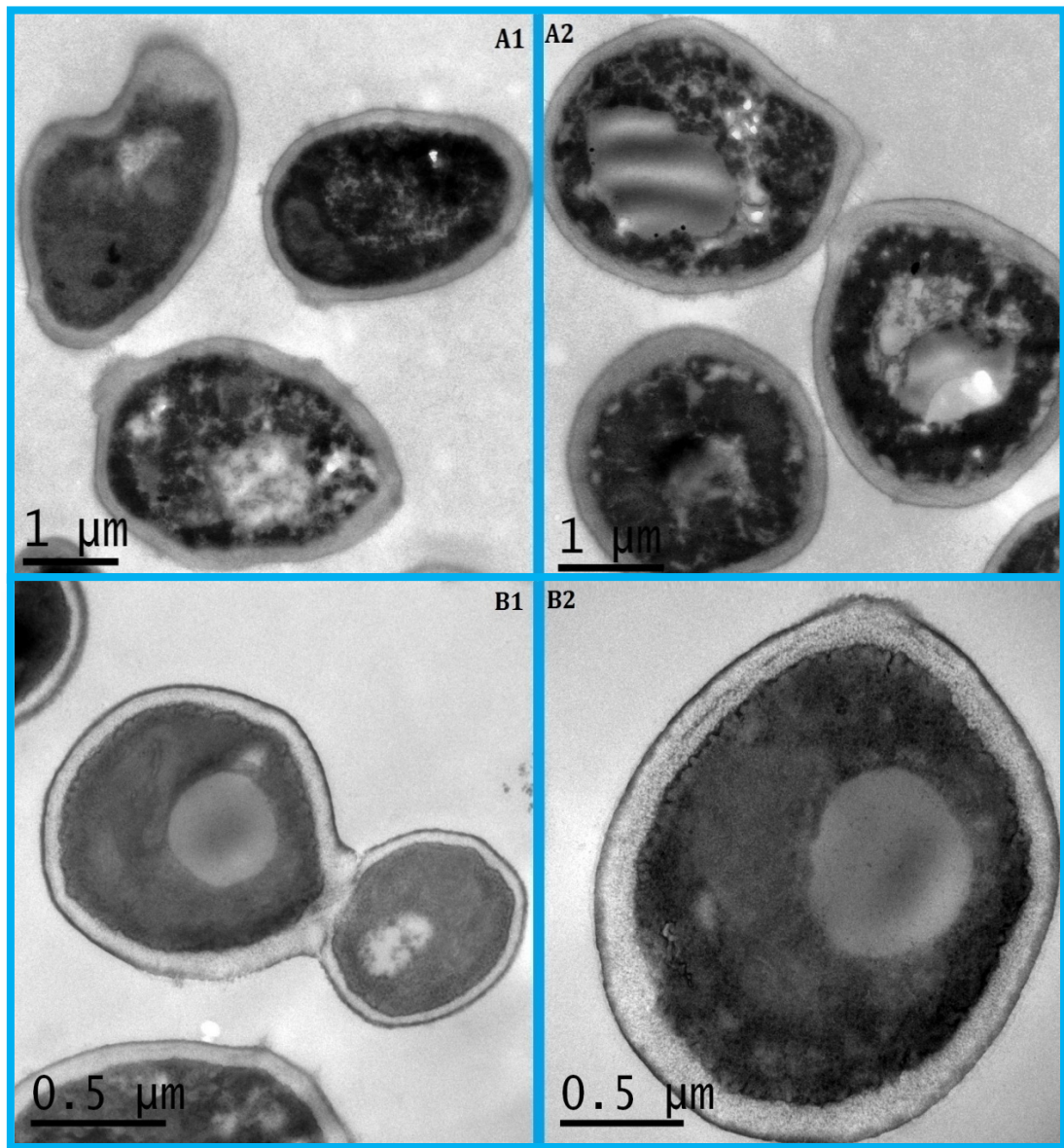
Figure 5-2: Electron micrographs of ultra-thin sections of *D. hansenii* cells grown overnight in M9 minimal medium in a 25ºC constant temperature room on an orbital shaker at 250 rpm at pH 3 (A1, 2) and pH 7 (B1, 2). Cell division by budding can be seen in B1. The cells were viewed by TEM (20000x A1 and A2) (200000x B1 and B2).
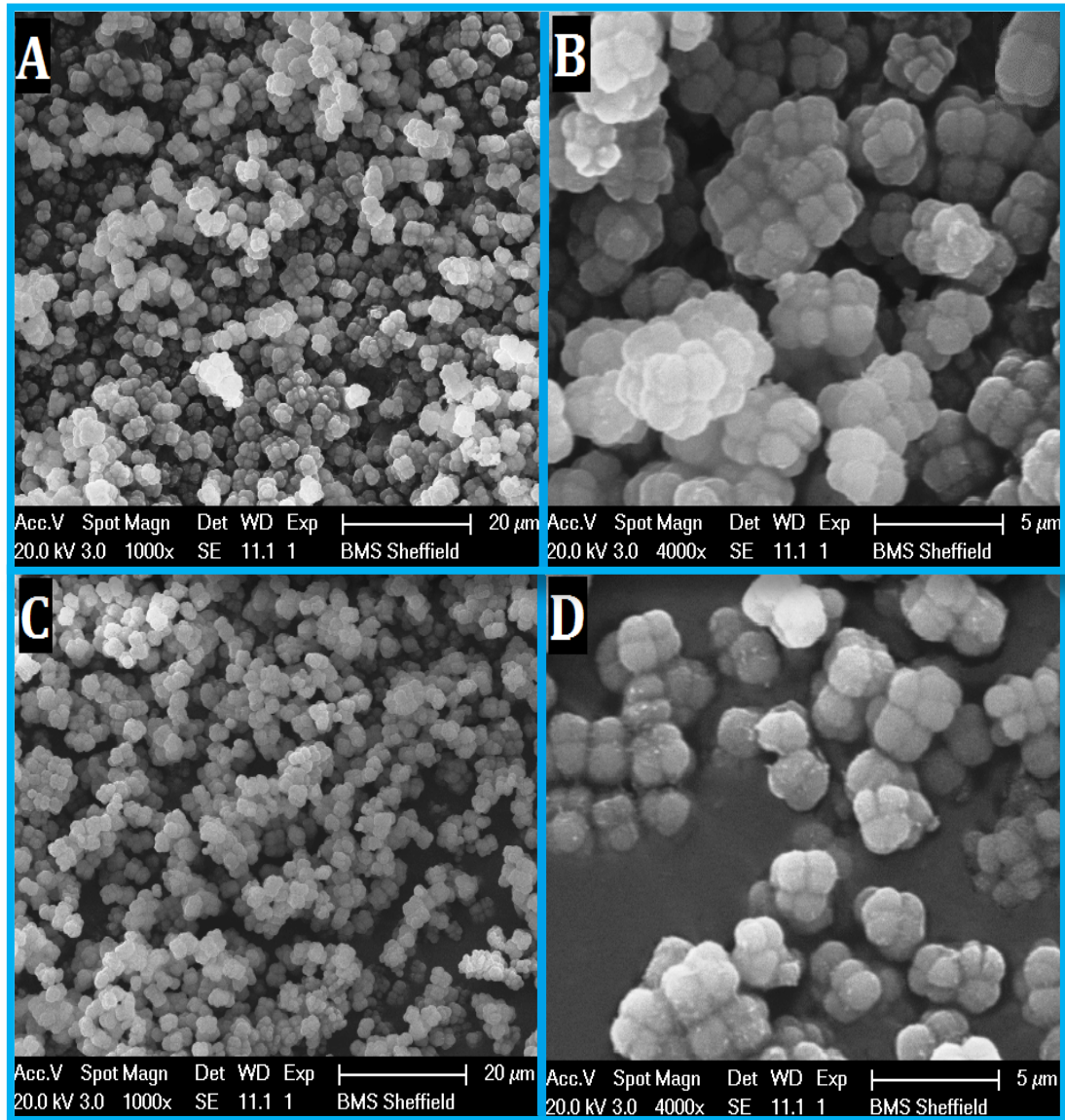
Figure 5-3: Electron micrographs of *M. luteus* cells grown overnight in M9 minimal medium in a 25°C constant temperature room on an orbital shaker at 250 rpm at pH 3 (A and B) and pH 7 (C and D). Note how *M. luteus* cells are arranged together in tetrads or clumps of tetrads. The cells were viewed by SEM (1000x A, C), (4000x B, D).
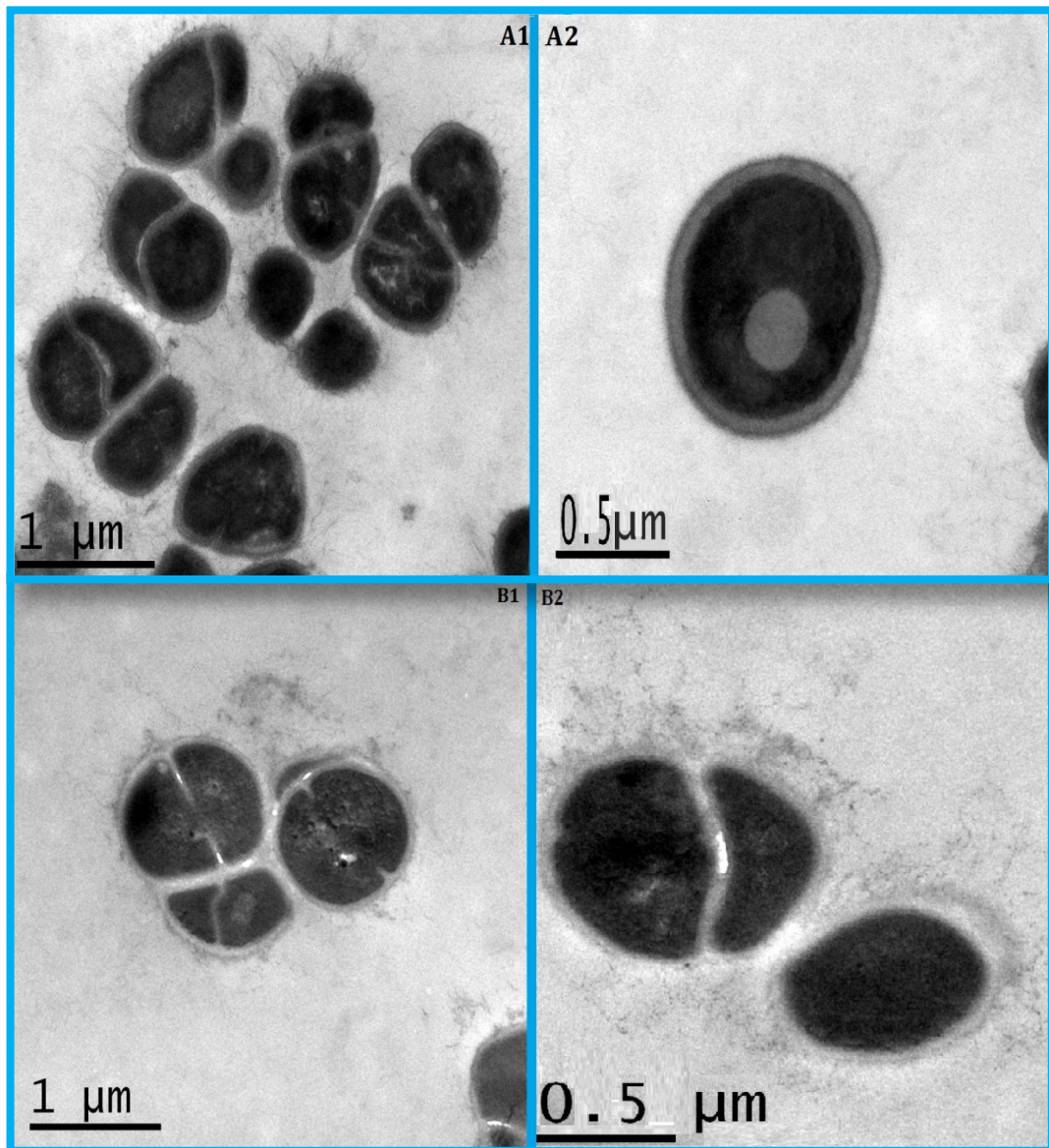
Figure 5-4: Electron micrographs of ultra-thin sections of *M. luteus* cells grown overnight in M9 minimal medium in a 25℃ constant temperature room on an orbital shaker at 250 rpm at pH 3 (A1, 2) and pH 7 (B1, 2). Note that the cell wall of the cells grown at pH 3 (A1and 2) is thicker than the cell wall of cells grown at pH 7 (B1 and 2). Also *M. luteus* cells clumped together more at pH 3(A1), and a large vacuole can seen in A2 in a cell grown at pH 3. The cells were viewed by TEM (20000x A1 and A2) (200000x B1 and B2).

5.2.2 Effect of External pH Medium on Growth Rate of *D. hansenii* and *M. luteus*

In section 3.2.7, the effect of external pH on the growth rates of *D. hansenii* (RV4) and *M. luteus* (LV1) was measured in ½ LB and M9 medium. To further investigate the effect of pH on growth of the two organisms, the same protocol was carried out with other two other media, YPD the standard medium for *D. hansenii* and LB the standard medium for *M. luteus*. Growth rates were produced for both strains at pH 3, 5 and 7. Growth was quantified by direct optical density (OD) readings at 600 nm measured with Unicam Helisα spectrophotometer.

Figure 5-5 shows very good growth of *D. hansenii* cells at pH 5 and pH 7 while lower growth was seen at pH 3, which differs from the results accrued when cells were grown in M9 and ½ LB medium (section 3.2.7), where growth was similar in all pH values in M9 and ½ medium. Growth at pH 3 in YPD medium is comparable to the growth at pH 3 in M9 and ½ LB medium (Table 5-1 and Figure 5-6). So, the biggest difference is the fast growth rates found at pH 5 and pH 7 in YPD medium in comparison to growth at the same pH in M9 and ½ LB medium.

M. luteus cells also grew best at neutral pH in LB medium (Figure 5-7), but significantly higher growth rates were found in LB medium at all pH values. The doubling times show clearly that *M. luteus* prefers enriched media rather than low nutrient media (Table 5-2 and Figure 5-8).
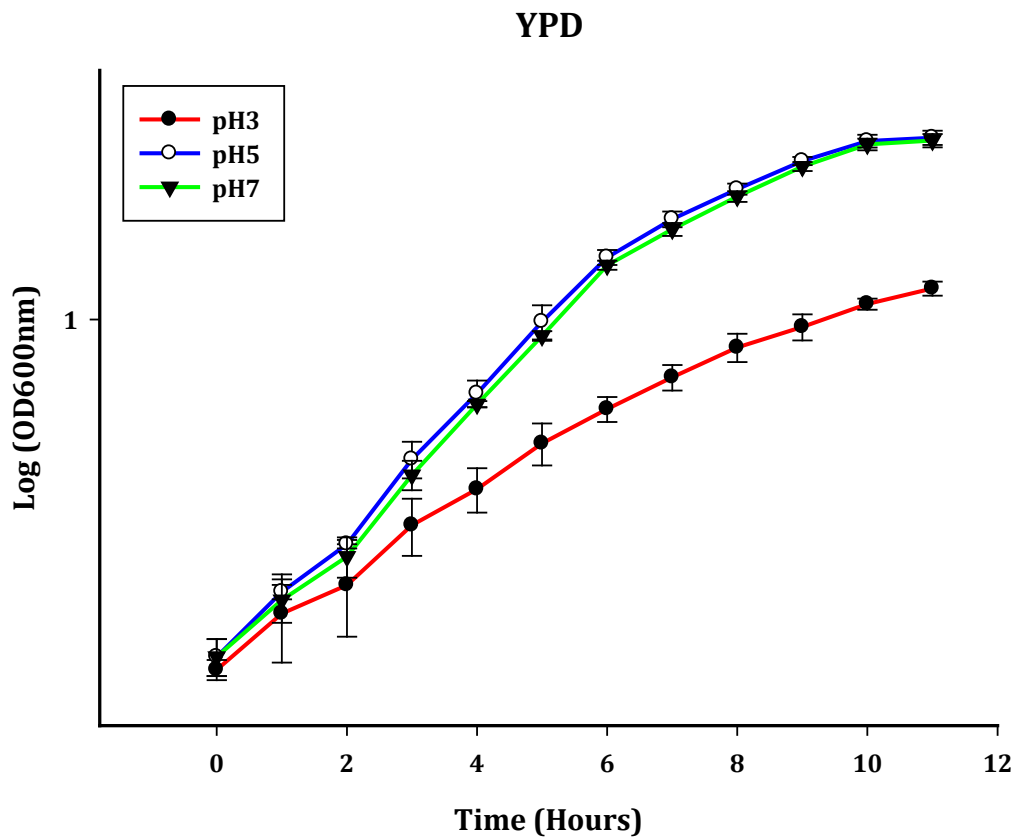
Figure 5-5: Growth curves for *D. hansenii*. Cells were incubated in YPD medium at pH 3, 5 and 7 and incubated in a 25°C constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.

Table 5-1: Effect of external pH on the doubling times of *D. hansenii* in M9, ½ LB and YPD medium. Each point represents the mean from four replicate samples.

Doubling Time of *D. hansenii* in Different Media (Minutes)			
	M9	1/2 LB	YPD
pH 7	189	156	120
pH 5	180	125	115
pH 3	182	162	195
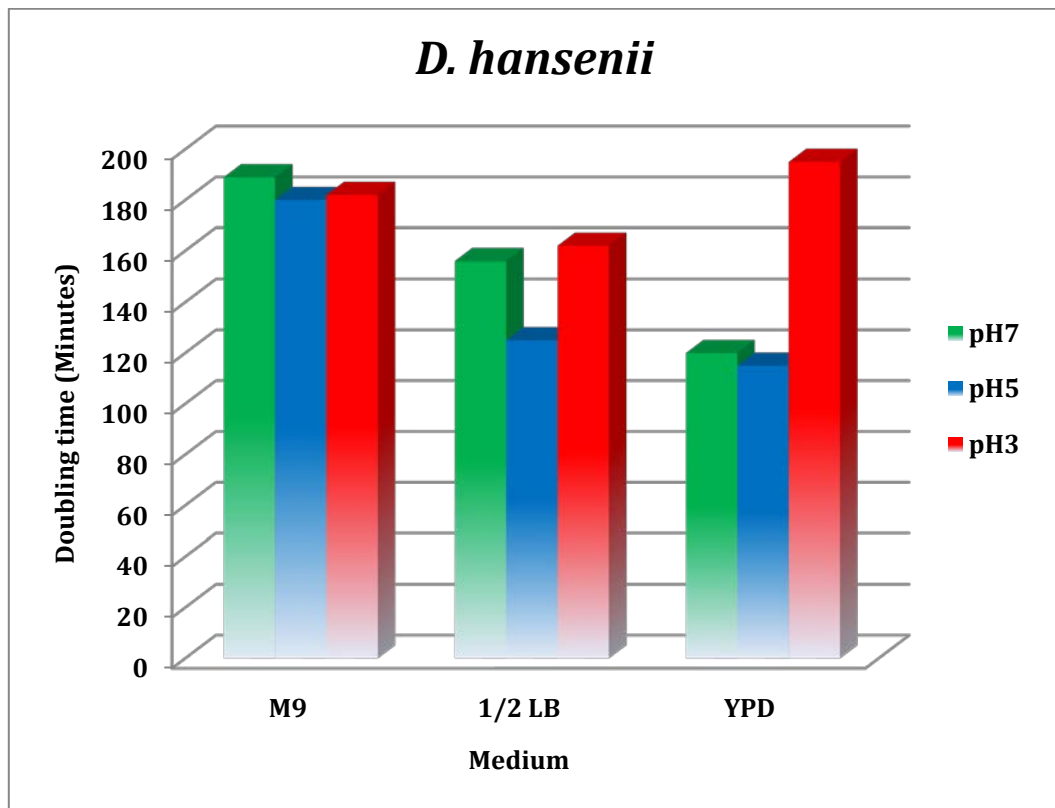
Figure 5-6: Effect of external pH on the doubling times of *D. hansenii* in M9, ½ LB and YPD medium. Each point represents the mean from four replicate samples.
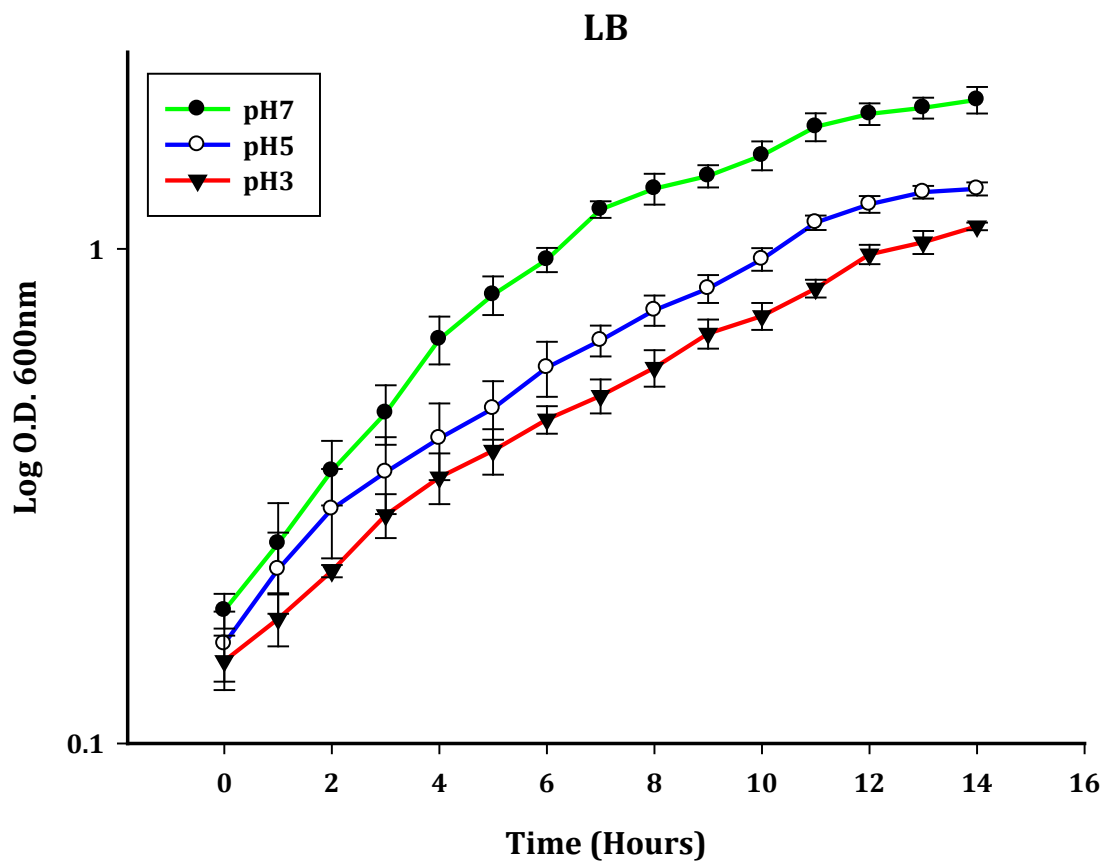
Figure 5-7: Growth curves for *M. luteus*. Cells were incubated in LB medium at pH 3, 5 and 7 in a 25ºC constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.

Table 5-2: Effect of external pH on the doubling times of *M. luteus* in M9, ½ LB and LB medium. Each point represents the mean from four replicate samples.

Doubling Time of *M. luteus* in Different Media (Minutes)			
	M9	**1/2 LB**	**LB**
pH 7	225	138	53
pH 5	293	246	114
pH 3	408	276	159
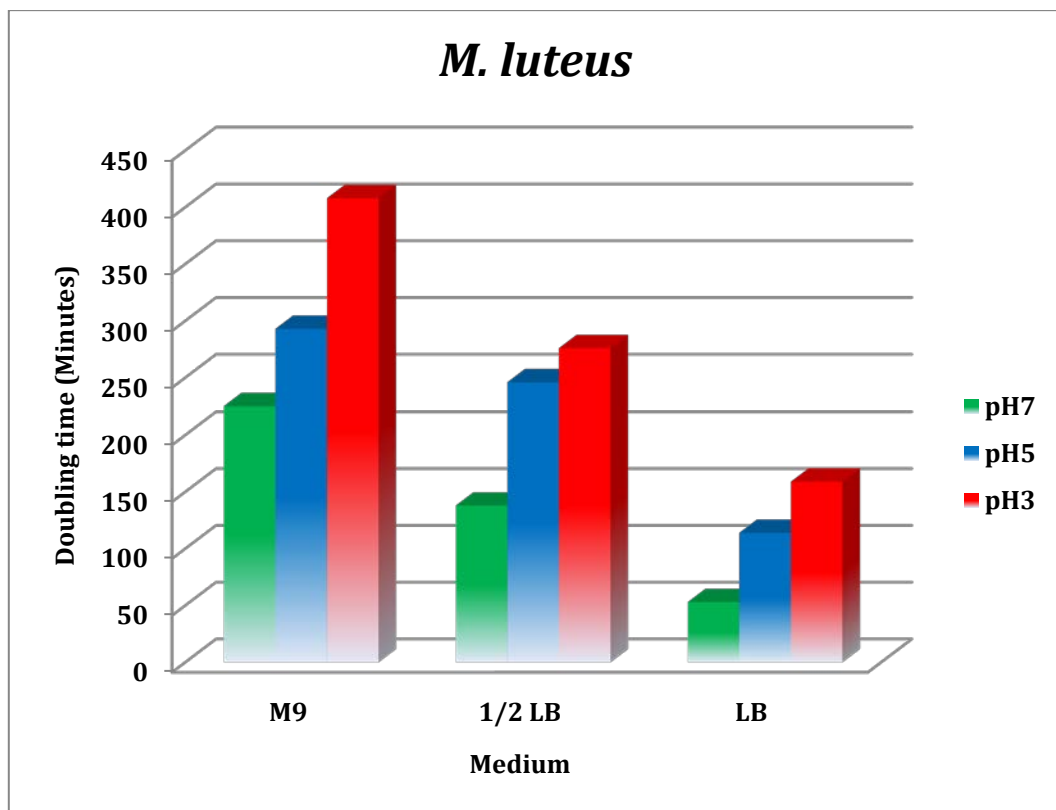
Figure 5-8: Effect of external pH on the doubling times of *M. luteus* in M9, ½ LB and LB medium. Each point represents the mean from four replicate samples.

5.2.3 Effect of High Salinity and External pH on the Growth Rate of *D. hansenii* and *M. luteus*

In order to investigate the effect of different high salinity concentrations and different external pH values on the growth rates of *D. hansenii* and *M. luteus*, growth curves were produced in M9 minimal medium for both strains, in YPD for *D. hansenii* and in LB medium for *M. luteus*. Growth of both microorganisms was quantified by direct optical density (OD) readings at 600 nm measured with the Unicam Helisα spectrophotometer.

M9 minimal medium, YPD and LB medium containing different NaCl concentrations (from 0.1 M to 3.5 M) were used in order to produce growth curves of *D. hansenii* and *M. luteus* at pH 7, 5 and 3 depending on the tolerance of the microbes to high salinity. M9 medium was used to indicate the effect of low nutrients on the growth rate of both strains simultaneously with the other two stresses (high salinity and low pH) (i.e. to demonstrate whether the two strains were capable of growing under stress conditions without the use of enriched medium components).

5.2.3.1 *Debaryomyces hansenii*

In general, *D. hansenii* cells show a good ability to adapt and grow in the three stresses (low nutrients, low pH and high salinity) at 0.1 M and 0.4 M NaCl in particular at pH 5 and pH 7 (Figure 5-9 and Figure 5-10). Figure 5-11 and Figure 5-12 show that *D. hansenii* cells do not grow well at pH 3 in 1 M and 1.5 M NaCl while the cells still adapted and thriving at both salinities at pH 5 and pH 7. The tolerance range of *D.*

hansenii cells reached its limit when the yeast cells were grown in 2 M and 2.5 M NaCl at all pH values in M9 minimal medium (Figure 5-13 and Figure 5-14).

Table 5-3 and Figure 5-15 show the doubling times of *D. hansenii* in M9 medium which confirm that the best growth of *D. hansenii* at all pH values was in 0.1 M NaCl and afterward the growth rate decreased with the rise of NaCl concentrations.

On the other hand, *D. hansenii* cells showed higher growth and adapt to grow and thrive in higher salinity concentrations and low pH when grown in YPD medium. Figure 5-16 shows that *D. hansenii* cells produced good growth at pH 5 and pH 7 while growth was lower at pH 3. However, when salinity was increased to 0.4 and 1 M NaCl the growth of *D. hansenii* cells improved and increased at all pH values (Figure 5-17 and Figure 5-18). The cells also show good ability to grow in 1.5 and 2 M NaCl at all pH levels (Figure 5-19 and Figure 5-20). Increasing the salinity to 2.5 M NaCl caused a drop in the growth of the cells grown at pH 3 while the cells grown at pH 5 and 7 still continue adapting and growing at this high level of salinity (Figure 5-21). Figure 5-22 shows that *D. hansenii* cells started to struggle to grow at all pH values when the NaCl concentration was increased to 3 M, while little or no growth was observed in 3.5 M NaCl at all pH values (Figure 5-23). The doubling times show that the best salinity for growth of *D. hansenii* in YPD medium is 1 M NaCl at all pH values (Table 5-4 and Figure 5-24).
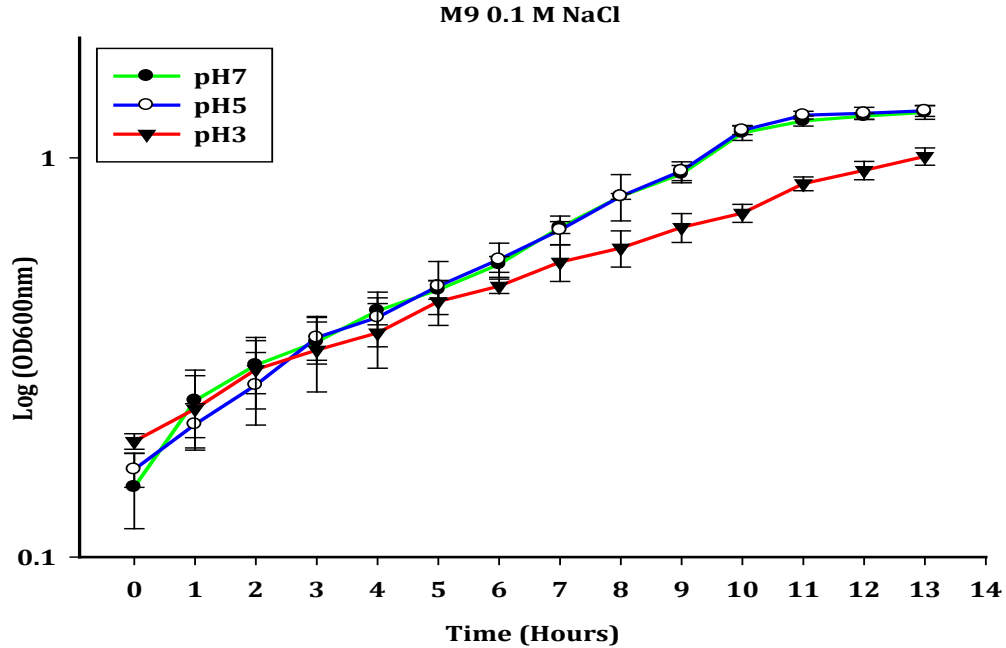
M9 0.1 M NaCl

Figure 5-9: Growth curves for *D. hansenii*. Cells were grown in M9 medium at pH 3, 5 and 7 in 0.1 M NaCl and incubated in a 25 °C constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.
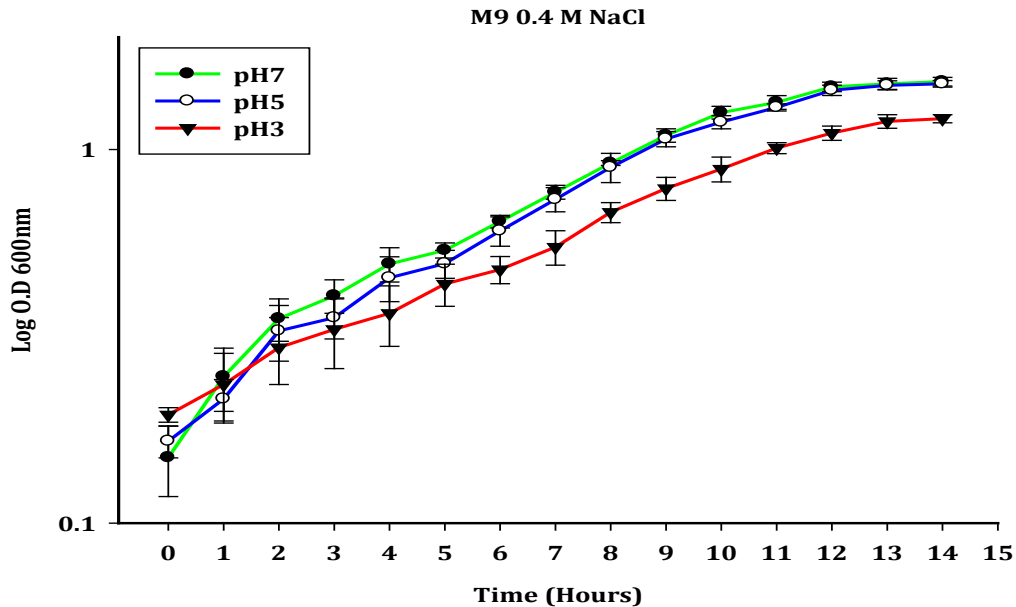
M9 0.4 M NaCl

Figure 5-10: Growth curves for *D. hansenii*. Cells were grown in M9 medium at pH 3, 5 and 7 in 0.4 M NaCl and incubated in a 25 °C constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.

1 M
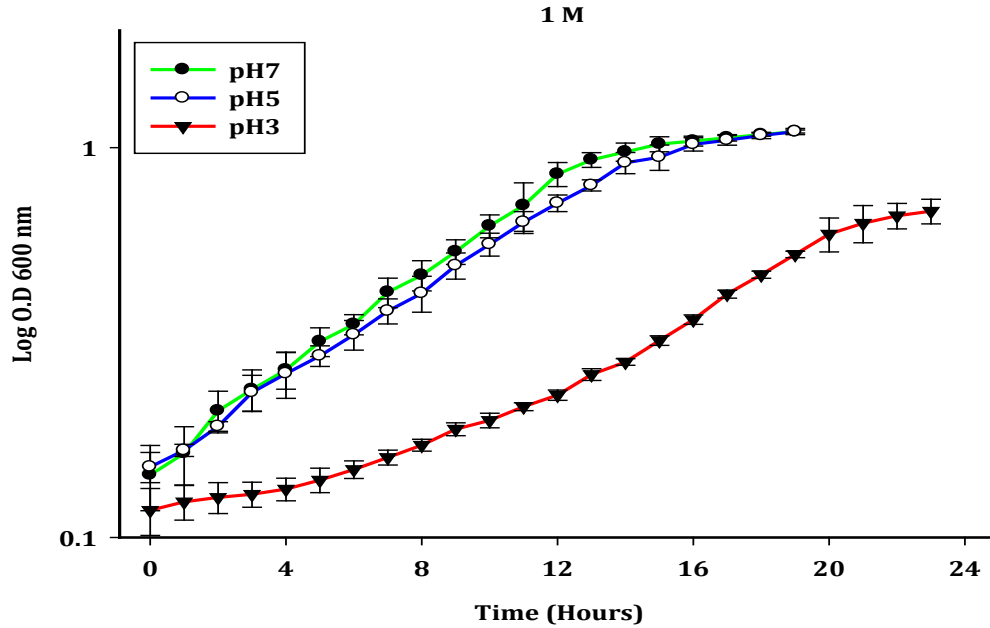
Figure 5-11: Growth curves for *D. hansenii*. Cells were grown in M9 medium at pH 3, 5 and 7 in 1 M NaCl and incubated in a 25 °C constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.

M9 1.5 M NaCl

Figure 5-12: Growth curves for *D. hansenii*. Cells were grown in M9 medium at pH3, 5 and 7 in 1.5 M NaCl and incubated in a 25 °C constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.

M9 2M NaCl

Figure 5-13: Growth curves for *D. hansenii*. Cells were grown in M9 medium at pH 3, 5 and 7 in 2 M NaCl and incubated in a 25 °C constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.

M9 2.5M NaCl

Figure 5-14: Growth curves for *D. hansenii*. Cells were grown in M9 medium at pH 3, 5 and 7 in 2.5 M NaCl and incubated in a 25 °C constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.

Table 5-3: Effect of different NaCl concentrations on the doubling times of *D. hansenii* in M9 medium at pH 7, 5 and 3. Each point represents the mean from four replicate samples.

Doubling Time of *D. hansenii* under Different Salinity in M9 Medium (Minutes)					
	0.1	**0.4**	**1**	**1.5**	**2**
pH 7	189	216	276	288	678
pH 5	180	240	282	296	660
pH 3	182	267	438	693	*

*: No growth was observed

Figure 5-15: Effect of different NaCl concentrations on the doubling times of *D. hansenii* in M9 medium at pH 7, 5 and 3. Each point represents the mean from four replicate samples.

YPD 0.1M NaCl
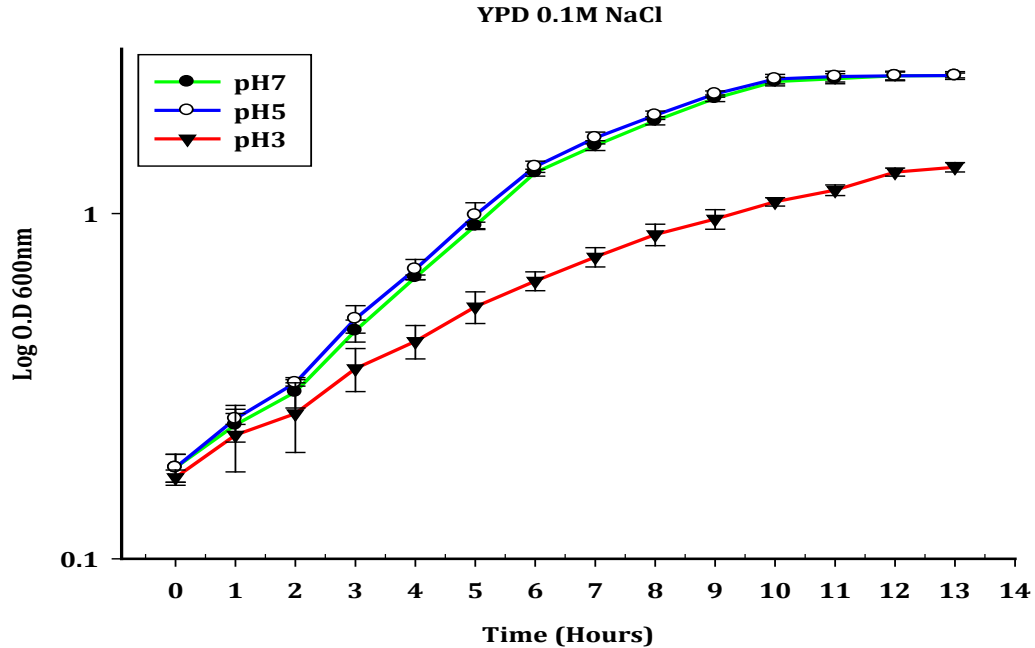
Figure 5-16: Growth curves for *D. hansenii*. Cells were grown in YPD medium at pH 3, 5 and 7 in 0.1 M NaCl and incubated in a 25 °C constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.

YPD 0.4M NaCl
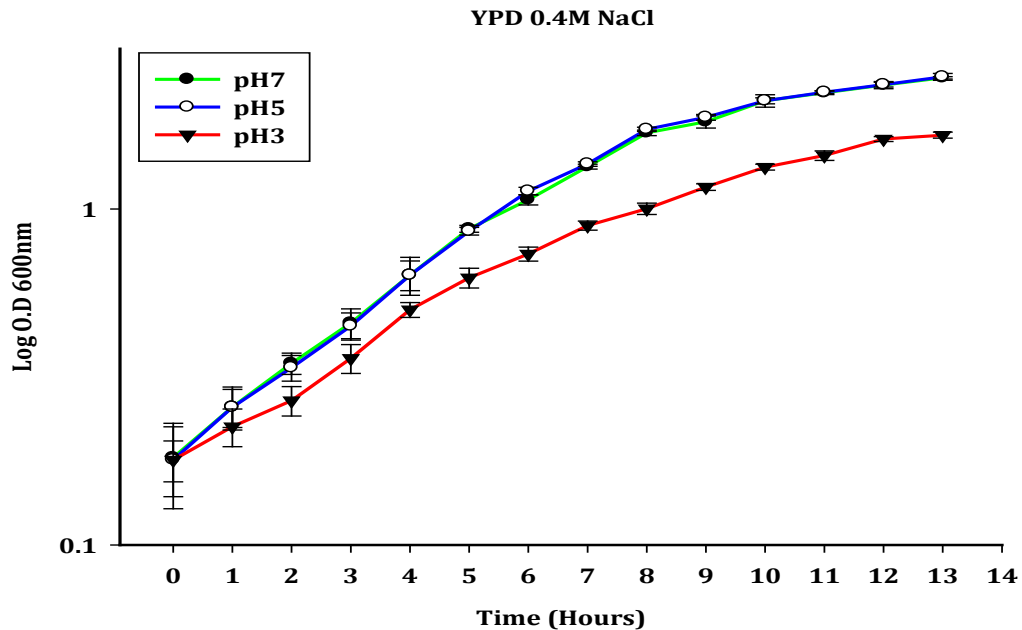
Figure 5-17: Growth curves for *D. hansenii*. Cells were grown in YPD medium at pH 3, 5 and 7 in 0.4 M NaCl and incubated in a 25 °C constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.
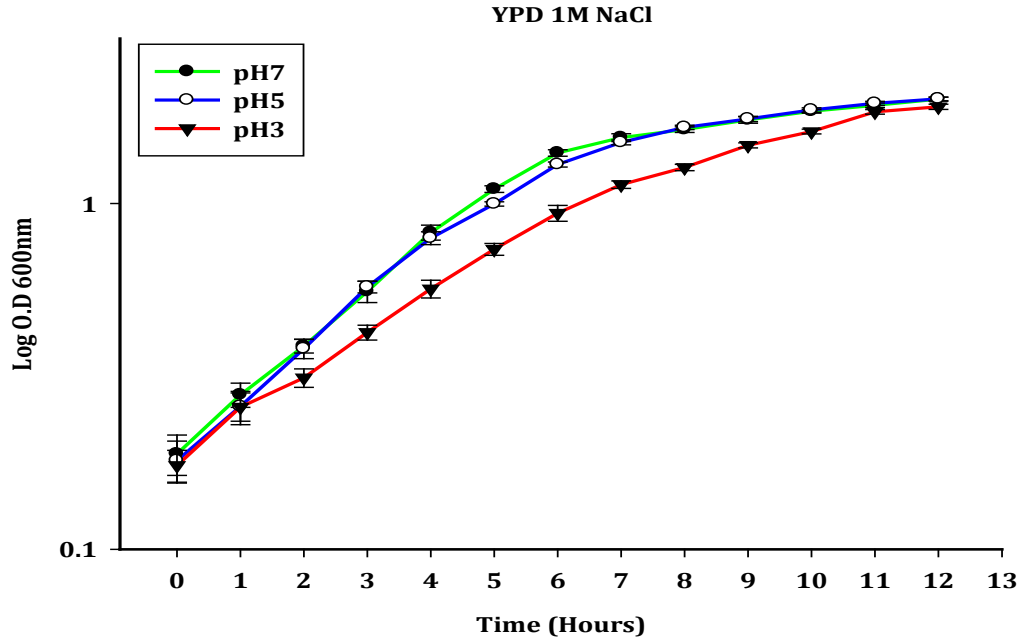
Figure 5-18: Growth curves for *D. hansenii*. Cells were grown in YPD medium at pH 3, 5 and 7 in 1 M NaCl and incubated in a 25 ℃ constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.

Figure 5-19: Growth curves for *D. hansenii*. Cells were grown in YPD medium at pH 3, 5 and 7 in 1.5 M NaCl and incubated in a 25 ℃ constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.

YPD 2M NaCl

Figure 5-20: Growth curves for *D. hansenii*. Cells were grown in YPD medium at pH 3, 5 and 7 in 2 M NaCl and incubated in a 25 °C constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.

YPD 2.5M NaCl

Figure 5-21: Growth curves for *D. hansenii*. Cells were grown in YPD medium at pH 3, 5 and 7 in 2.5 M NaCl and incubated in a 25 °C constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.

YPD 3M NaCl

Figure 5-22: Growth curves for *D. hansenii*. Cells were grown in YPD medium at pH 3, 5 and 7 in 3 M NaCl and incubated in a 25 °C constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.
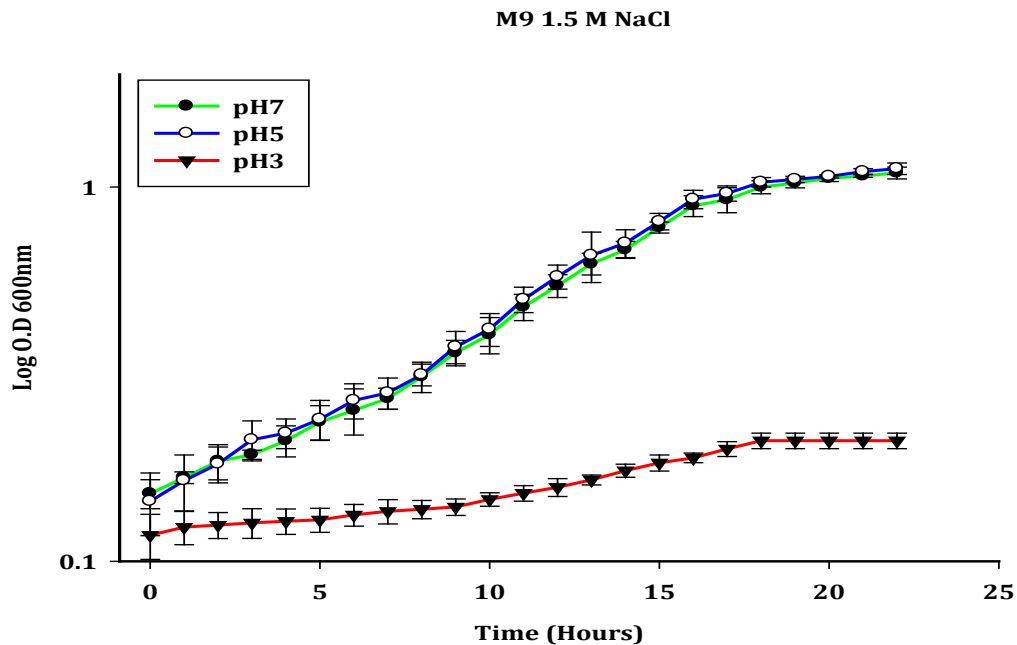
YPD 3.5M NaCl

Figure 5-23: Growth curves for *D. hansenii*. Cells were grown in YPD medium at pH 3, 5 and 7 in 3.5 M NaCl and incubated in a 25 °C constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.

Table 5-4: Effect of different NaCl concentrations on the doubling times of *D. hansenii* in YPD medium at pH 7, 5 and 3. Each point represents the mean from four replicate samples.

Doubling Time of *D. hansenii* under Different Salinity in YPD medium (Minutes)							
	0.1	**0.4**	**1**	**1.5**	**2**	**2.5**	**3**
pH 7	120	101	89	135	295	336	588
pH 5	115	100	85	128	288	328	588
pH 3	195	145	111	171	321	473	655

Figure 5-24: Effect of different NaCl concentrations on the doubling times of *D. hansenii* in YPD medium at pH 7, 5 and 3. Each point represents the mean from four replicate samples.

5.2.3.2   *Micrococcus luteus*

Generally, the growth curves for *M. luteus* in M9 medium in different pH values and at various NaCl concentrations showed the limited ability of *M. luteus* to adapt and thrive in low nutrient medium at low pH values. However, the bacterial cells were able to adapt and grow at pH 3, 5 and 7 in 0.1 M NaCl (Figure 5-25). Figure 5-26 shows that increasing the NaCl concentration to 0.4 M partially inhibited growth at all pH values. *M. luteus* cells were unable to adapt when grown in the presence of 1 M NaCl at all pH values in M9 medium (Figure 5-27). The doubling times show that the best growth of *M. luteus* cells in M9 minimal medium was in 0.1 M NaCl at pH7 (Table 5-5 and Figure 5-28).

Unlike in M9 medium, *M. luteus* cells showed good adaptation and higher growth in LB medium at different pH values and different NaCl concentrations. The best growth for *M. luteus* cells occurred at pH 3 in 0.1 M NaCl (Figure 5-29), while the highest growth at pH 7 and pH 5 occurred when cells were grown in 0.4 M NaCl (Figure 5-30). Figure 5-31 shows that *M. luteus* was still able to adapt and thrive in 1 M NaCl at pH 7, 5 and 3. However, when *M. luteus* cells were grown in 1.5 M NaCl, the growth at pH 3 decreased while the cells continue to adapt and grow at pH 7 and pH 5 (Figure 5-32). The same happened in 2 and 2.5 M NaCl (Figure 5-33 and Figure 5-34). At 3 M NaCl, *M. luteus* was unable to grow at all three pH values (Figure 5-35).

Table 5-6 and Figure 5-36 show the calculation of doubling times for *M. luteus* cells grown in LB medium at pH 7, 5 and 3.
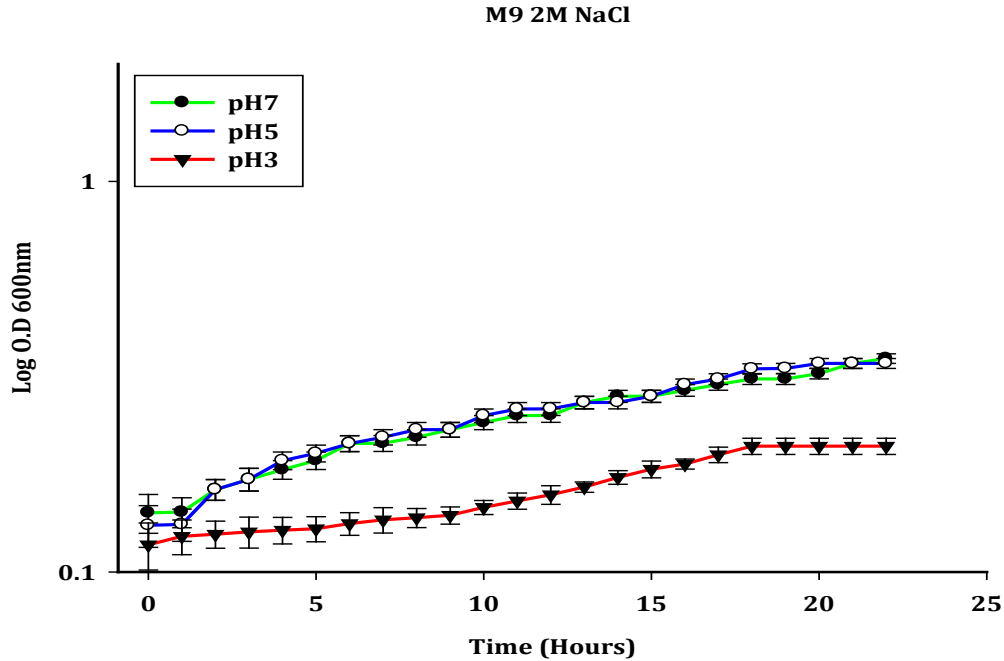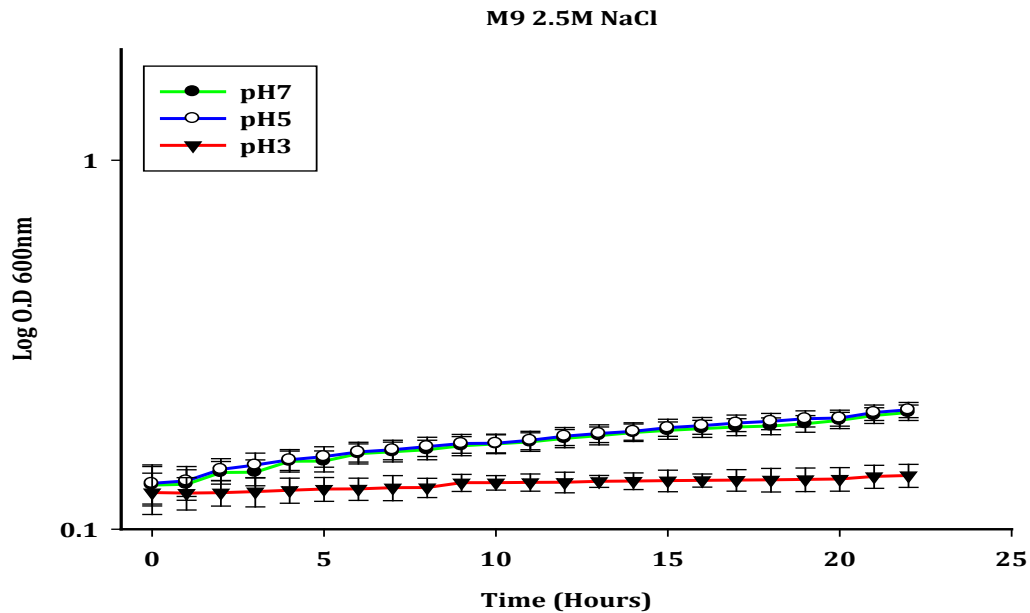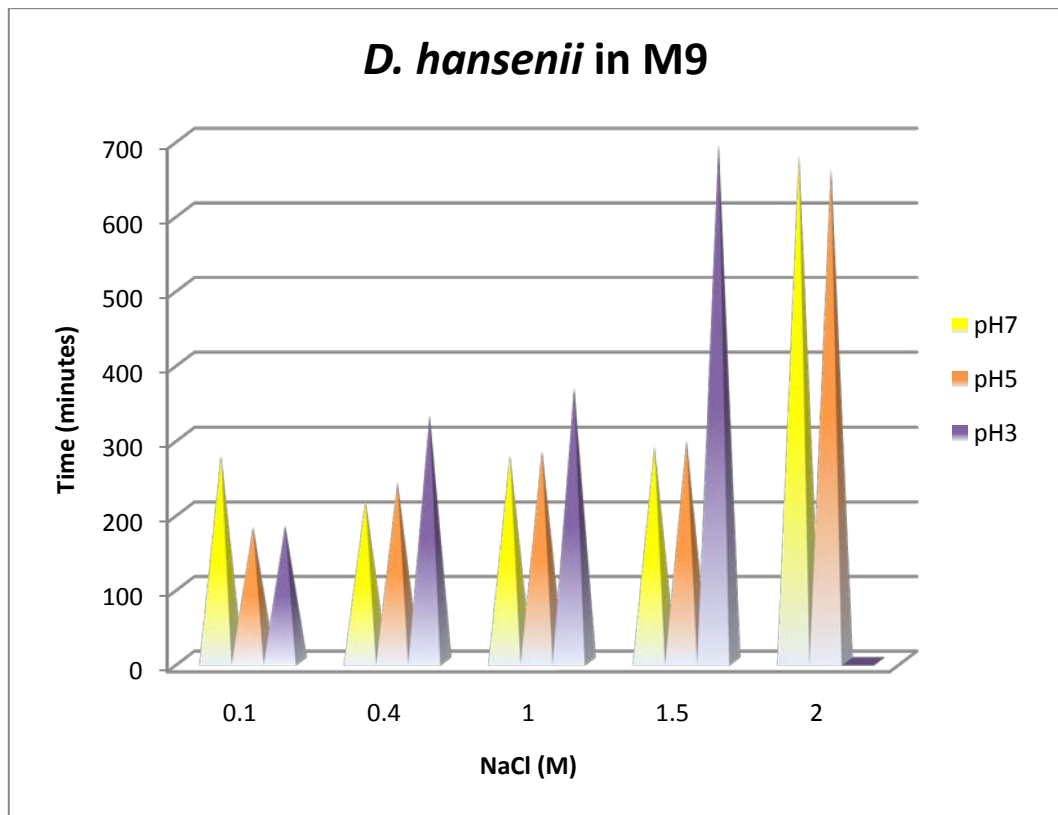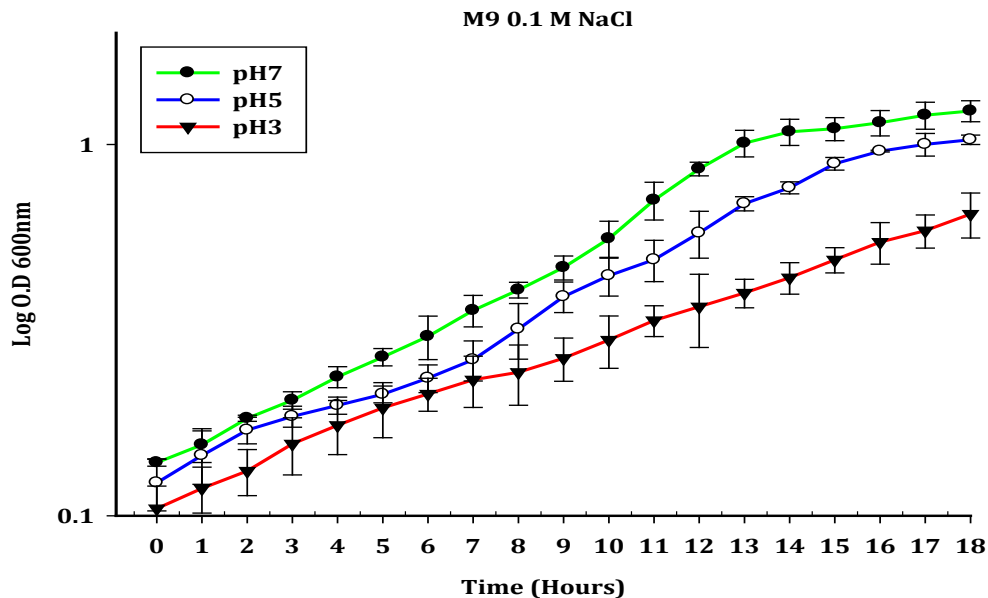
M9 0.1 M NaCl

Figure 5-25: Growth curves for *M. luteus*. Cells were grown in M9 medium at pH 3, 5 and 7 in 0.1 M NaCl and incubated in a 25°C constant temperature room on an orbital shaker at 250 rpm overnight. The OD for each strain was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.

M9 0.4NaCl
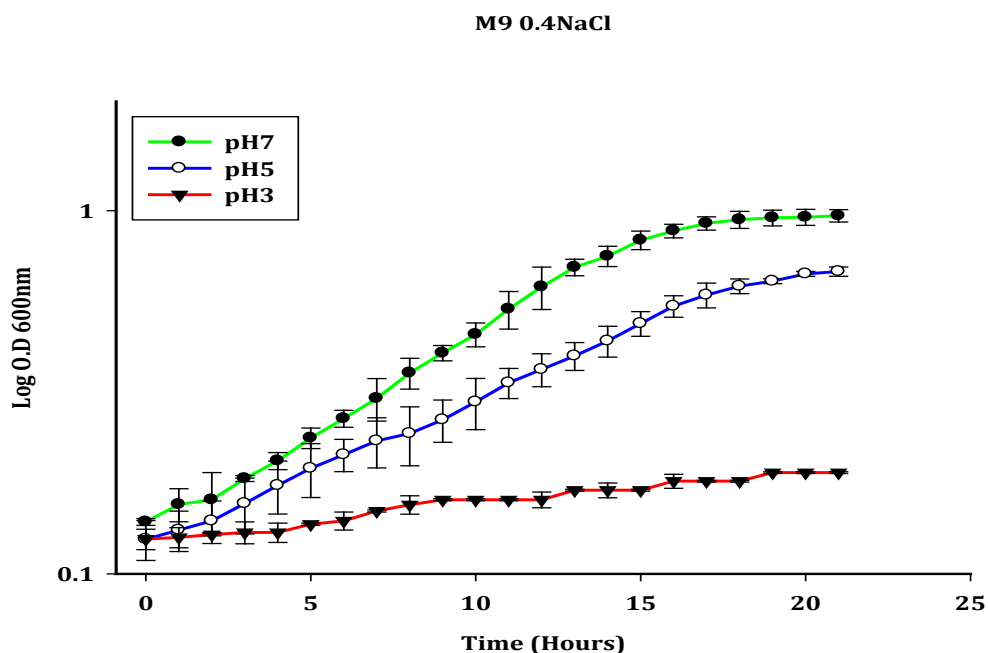
Figure 5-26: Growth curves for *M. luteus*. Cells were grown in M9medium at pH 3, 5 and 7 in 0.4 M NaCl and incubated in a 25°C constant temperature room on an orbital shaker at 250 rpm overnight. The OD for each strain was measured at 600 nm. Data points are the mean of four replicates plus or minus standard deviation.
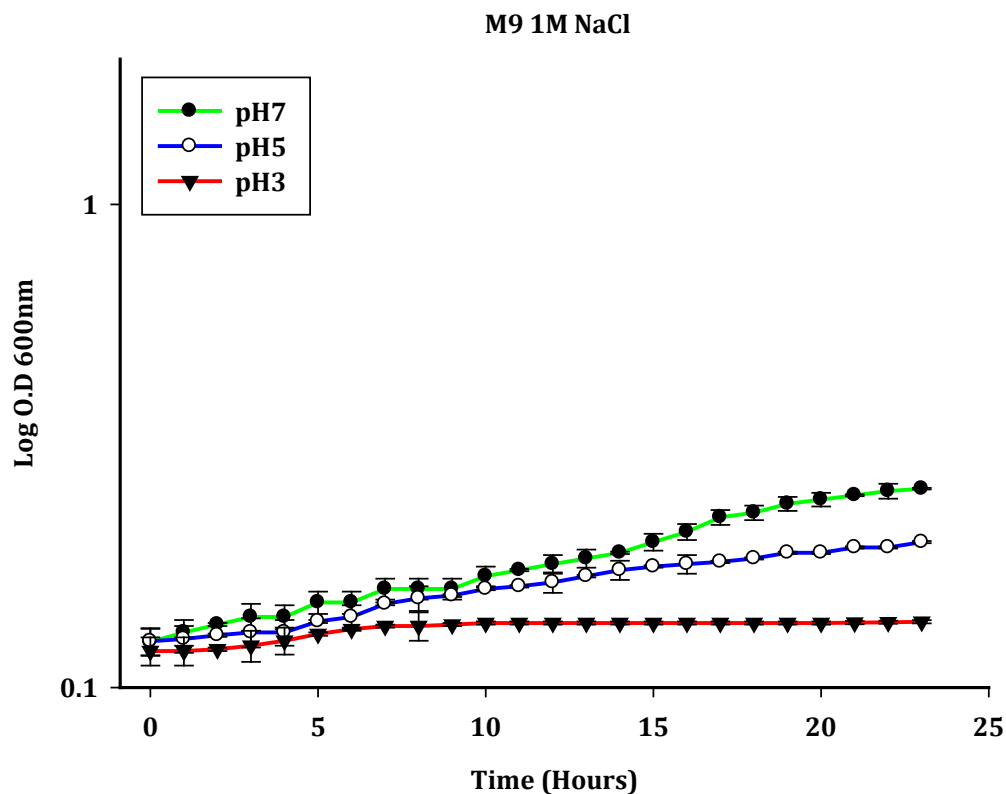
Figure 5-27: Growth curves for *M. luteus*. Cells were grown in M9 medium at pH 3, 5 and 7 in 1 M NaCl incubated in a 25°C constant temperature room on an orbital shaker at 250 rpm overnight. The OD for each strain was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.

Table 5-5: Effect of different NaCl concentrations on the doubling times of *M. luteus* in M9 medium at pH 3, 5 and 7. Each point represents the mean from four replicate samples.

Doubling Times of *M. luteus* under Different Salinities in M9 Medium (Minutes)			
	0.1	**0.4**	**1**
pH 7	225	239	720
pH 5	293	372	765
pH 3	408	870	*

*: No growth was observed
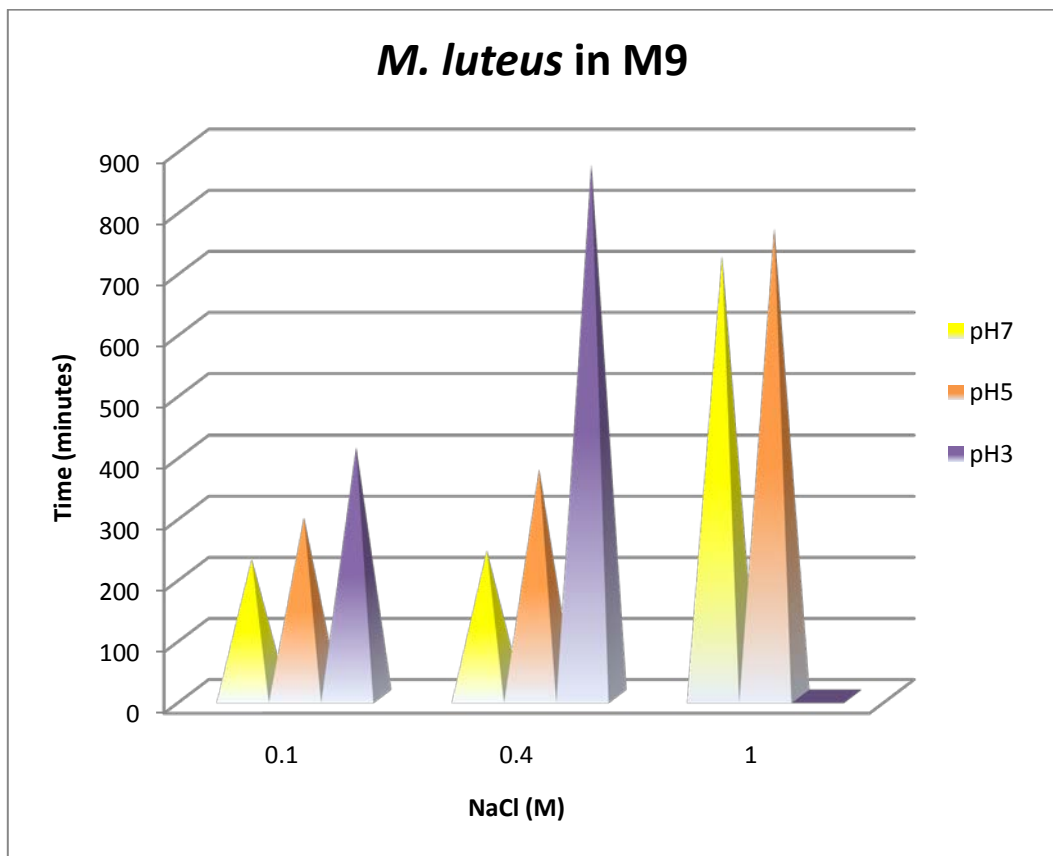
Figure 5-28: Effect of different NaCl concentrations on the doubling times of *M. luteus* in M9 medium at pH 3, 5 and 7. Each point represents the mean from four replicate samples.
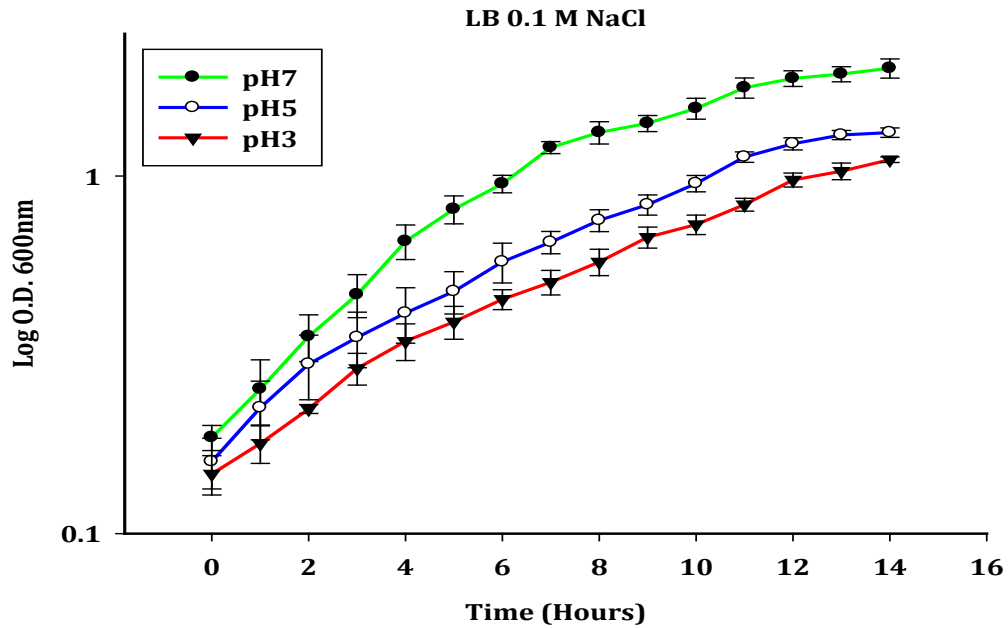
Figure 5-29: Growth curves for *M. luteus*. Cells were grown in LB medium at pH 3, 5 and 7 in 0.1 M NaCl and incubated in a 25ºC constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.
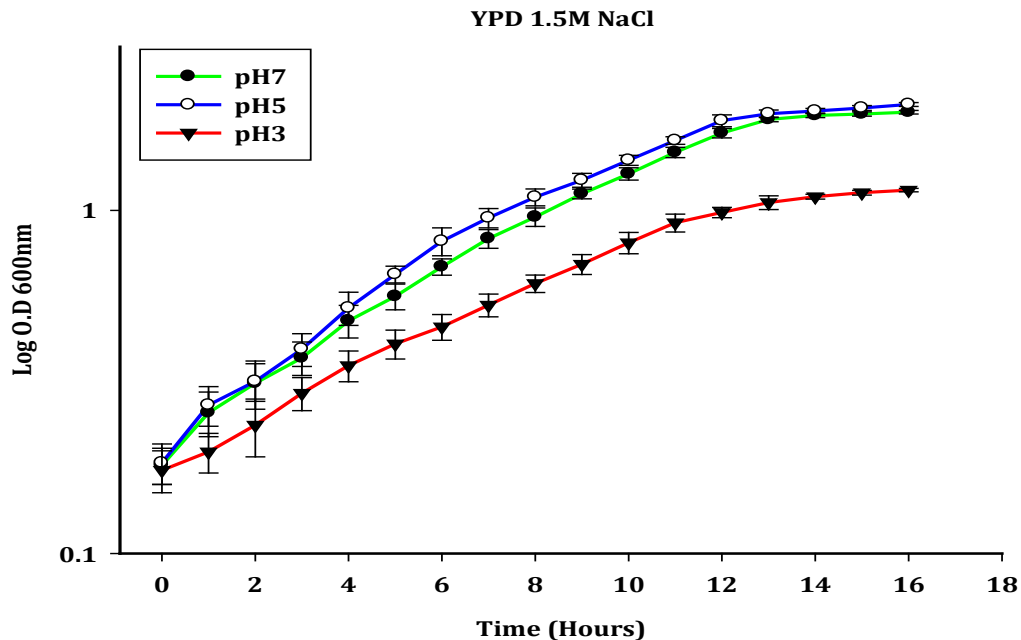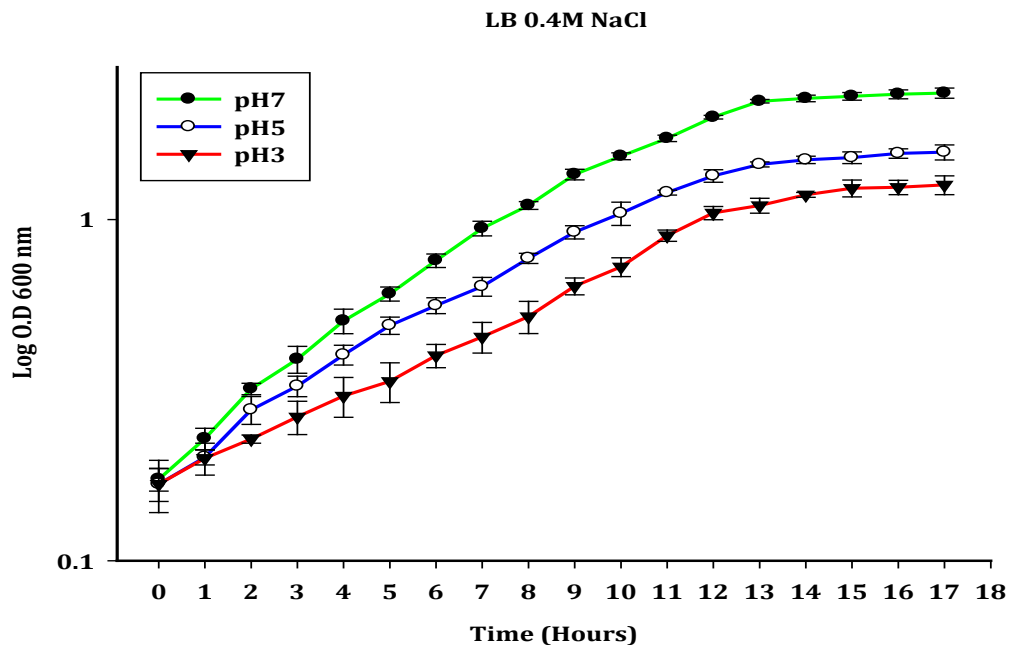

Figure 5-30: Growth curves for *M. luteus*. Cells were grown in LB medium at pH 3, 5 and 7 in 0.4 M NaCl and incubated in a 25ºC constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.
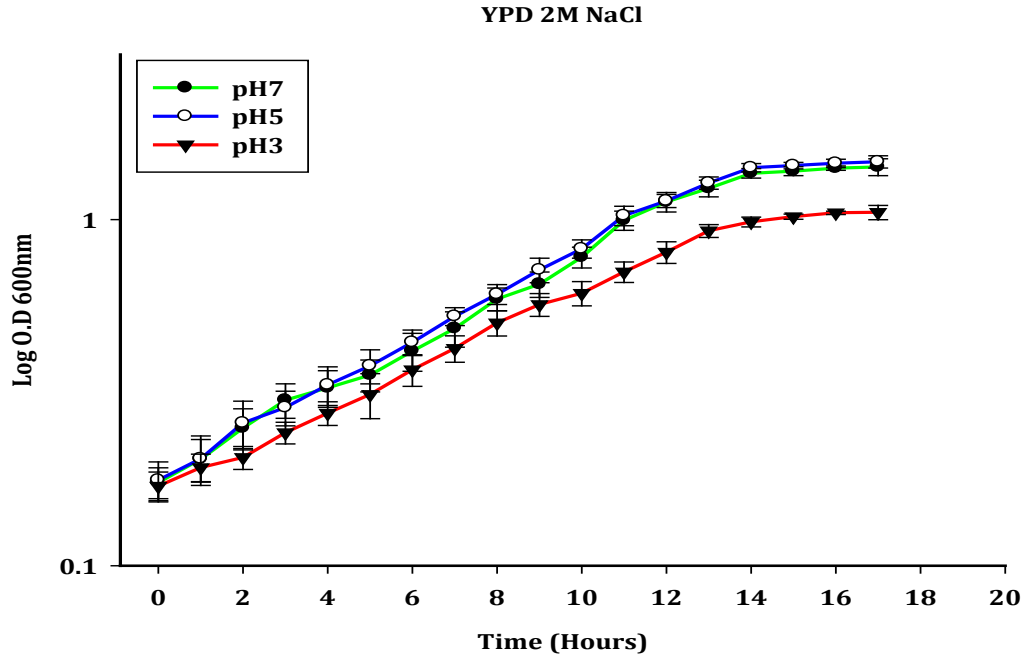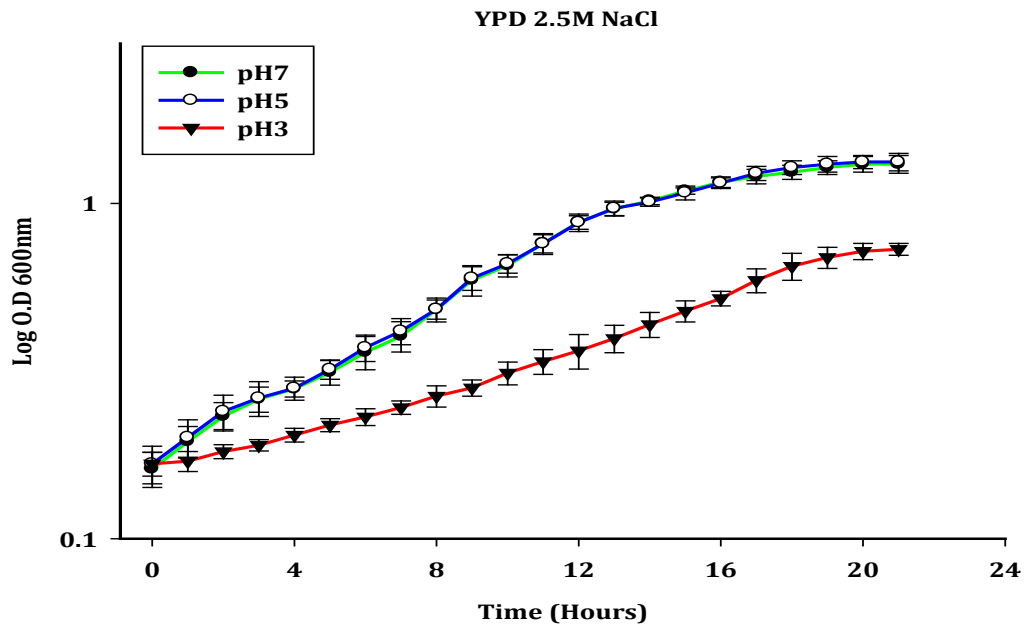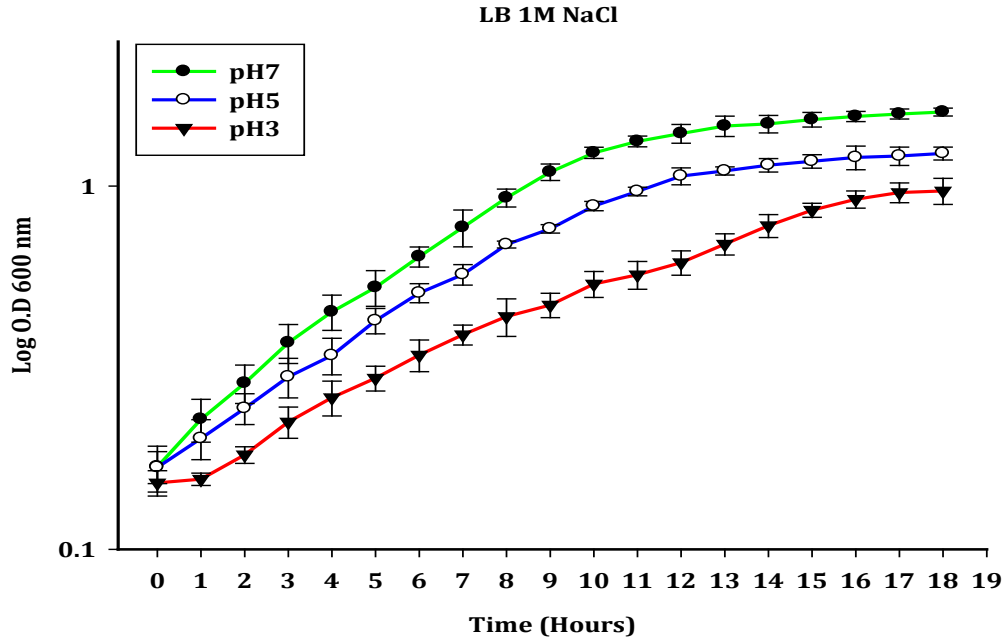
Figure 5-31: Growth curves for *M. luteus*. Cells were grown in LB medium at pH 3, 5 and 7 in 1 M NaCl and incubated in a 25°C constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.
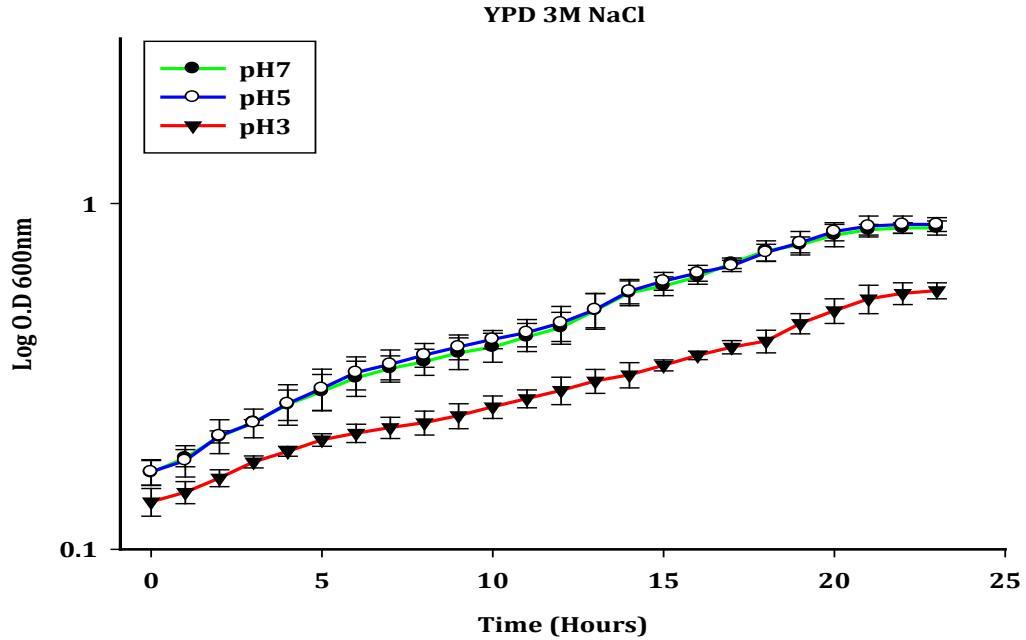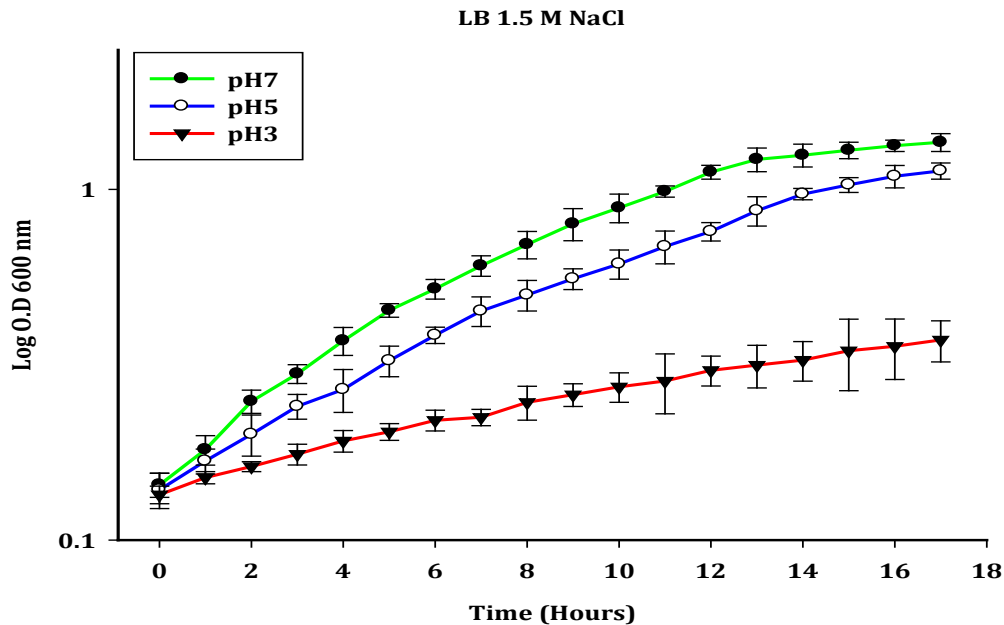

Figure 5-32: Growth curves for *M. luteus*. Cells were grown in LB medium at pH 3, 5 and 7 in 1.5 M NaCl and incubated in a 25°C constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.
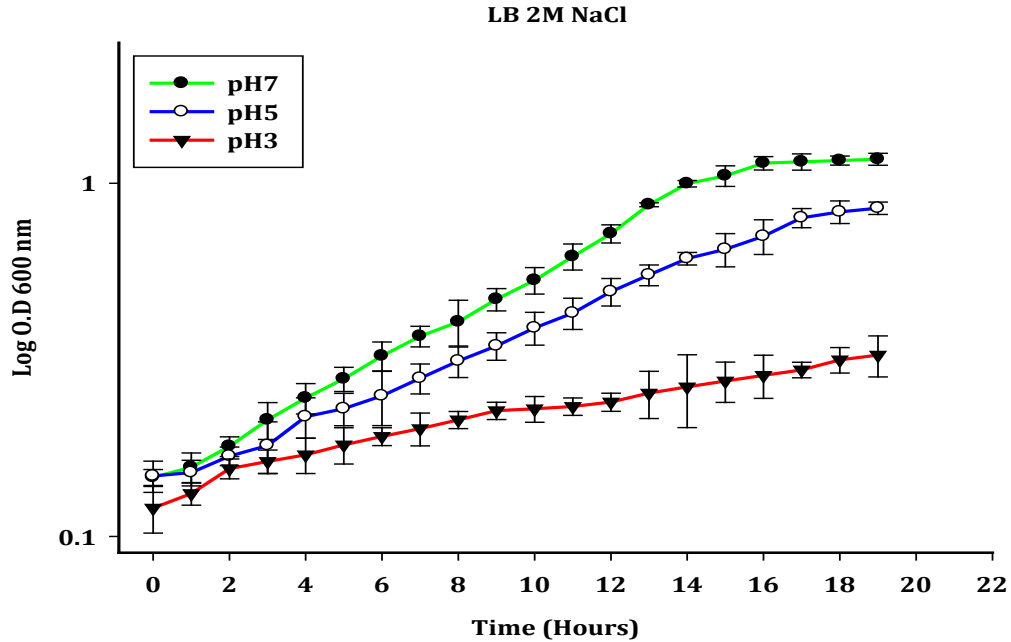
LB 2M NaCl

Figure 5-33: Growth curves for *M. luteus*. Cells were grown in LB medium at pH 3, 5 and 7 in 2 M NaCl and incubated in a 25°C constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.
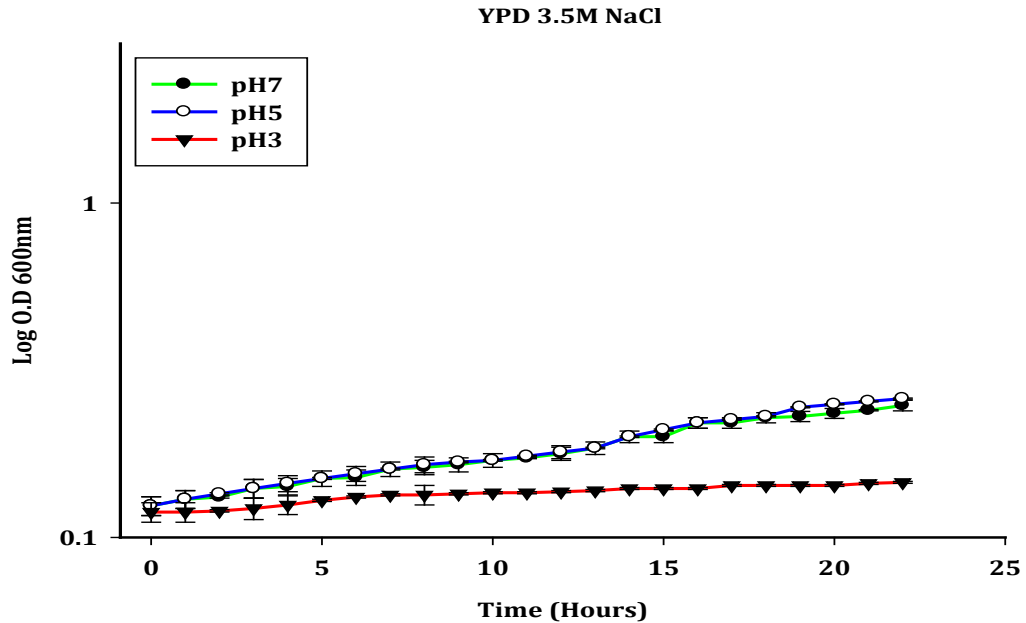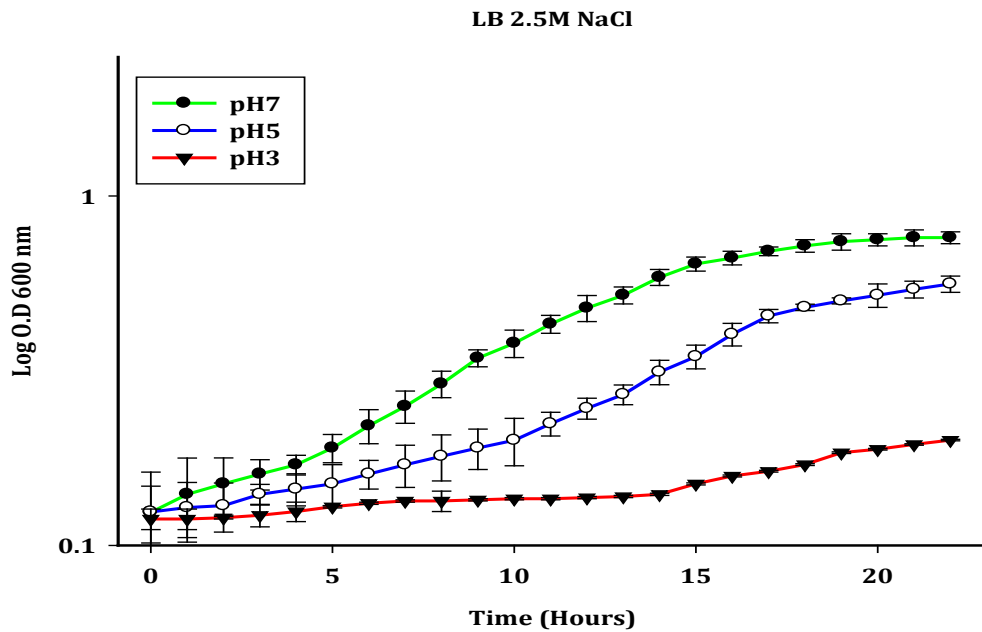

LB 2.5M NaCl

Figure 5-34: Growth curves for *M. luteus*. Cells were grown in LB medium at pH 3, 5 and 7 in 2.5 M NaCl and incubated in a 25°C constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.
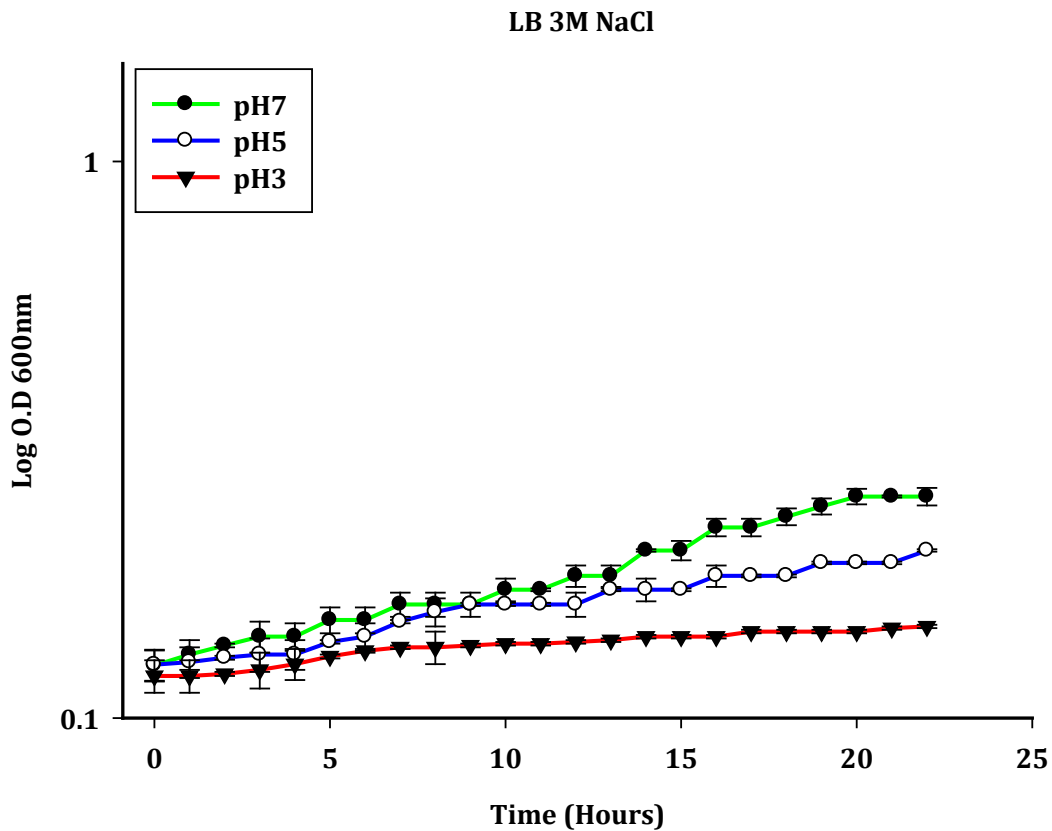
Figure 5-35: Growth curves for *M. luteus*. Cells were grown in LB medium at pH 3, 5 and 7 in 3 M NaCl and incubated in a 25°C constant temperature room on an orbital shaker at 250 rpm overnight. The OD was measured at 600 nm. Data points are the means of four replicates plus or minus standard deviation.
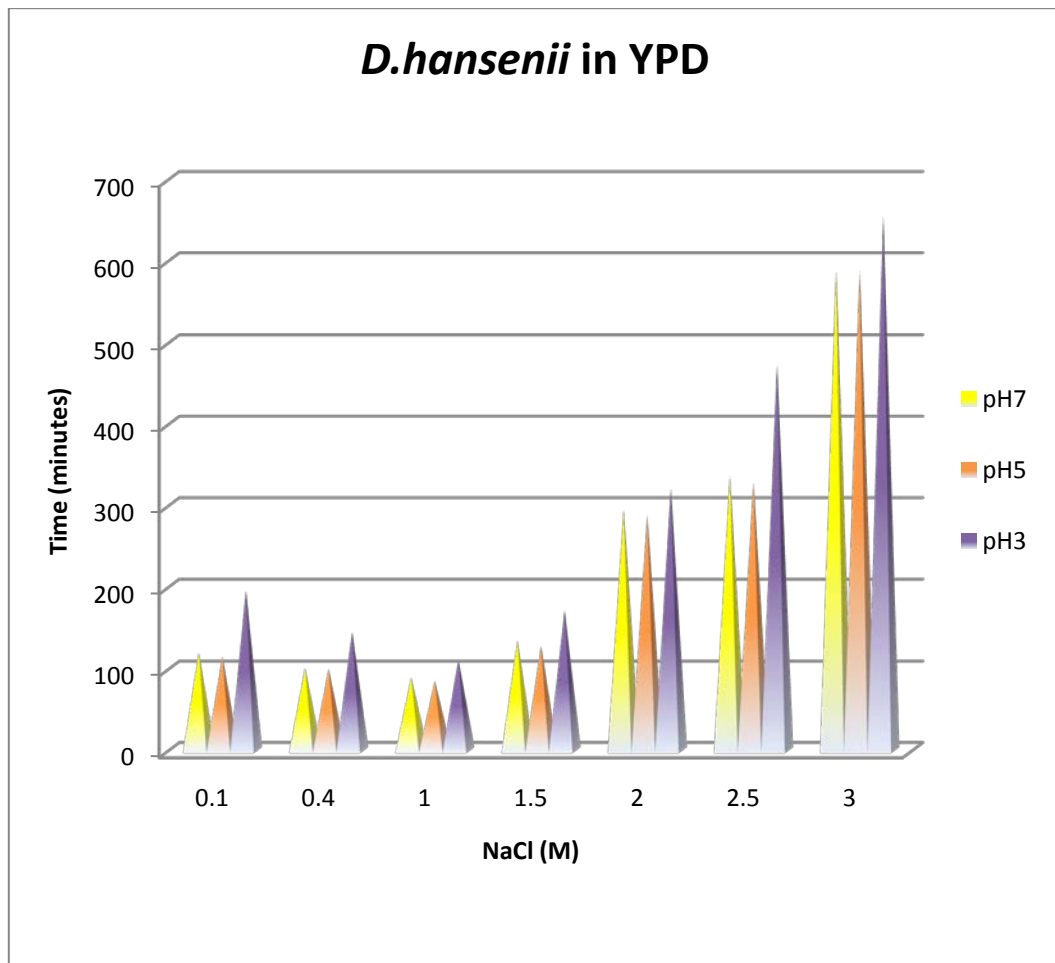
Table 5-6 : Effect of different NaCl concentrations on the doubling times of *M. luteus* in LB medium at pH 7, 5 and 3. Each point represents the mean from four replicate samples.

| Doubling Time of *M. luteus* under Different Salinities in LB medium (Minutes)						
	0.1	**0.4**	**1**	**1.5**	**2**	**2.5**
pH 7	53	50	68	148	285	421
pH 5	114	105	106	178	342	673
pH 3	159	196	284	395	587	*

*: No growth was observed
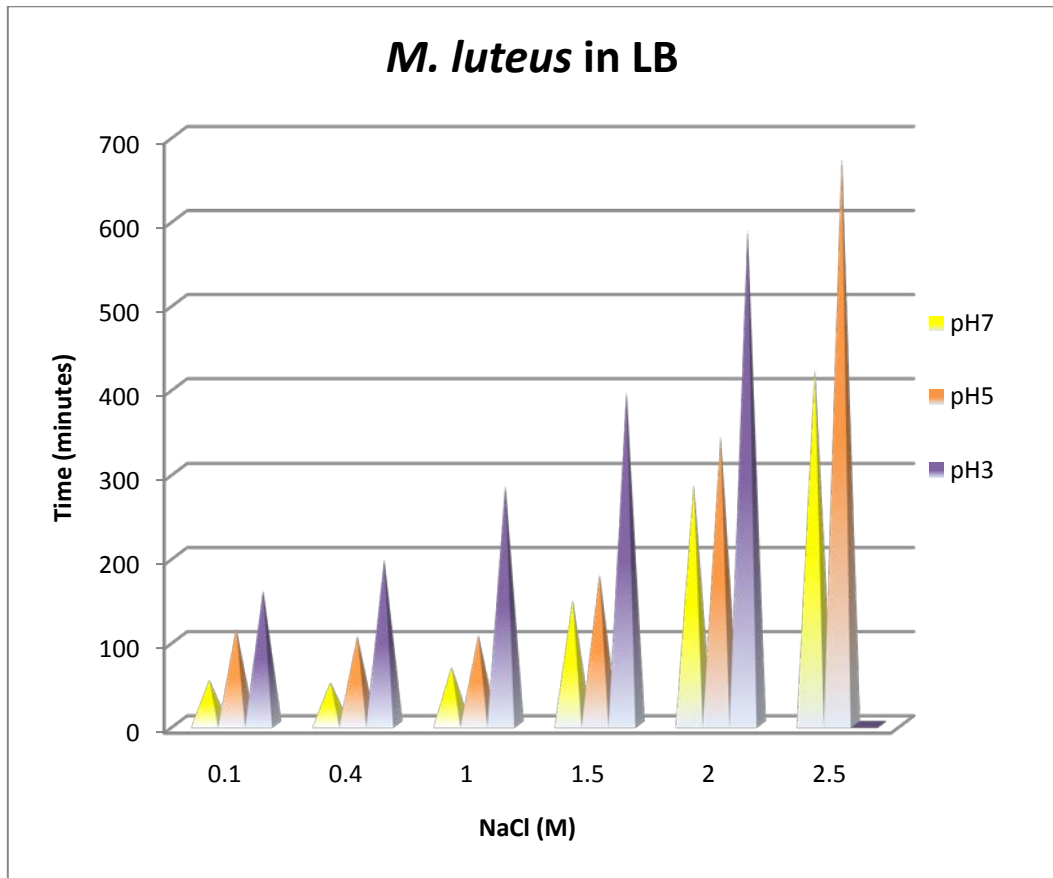
Figure 5-36: Effect of different NaCl concentrations on the doubling times of *M. luteus* in LB medium at pH 7, 5 and 3. Each point represents the mean from four replicate samples.

5.2.4 Effect of High Salinity and External pH Medium on the Final Biomass of *D. hansenii* and *M. luteus* Cells

To further investigate the growth of *D. hansenii* and *M. Luteus* under stress conditions, the incubation period was increased to include overnight incubation (minimum of 24 hours) to observe the final biomass level attained. The overnight cultures of both strains show similar results to growth curves discussed in the previous section, but with more clarity and the results are easier to summarize in graphs. In general, both strains required at least 0.1 M NaCl to grow and thrive in M9, YPD and LB medium, their cells showed little or no growth when grown on those media with no NaCl added (Figures 5-37, 5-38, 5-39 and 5-40).

Figure 5-37 shows that the highest biomass attained for *D. hansenii* grown in M9 medium was in 0.4 M NaCl medium at all pH values, but when the cells were grown in YPD medium, the greatest biomass was observed in 1 M NaCl at all three pH values (Figure 5-38). *M. luteus* cells did not grow well above 0.4 M NaCl when grown in M9 medium at all three pH values; biomass produced was steadily inhibited by an increase in NaCl concentrations and biomass also dropped with decreasing pH 7 at 1 M NaCl (Figure 5-39). On the other hand, *M. luteus* biomass production was stimulated when the NaCl concentration was increased to 0.4 M at all three pH values in LB medium. Furthermore in LB medium *M. luteus* cells were able to adapt and grow at pH 3 until the salinity reached 2 M and at pH 7 and pH 5 the maximum salinity tolerated was 2.5 M NaCl (Figure 5-40).
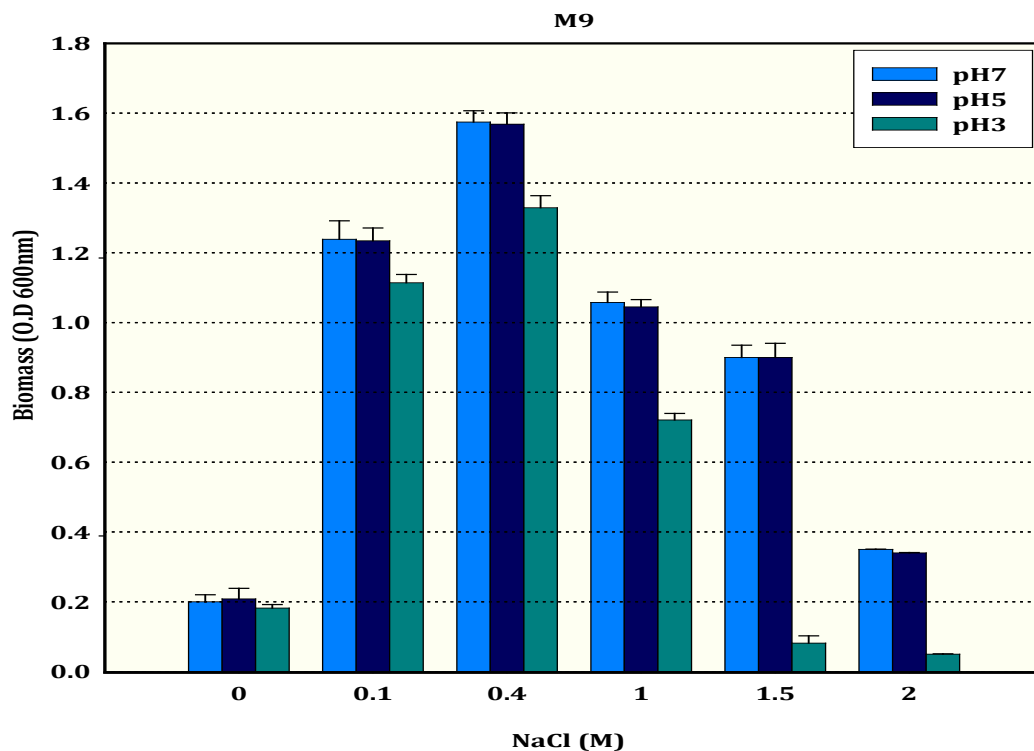
Figure 5-37: Effect of different NaCl concentrations on the biomass attained by overnight cultures of *D. hansenii* in M9 medium at pH 7, 5 and 3.
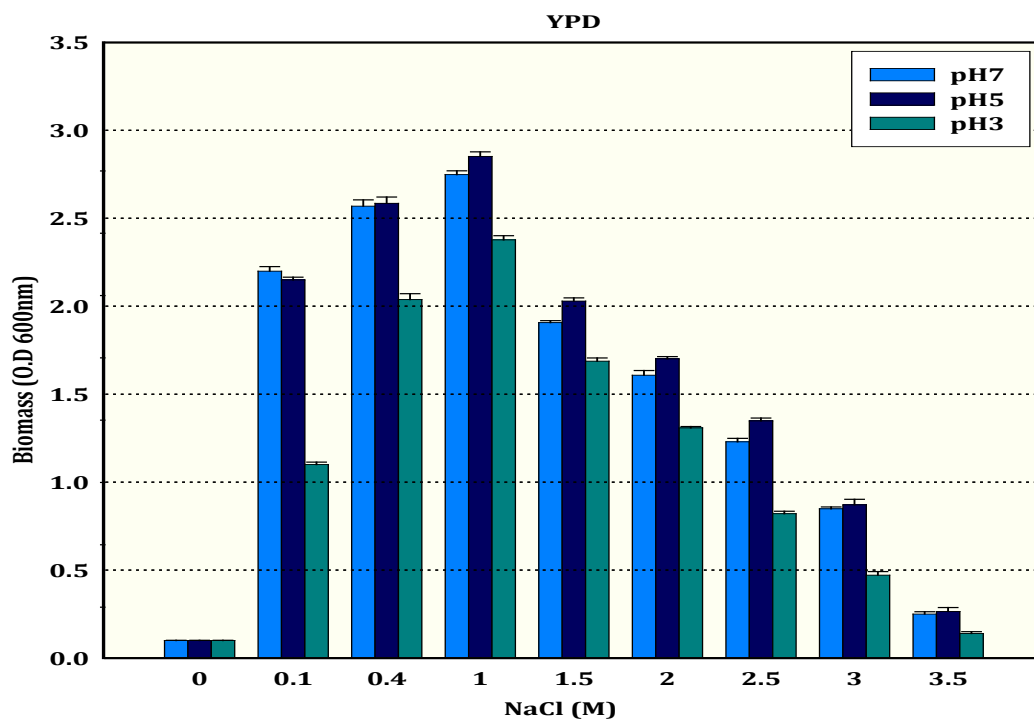
Figure 5-38: Effect of different NaCl concentrations on the biomass attained by overnight cultures of *D. hansenii* in YPD medium at pH 7, 5 and 3.
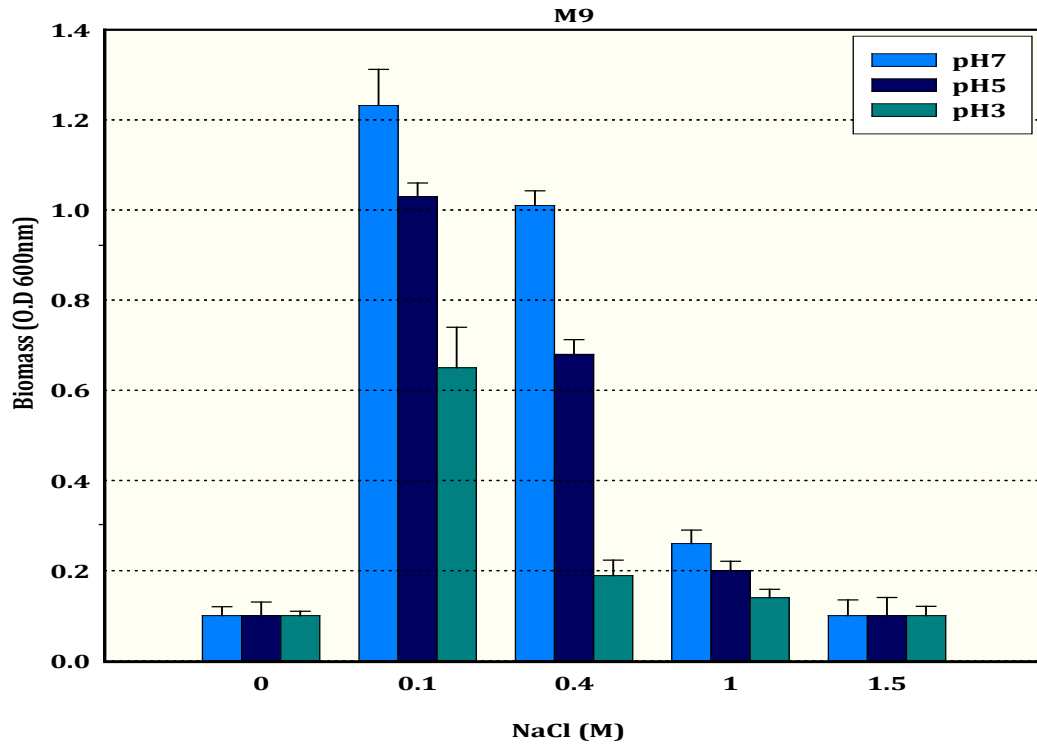
Figure 5-39: Effect of different NaCl concentrations on the biomass attained by overnight cultures of *M. luteus* in M9 medium at pH 7, 5 and 3.
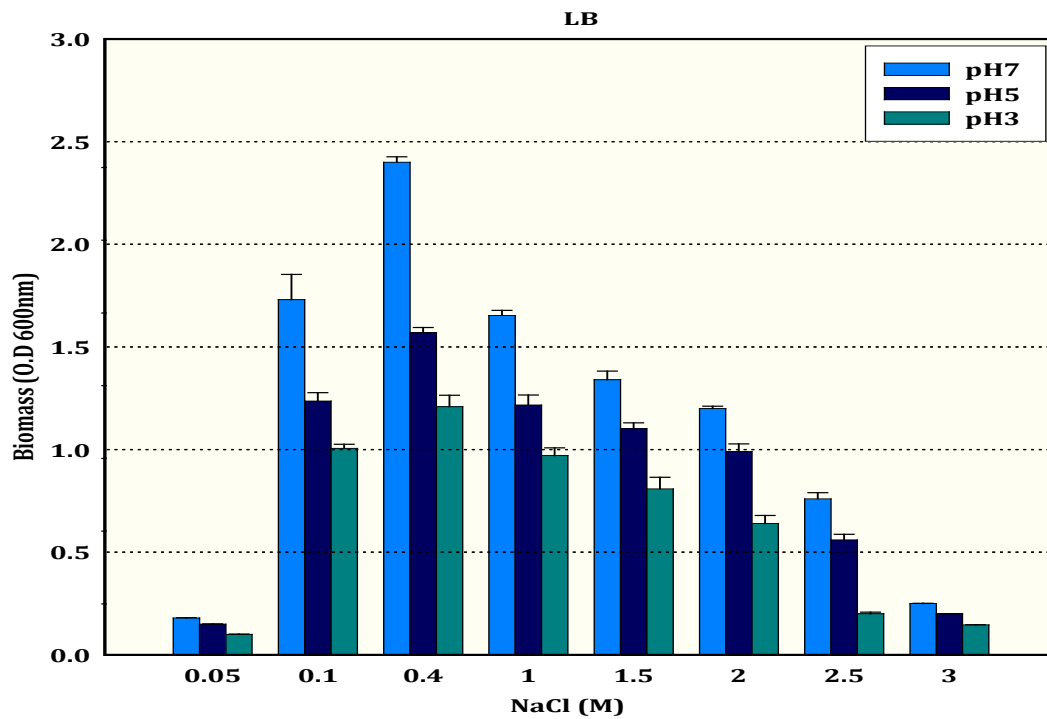
Figure 5-40: Effect of different NaCl concentrations on the biomass attained by overnight cultures of *M. luteus* in LB medium at pH 7, 5 and 3.

5.2.5 Measurement of External pH After Overnight Growth of *D. hansenii* and *M. luteus*

The external pH of the overnight cultures of *D. hansenii* and *M. luteus* was measured in order to determine how much it changed during growth of the strains under pH and salinity stress in rich and minimal media.

D. hansenii showed different behaviour according to the medium used. When the cells were grown in M9 minimal medium the external pH did not change in pH 7 and pH 3 cultures at all NaCl concentrations. However, when they were grown at pH 5 the pH decreased to 4 or less with a rise in NaCl concentration up to 1 M NaCl. Above this salinity, the external pH returned again to pH 5 (Figure 5-41). In rich YPD medium when *D. hansenii* was grown at neutral pH the external pH was stable at 7 in 0.4 M NaCl then decreased with increases of NaCl concentration up to 1.5 M, before the opposite trend of increasing pH again at higher salinities (Figure 5-42). For *D. hansenii* cells grown in M9 medium, the results showed that cells grown at pH 5 raised the pH from 5 to 6 in 0.4 M NaCl and then reduced the pH to 5 again and keep reducing it until the external pH reached a pH 4 in 2 M NaCl and then increased again towards 5 at the highest salinities (Figure 5-42). Finally, the external pH in YPD medium at all salinity concentrations set to pH 3 did not change (Figure 5-42), this agrees with the results in M9 medium (Figure 5-41).

Contrary to *D. hansenii*, the external pH in *M. luteus* cultures did not decrease under all conditions studied in both media, as *M. luteus* cells always tended to increase the external pH value. Figure 5-43 shows that *M. luteus* cells made slight changes to the external pH

in M9 medium at pH 7 and pH 3, but when grown at pH 5, they tended to increase the external pH from 5 to 6 in normal and high NaCl concentrations. On the other hand, *M. luteus* cells grown in LB medium raised the external pH in all cases, they increased the external pH from 7 to close to 9 at low NaCl concentrations, but the effect was less pronounced at high salinities. The same pH changes happened when cells were grown at pH 5 or pH 3, but the effect was smaller at pH 3 (Figure 5-44).

An NMR experiment was performed in order to find out how *M. luteus* cells changed the external pH from acidity to alkalinity when grown in LB medium at low pH values. Figure 5-45 shows that *M. luteus* cells released approximately 10 mM ammonium to increase the external pH and adapt to the acidity stress.
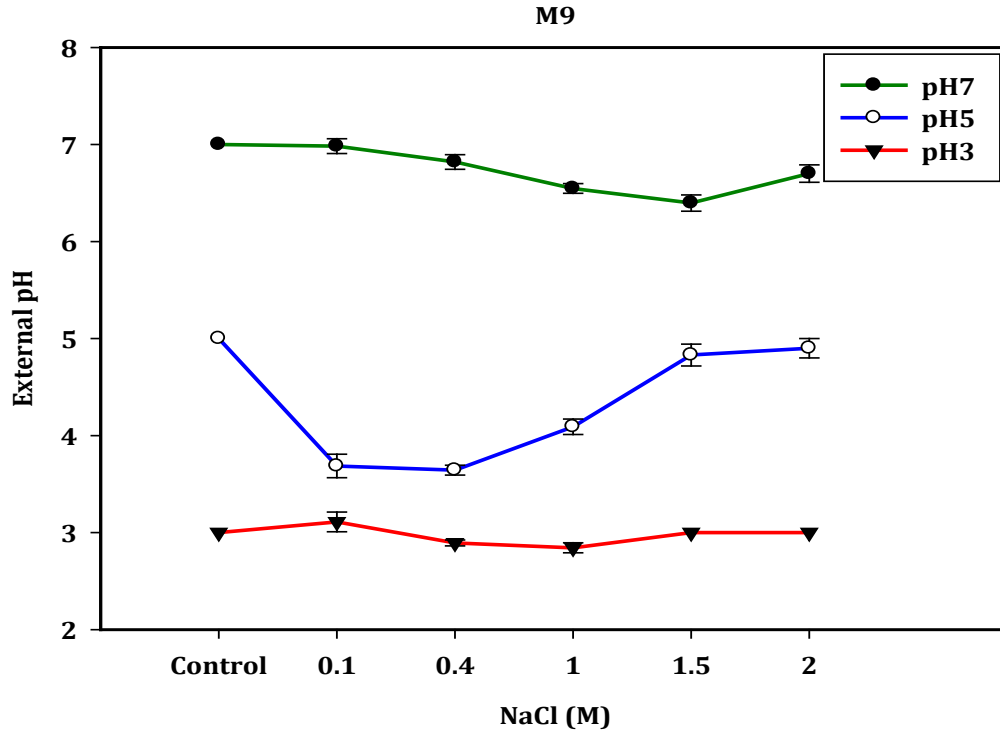
Figure 5-41: External pH of *D. hansenii* cultures grown overnight in M9 medium at pH 7, 5 and 3. (Control: M9 medium has not been inoculated with microorganisms).
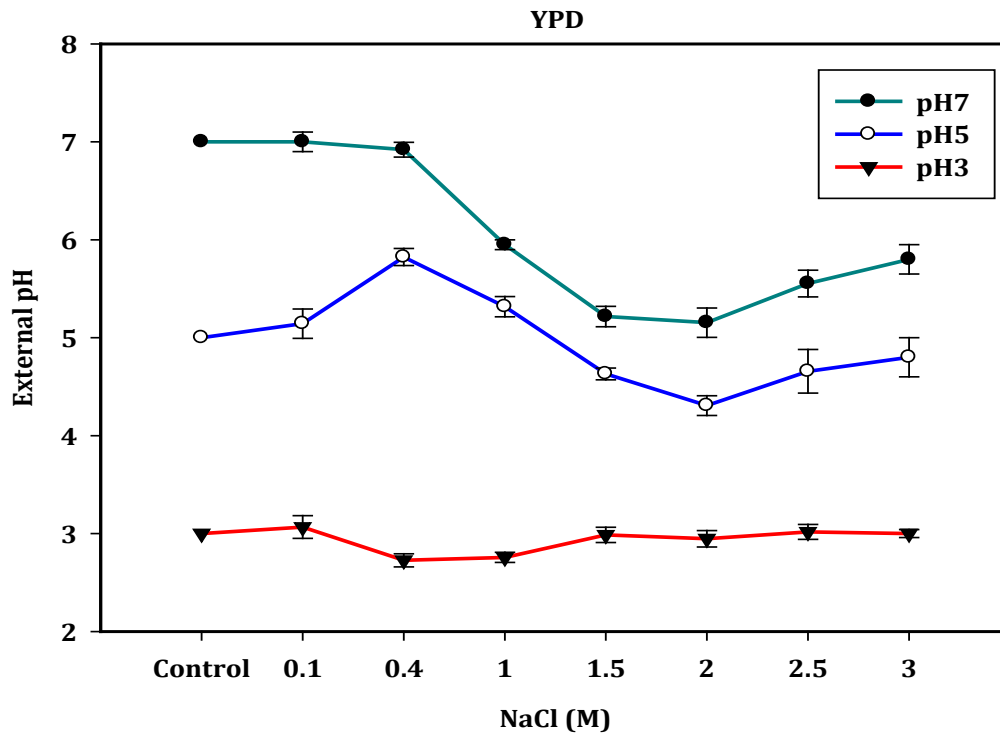
Figure 5-42: External pH of *D. hansenii* cultures grown overnight in YPD medium at pH 7, 5 and 3. (Control: YPD medium has not been inoculated with microorganisms).
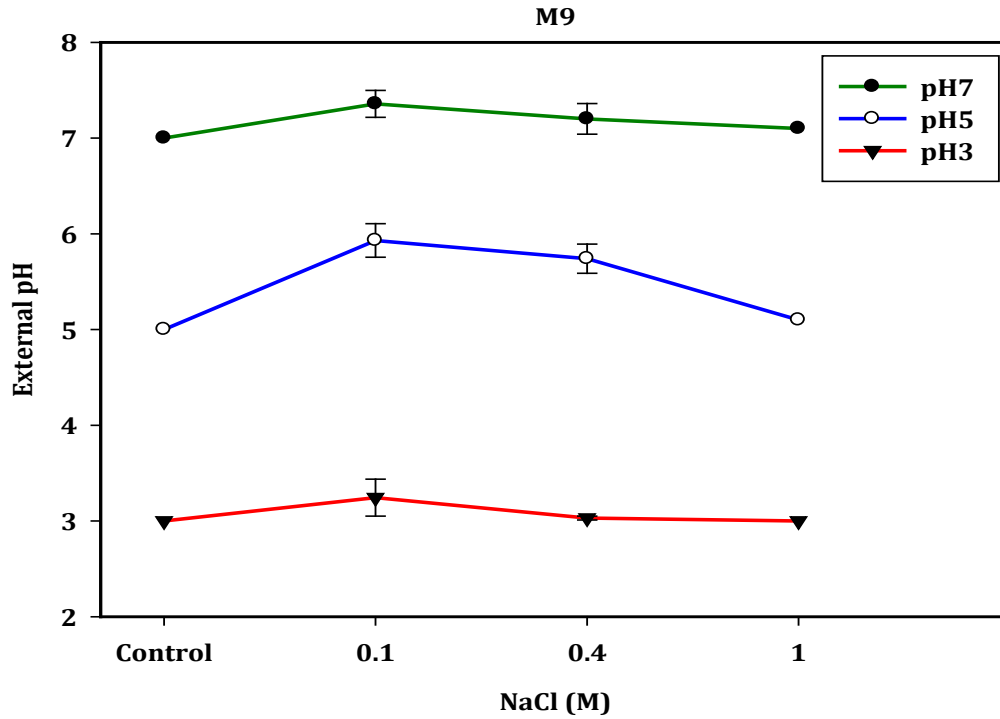
Figure 5-43: External pH of *M. luteus* cultures grown overnight in M9 medium at pH 7, 5 and 3. (Control: M9 medium has not been inoculated with microorganisms).
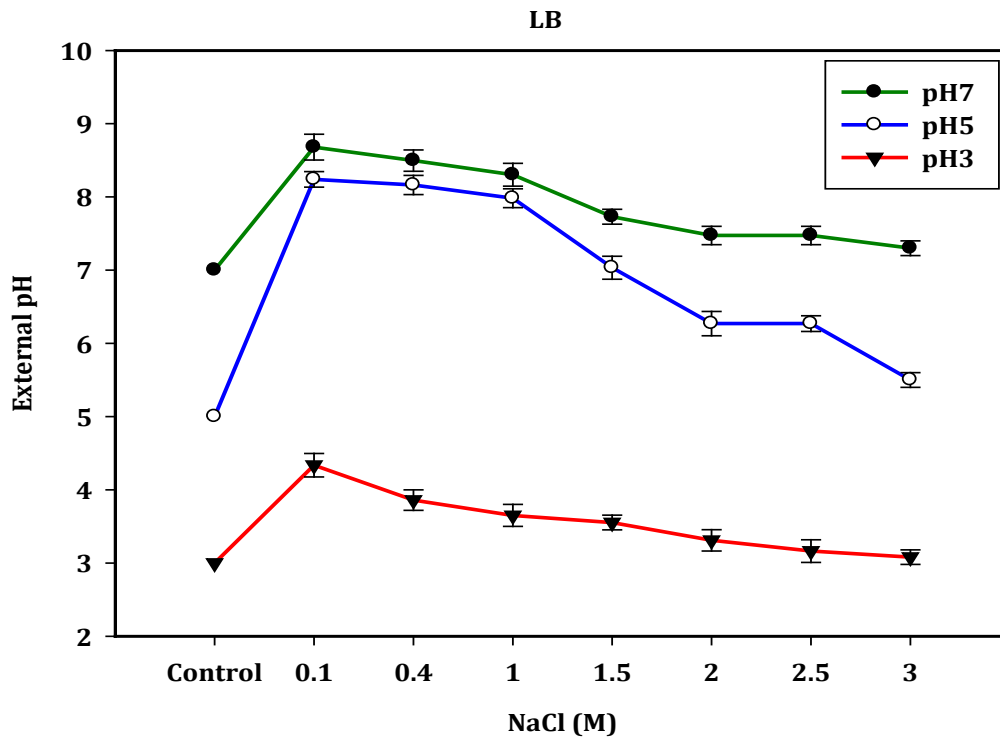
Figure 5-44: External pH of *M. luteus* cultures grown overnight in LB medium at pH 7, 5 and 3. (Control: LB medium has not been inoculated with microorganisms).
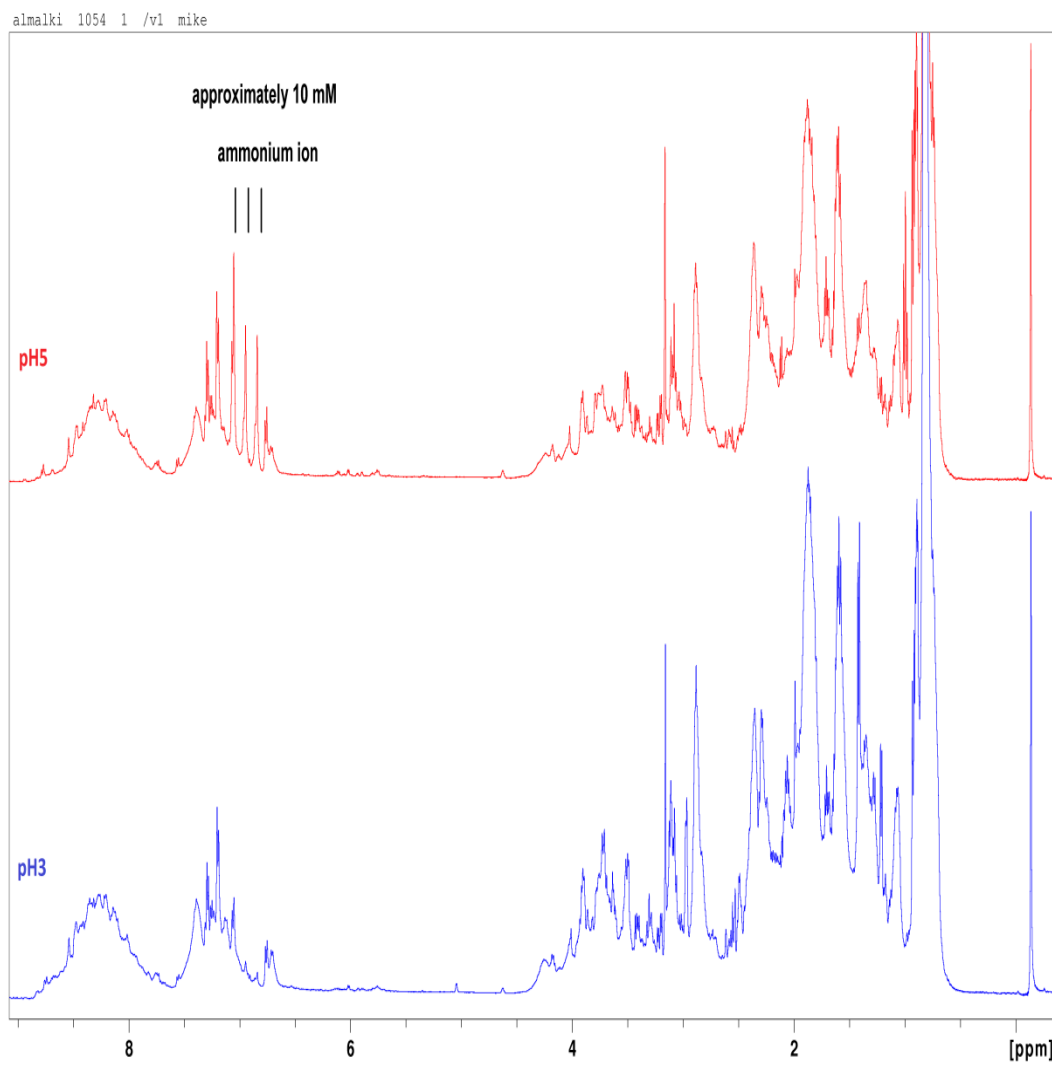
Figure 5-45: One-dimensional 1H-NMR spectra of culture supernatant derived from *M. luteus* cells grown in LB medium at pH 5 and pH 3.

5.2.6 **Effect of Salinity and pH on Respiration Rate of *D. hansenii* and *M. luteus***

Cells of *D. hansenii* and *M. luteus* grown in YPD and LB medium, respectively, at different salinity (0.1, 0.4, 1.0 1.5, 2.0 and 2.5 M NaCl) and pH (3, 5 and 7), were harvested by centrifugation then the pellets re-suspended in YPD and LB media of the same salinity and pH. Then oxygen uptake of bacterial strains was immediately determined by the oxygen electrode as described in section 2.11.

Figure 5-46 shows that the respiration rate of *D. hansenii* at pH 3 was the highest while the lowest rate occurred at pH 7. Furthermore, respiration rate in all cases increased when salinity is increased from 0.1 to 0.4 M NaCl, and then decreased again at higher salinities for all pH values. The decrease in respiration rate at high salinities was particularly pronounced at pH 3.

On the other hand, the effect of pH on respiration rates in *M. luteus* was quite different from that in *D. hansenii*; the respiration rate was higher at pH 7 while the lowest rates occurred at pH 3 in *M. luteus* (Figure 5-47). Respiration rate at pH 7 increased slightly when NaCl concentration was increased from 0.1 to 0.4 M, and then decreased with any increase of NaCl concentrations (Figure 5-47). However, at pH 5 and pH 3 when NaCl concentrations increased from 0.1 to 1 M through 0.4 M NaCl, the respiration rates increased. When salinity was increased beyond 1 M NaCl, the respiration rates decrease again at both pH values (Figure 5-47).
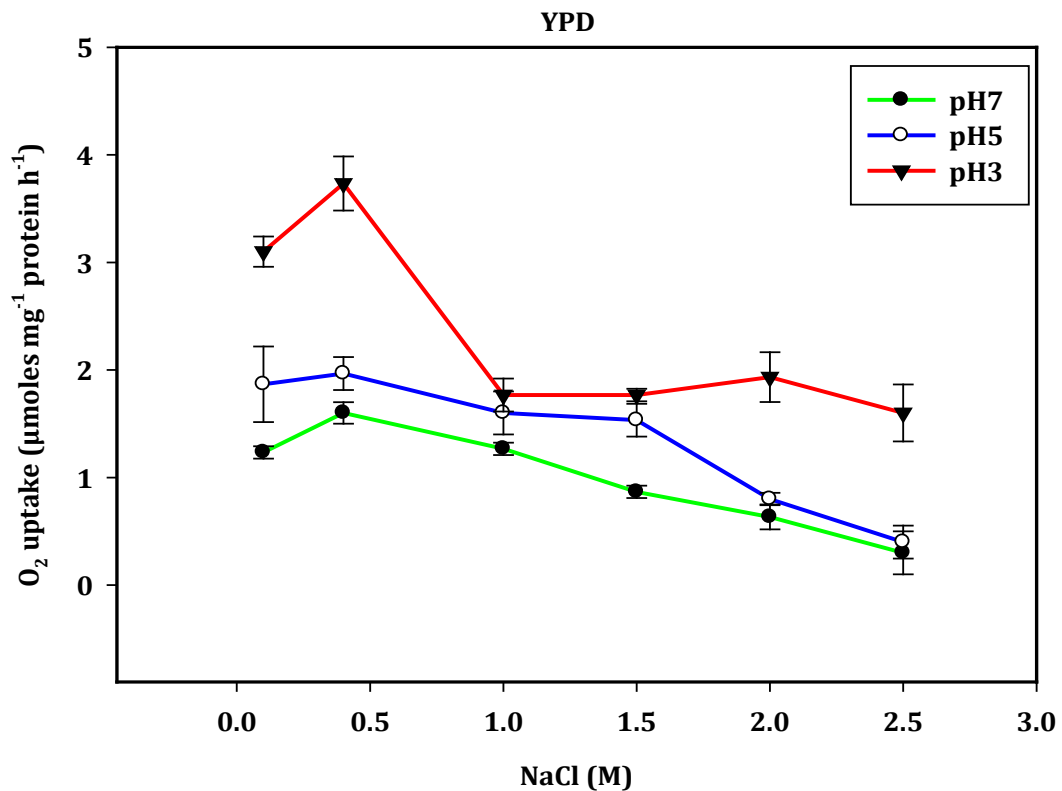
Figure 5-46: Respiration rate of *D. hansenii* subjected to different salinity concentrations (0.1, 0.4, 1.0, 1.5, 2.0 and 2.5 M NaCl) after overnight growth in YPD medium of the same salinity at pH 7, 5 and 3 at 25°C on an orbital shaker at 250 rpm. Measurement of respiration rate took place after adaptation to several NaCl concentrations at different pH values. 5 ml of cells were harvested by centrifugation and resuspended in 20 ml of fresh YPD medium of the same NaCl concentration and pH value. The O_2 uptake was measured as described in section 2.11. Data points are the means of three replicates plus or minus standard deviation.
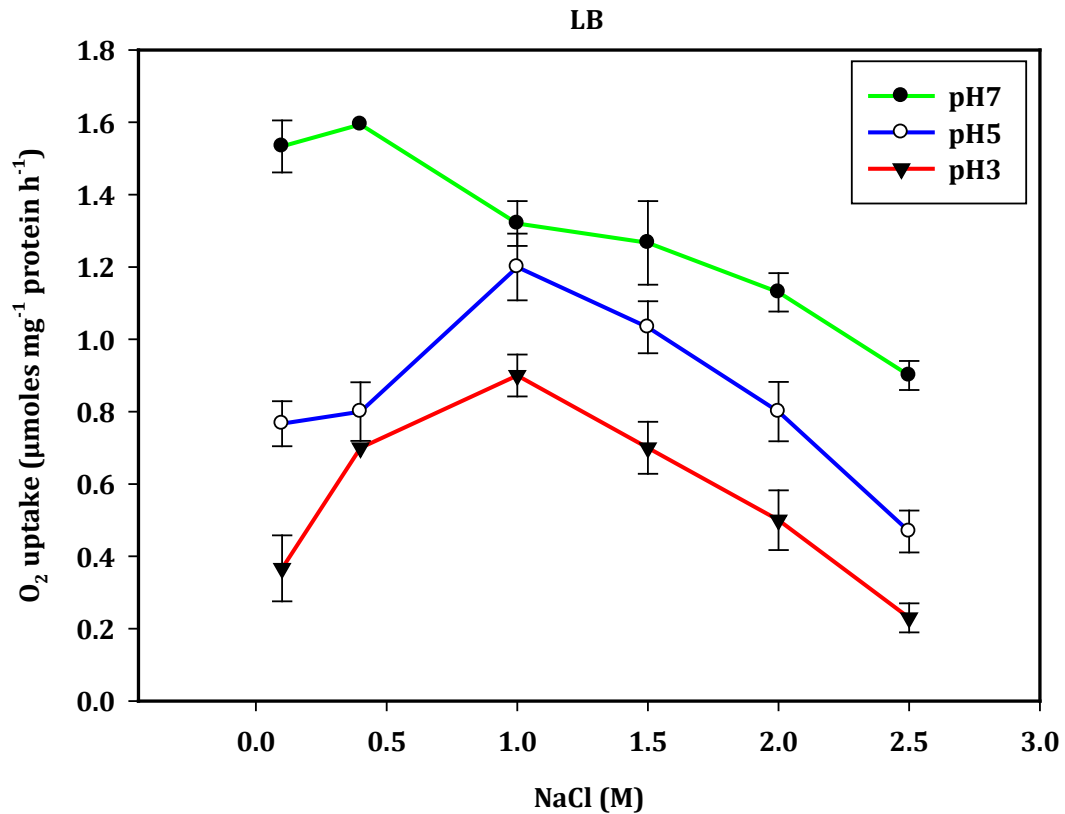
Figure 5-47: Respiration rate of *M. luteus* subjected to different salinity concentrations (0.1, 0.4, 1.0, 1.5, 2.0 and 2.5 M NaCl) after overnight growth in LB medium of the same salinity at pH 7, 5 and 3 at 25°C on an orbital shaker at 250 rpm. Measurement of respiration rate took place after adaptation to several NaCl concentrations at different pH values. 5 ml of cells were harvested by centrifugation and resuspended in 20 ml of fresh LB medium of the same NaCl concentration and pH value. The O_2 uptake was measured as described in section 2.11. Data points are the means of three replicates plus or minus standard deviation.

5.2.7 Determination of Compatible Solutes (Osmolytes) by NMR Spectroscopy

In order to identify the compatible solutes accumulated by *D. hansenii* and *M. luteus* when exposed to different salinity concentrations and different external pH values, NMR was used as described in section 2.18. The importance of the medium used was investigated by comparing rich LB medium (*M. luteus*) and rich YPD medium (*D. hansenii*) with M9 minimal salts medium.

Figure 5-48, Figure 5-49 and Figure 5-50 show the variety of compatible solutes accumulated by *D. hansenii* when yeast cells were grown in M9 minimal medium at pH 7, 5 and 3 in different salinities (0.1, 0.4, 1 M NaCl). From the spectra it is clear that glycerol, arabitol, glycine betaine (betaine) and alanine were found under all conditions but at varying concentrations depending on NaCl concentration and pH value, for example, alanine was quite low at all pH values in 0.1 and 0.4 M NaCl (Figure 5-48 and Figure 5-49). Moreover, all compatible solutes increased with increasing salinity from 0.1 - 1 M NaCl (Figure 5-48 and Figure 5-50). However, glycerol was the compatible solute accumulated to the highest amount in all spectra and in particular at pH 3, 1 M NaCl there was more glycerol (Figure 5-50).

Similar results occurred when *D. hansenii* cells were grown in YPD medium (Figures 5-51, 5-52, 5-53 and 5-54). In YPD medium the highest amount of glycerol was found when yeast cells were grown in 0.4 M NaCl at pH 3 (Figure 5-52). So, it is very clear that glycerol is the main compatible solute accumulated by *D. hansenii* cells when they were grown under different stresses.
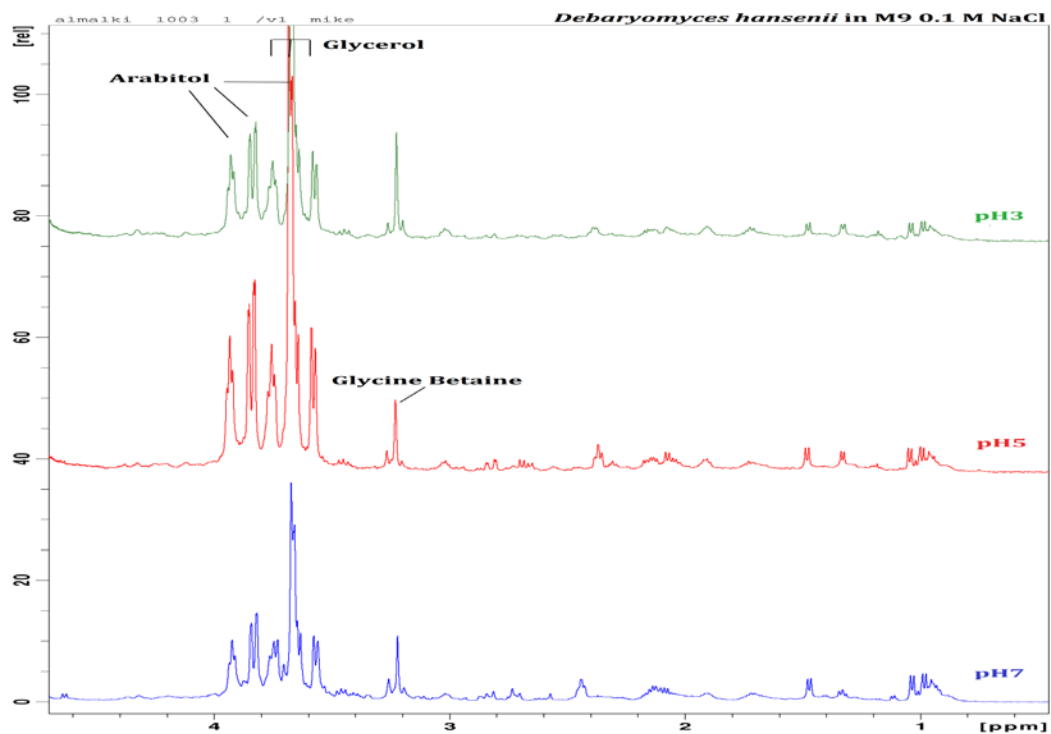
Figure 5-48: One-dimensional 1H-NMR spectra of cell extracts derived from *D. hansenii* cells grown in M9 minimal medium in 0.1 M NaCl at pH 7, 5 and 3.
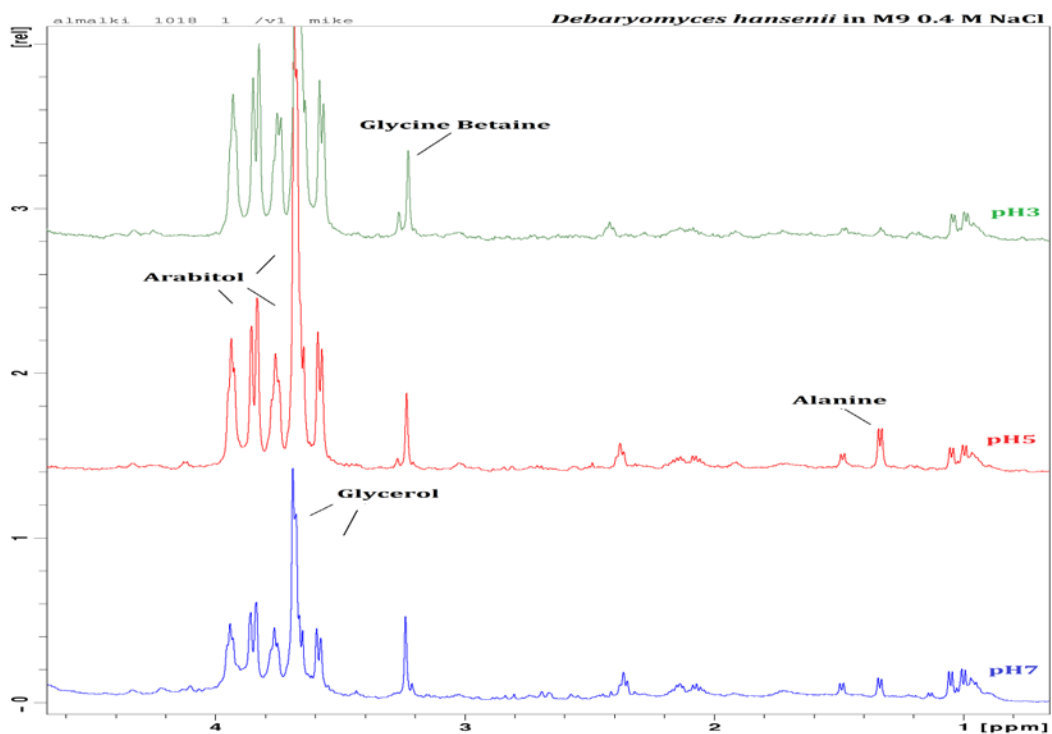
Figure 5-49: One-dimensional 1H-NMR spectra of cell extracts derived from *D. hansenii* cells grown in M9 minimal medium in 0.4 M NaCl at pH 7, 5 and 3.
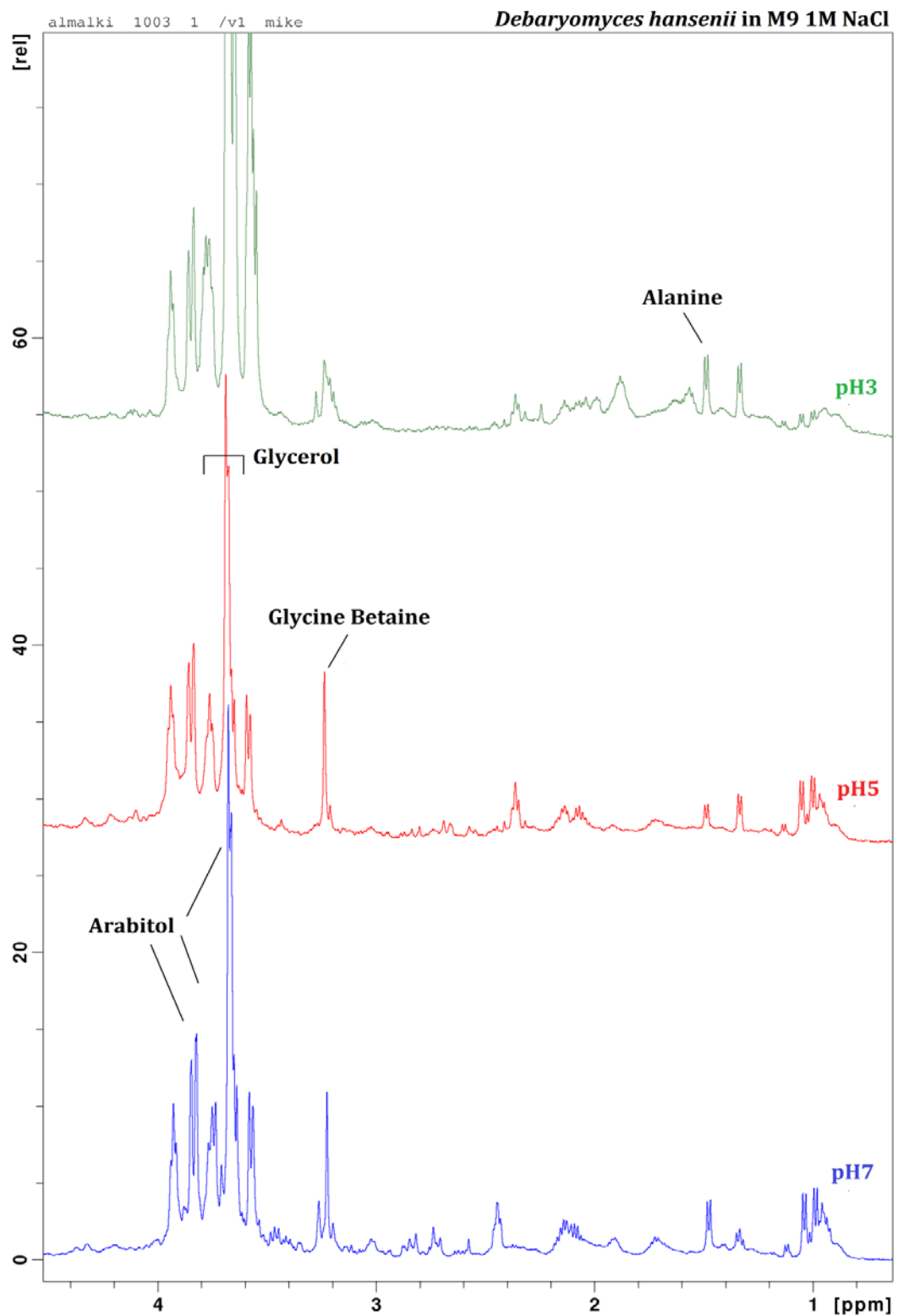
Figure 5-50: One-dimensional 1H-NMR spectra of cell extracts derived from *D. hansenii* cells grown in M9 minimal medium in 1.0 M NaCl at pH 7, 5 and 3.
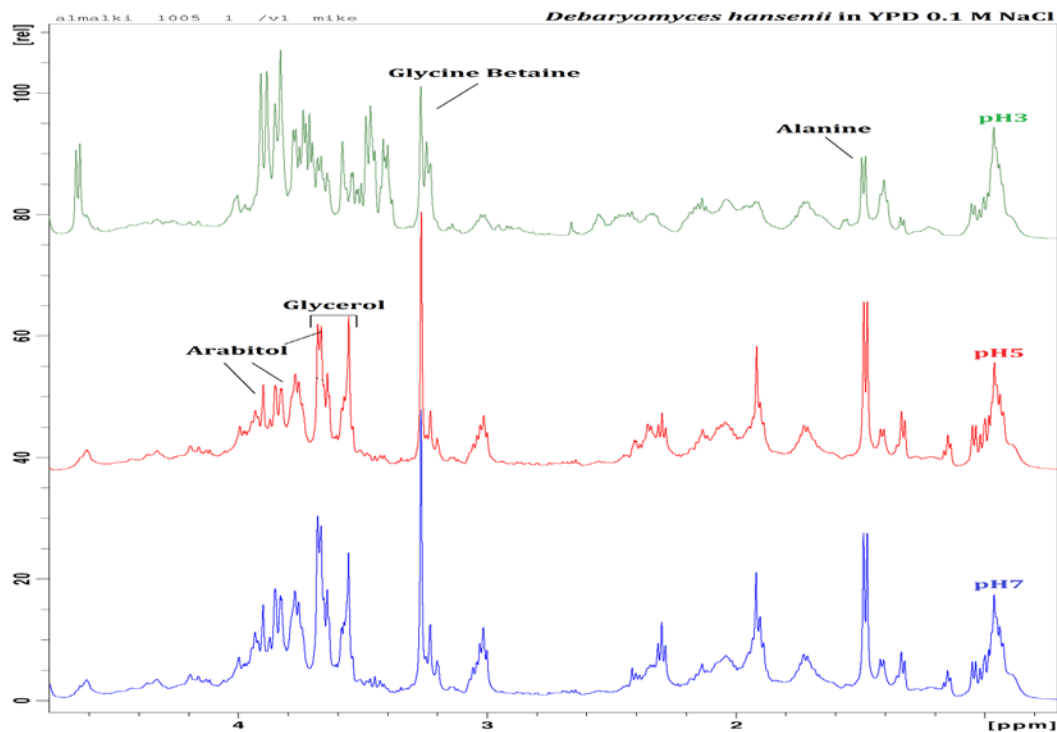
Figure 5-51: One-dimensional 1H-NMR spectra of cell extracts derived from *D. hansenii* cells grown in YPD medium in 0.1 M NaCl at pH 7, 5 and 3.
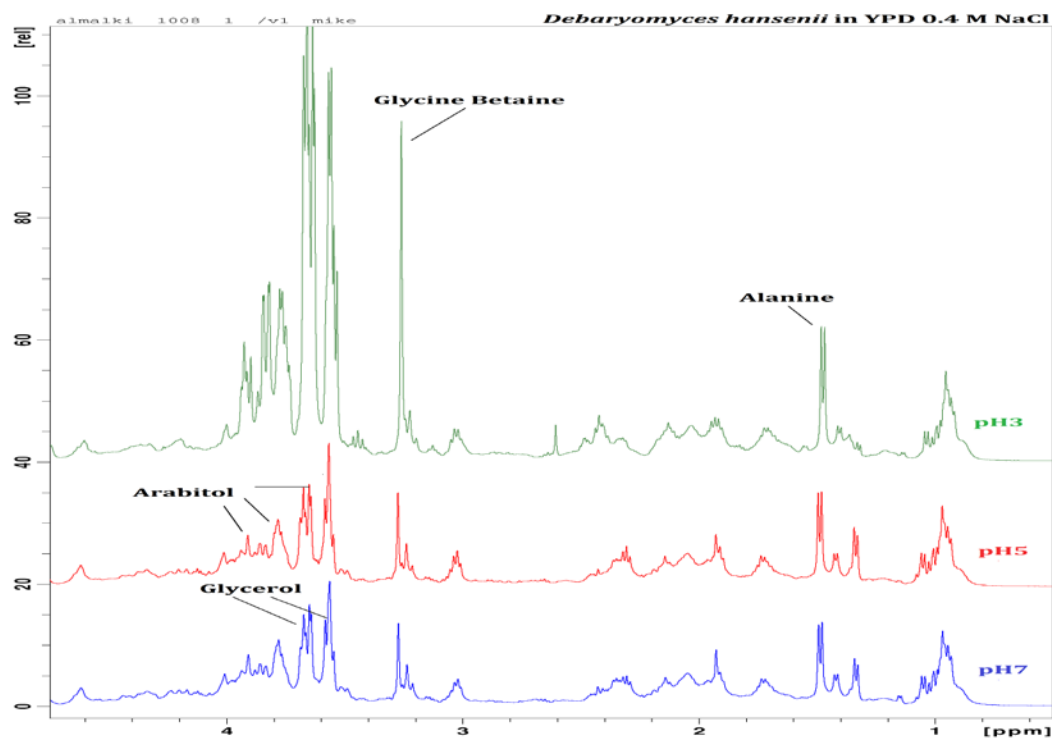
Figure 5-52: One-dimensional 1H-NMR spectra of cell extracts derived from *D. hansenii* cells grown in YPD medium in 0.4 M NaCl at pH 7, 5 and 3.
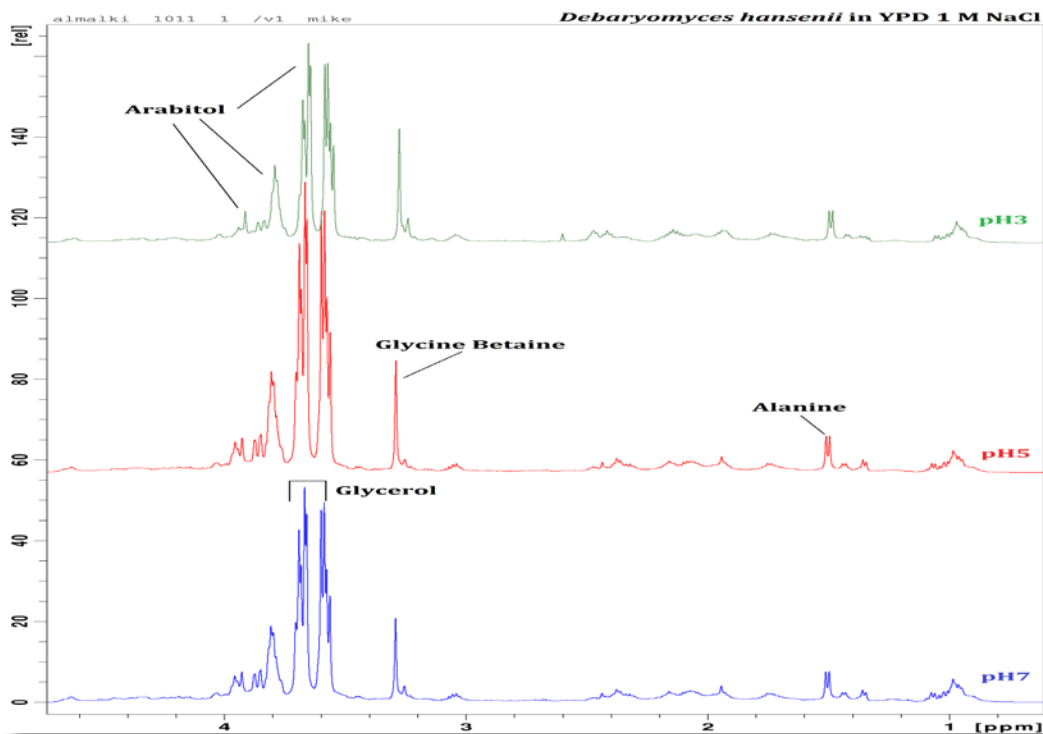
Figure 5-53: One-dimensional 1H-NMR spectra of cell extracts derived from *D. hansenii* cells grown in YPD medium in 1.0 M NaCl at pH 7, 5 and 3.
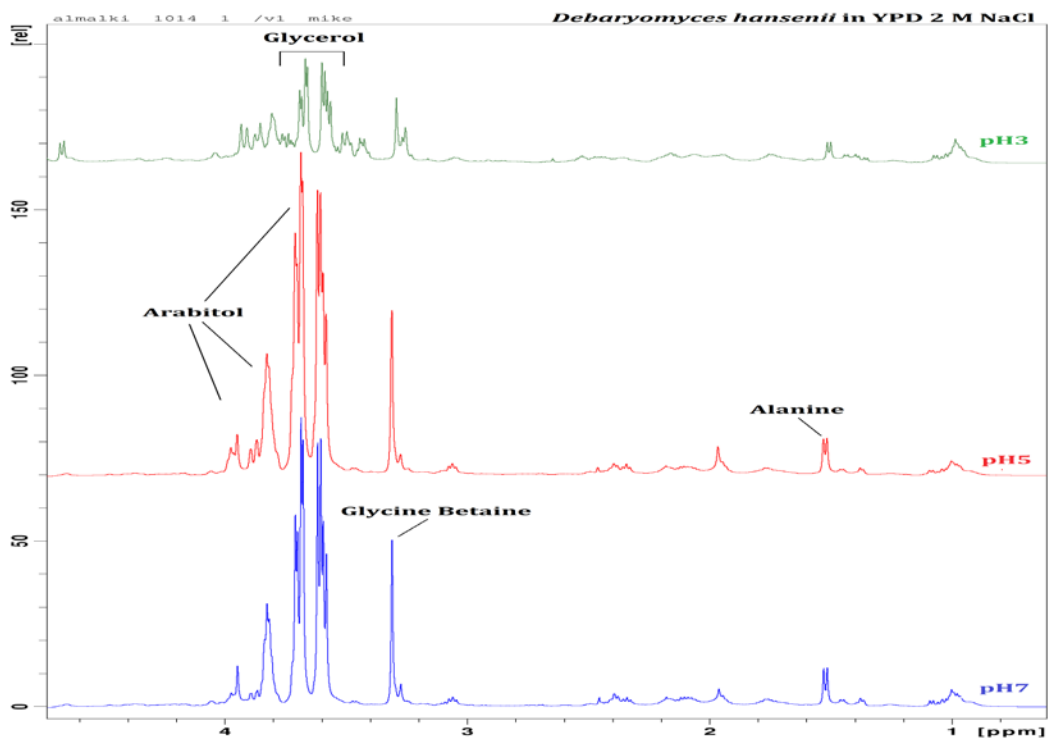
Figure 5-54: One-dimensional 1H-NMR spectra of cell extracts derived from *D. hansenii* cells grown in YPD medium in 2 M NaCl at pH 7, 5 and 3.

Figures 5-55, 5-56 and 5-57 show the range of compatible solutes found in *M. luteus* when cells were grown in M9 minimal medium at different pH values and different salinity concentrations (0.1, 0.4 and 1 M NaCl). However, betaine was the main compatible solute which was present under all conditions. In contrast, glutamic acid (glutamate) was only found in cells grown in 0.1 M NaCl at pH 5 and pH 7 (Figure 5-55), and proline was only present when cells were grown in 1 M NaCl at pH 5 and pH 7 (Figure 5-57).

When *M. luteus* cells were grown in LB medium with different NaCl concentrations at different pH values (Figures 5-58, 5-59 and 5-60), betaine was again found in all spectra, the lowest amount of betaine was found at pH 3 (Figure 5-58), and almost equal amounts at pH 5 and pH 7 (Figure 5-59 and Figure 5-60) respectively. Quite low amounts of proline were present at pH 5 and pH 7 at all NaCl concentrations (Figure 5-59 and Figure 5-60). Furthermore, glutamate is present, but only at 2 M NaCl concentration at pH 5 and pH 7 (Figure 5-59 and Figure 5-60).
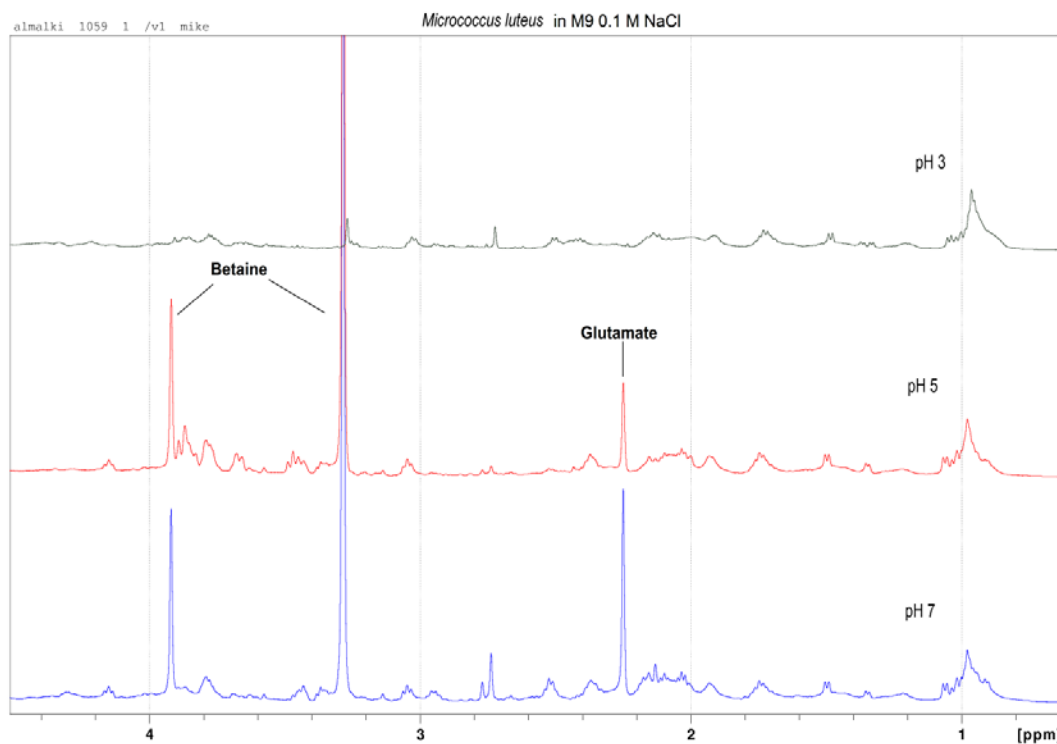
Figure 5-55: One-dimensional 1H-NMR spectra of cell extracts derived from *M. luteus* cells grown in M9 minimal medium in 0.1 M NaCl at pH 7, 5 and 3.
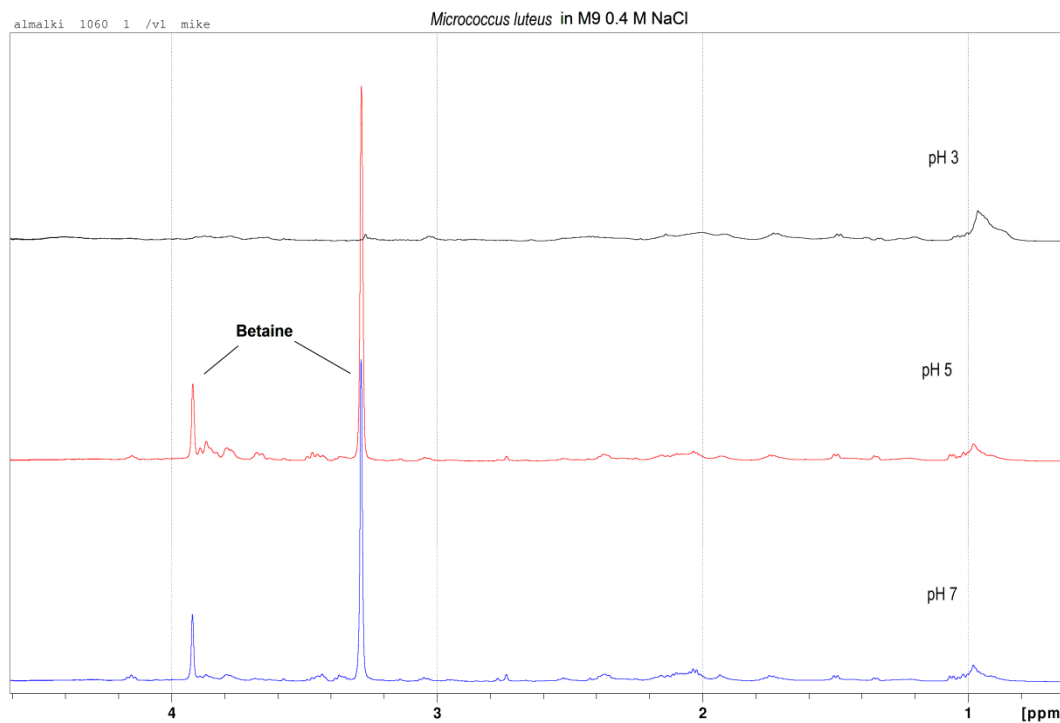
Figure 5-56: One-dimensional 1H-NMR spectra of cell extracts derived from *M. luteus* cells grown in M9 minimal medium in 0.4 M NaCl at pH 7, 5 and 3.
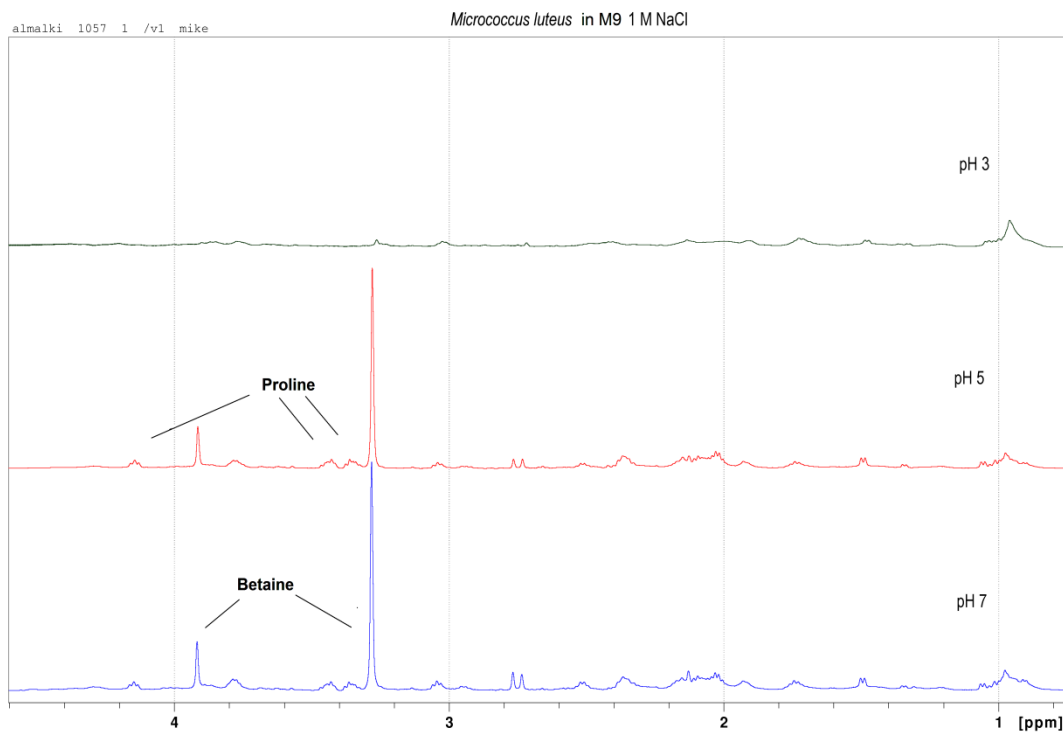
Figure 5-57: One-dimensional 1H-NMR spectra of cell extracts derived from *M. luteus* cells grown in M9 minimal medium in 1.0 M NaCl at pH 7, 5 and 3.
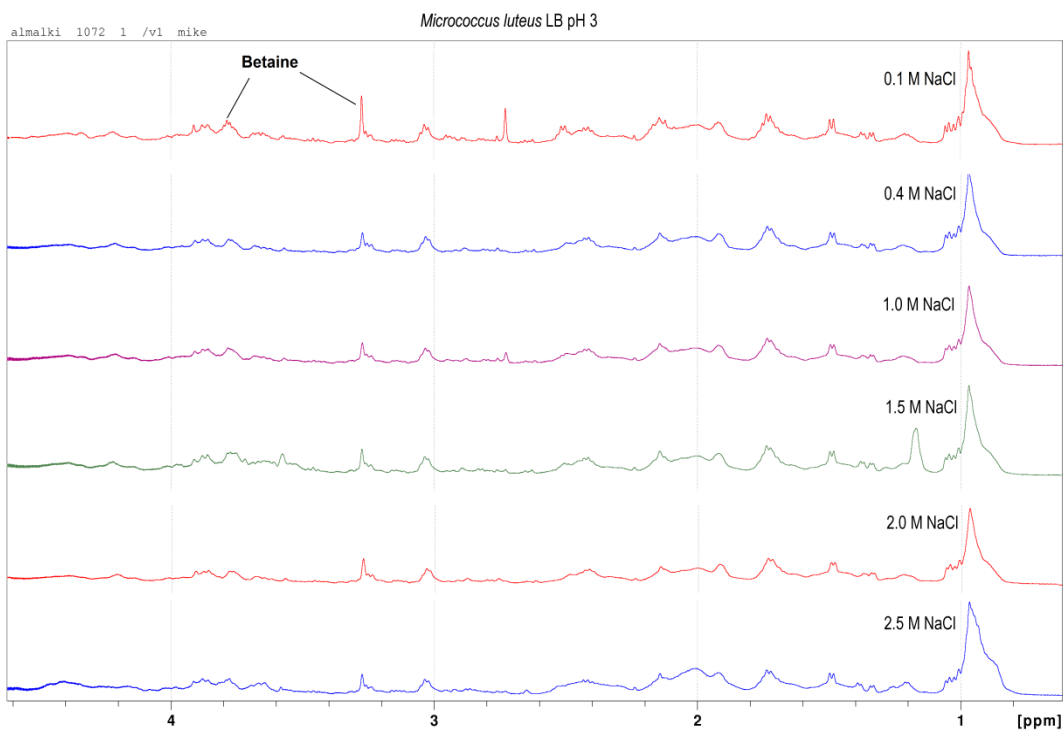
Figure 5-58: One-dimensional 1H-NMR spectra of cell extracts derived from *M. luteus* cells grown in LB medium in different NaCl concentrations at pH 3.
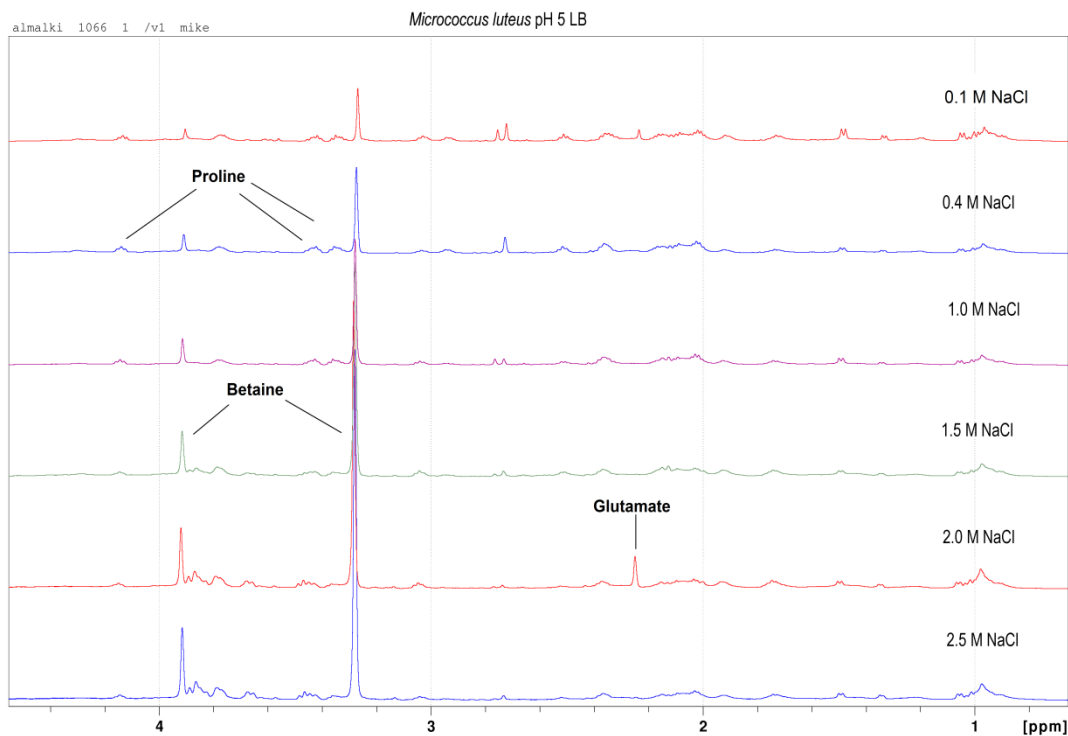
Figure 5-59: One-dimensional 1H-NMR spectra of cell extracts derived from *M. luteus* cells grown in LB medium in different NaCl concentrations at pH 5.
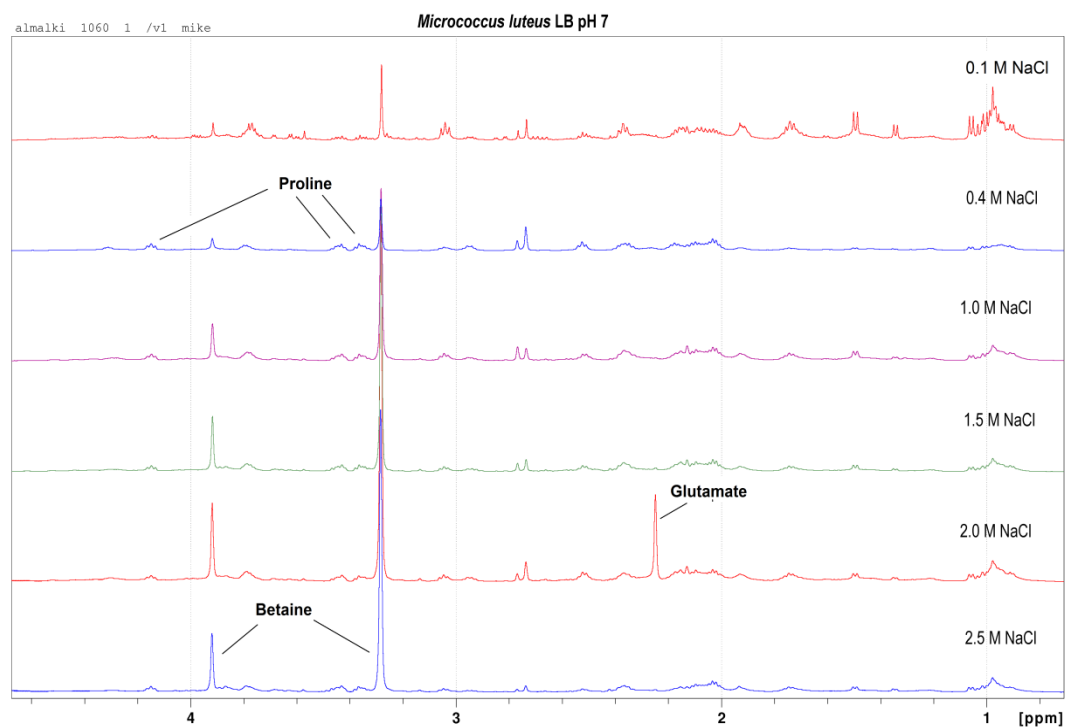
Figure 5-60: One-dimensional 1H-NMR spectra of cell extracts derived from *M. luteus* cells grown in LB medium in different NaCl concentrations at pH 7.

5.2.8 Effect of Sulphur, Aluminium and Iron on the Growth of *D. hansenii* and *M. luteus*

As mentioned in chapter three, it was assumed that even today, the Rivelin and Limb Valleys in Sheffield would remain polluted acidic environments because they have a long history of industrial activity dating back to the Industrial Revolution. Therefore, both rivers water metallic contents were analysed as and the results were presented in chapter three (section 3.2.2).

However, in the present chapter three elements (iron, sulphur and aluminium) which exist in both rivers water were chosen in order to investigate their effect on the growth of *D. hansenii* and *M. luteus*. In addition, experiments were performed to examine the ability of *D. hansenii* and *M. luteus* in to utilize these elements by growing them in M9 minimal medium in the presence of the elements as described in section 2.15.

Figure 5-61 shows that *D. hansenii* was able to grow and thrive in all M9 media containing iron, sulphur and aluminium as well as in the control (M9 minimal medium). It is clear that iron showed most inhibition of growth, but this was still a very small effect.

M. luteus showed a good ability to grow in the presence of iron, sulphur and aluminum in M9 minimal medium (Figure 5-62). Again iron was the most effective inhibitor of *M. luteus* growth while aluminium and sulphur were less effective.
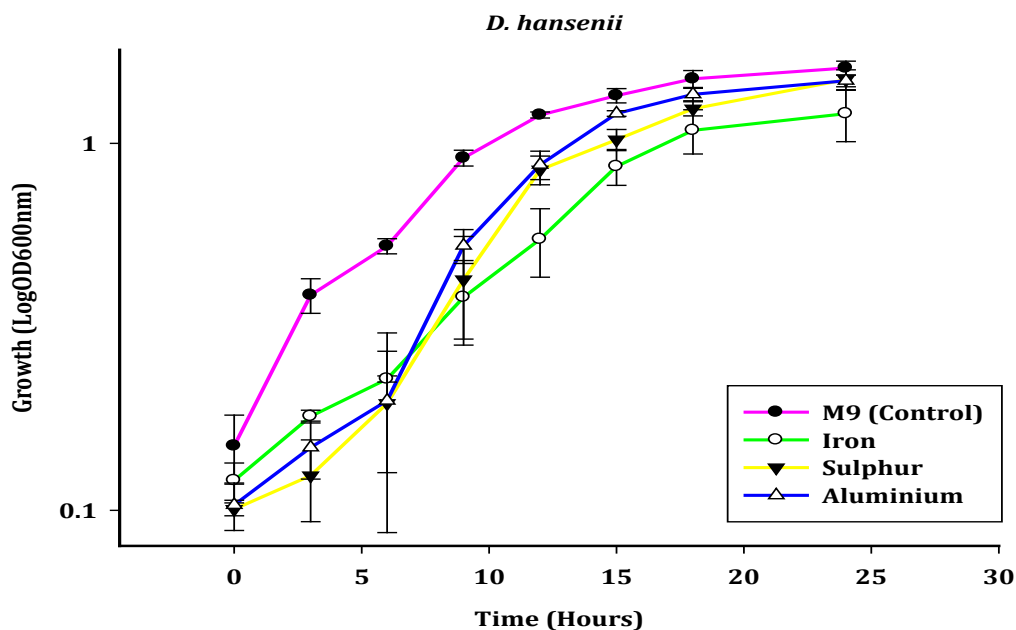
Figure 5-61: Effect of iron, sulphur and aluminium on the growth of *D. hansenii*. The cells were grown in M9 medium at pH 7 and incubated in a 25°C constant temperature room on an orbital shaker at 250 rpm overnight. The O.D was measured at 600 nm every 3 hours. Data points are the means of four replicates plus or minus standard deviation.
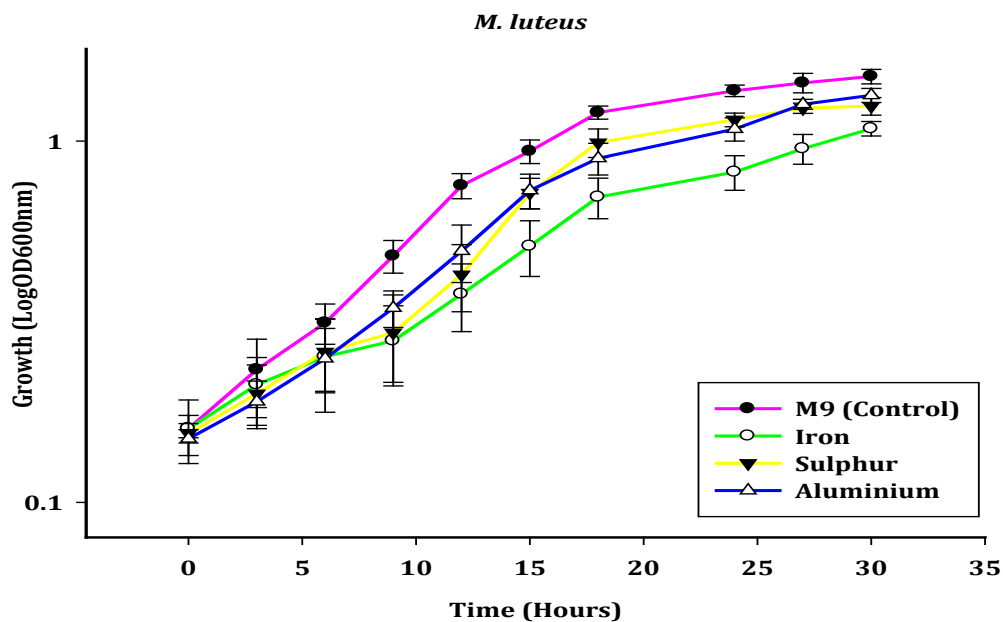
Figure 5-62: Effect of sulphur, iron and aluminium on the growth of *M luteus*. The cells were grown in M9 medium at pH 7 and incubated in a 25°C constant temperature room on an orbital shaker at 250 rpm overnight. The O.D was measured at 600 nm every 3 hours. Data points are the means of four replicates plus or minus standard deviation.

5.3 Conclusions

In the current chapter, low nutrient and rich nutrient media with different pH values (pH 7, 5 and 3) and different NaCl concentrations (0.1, 0.4, 1.0, 1.5, 2.0, 2.5, 3 M) were used to determine the ability of eukaryotic yeast strain (*Debaryomyces hansenii*) and prokaryotic bacterial strain (*Micrococcus luteus*) to grow under different extreme conditions. Furthermore, generation (doubling) times, respiration rates and the presence of compatible solutes were all determined. Finally, the effects of three elements (iron, sulphur and aluminium) on growth were also investigated.

D. hansenii and *M. luteus* were grown in low nutrient (M9 minimal medium) under acid stress, and then electron microscopy (SEM and TEM) was used in order to investigate any changes that may have occurred to the cells structure. It was a good achievement as recorded in the beginning of the current chapter to obtain many high resolution microscope images of *D. hansenii* (Figure 5-1 and Figure 5-2) and *M. luteus* (Figure 5-3 and Figure 5-4) which confirm and support the phenotypic and phylogenic identification in chapters three and four. Also, SEM images showed that *D. hansenii* cells grown at pH 7 were larger than cells grown at pH 3 in low nutrient medium (Figure 5-1). While TEM images showed that the cell walls of *M. luteus* cells grown at pH 3 were thicker than the cell walls of cells grown at pH 7, and that the *M. luteus* cells grown at pH 3 tended to clump together more than in cells grown at pH 7 (Figure 5-4).

Despite the changes which were observed by the use of SEM and TEM, it was quite hard to investigate more significant changes using these types of electron microscope. Using Environmental Scanning Electron Microscope (ESEM) may lead

to the discovery of more significant changes happening to microbial cells when grown under stress. ESEM can examine microbial cells directly while they are still subject to the environmental stresses while in SEM and TEM the cells must be re-suspended in a phosphate buffer then followed by many other fixation steps as described in section 2.19. These additional steps may obscure the effects of environmental stresses on the microbial cells.

In growth curves experiments, *D. hansenii* shows a good ability to grow and thrive under many extreme conditions such as low nutrients, high salinity and low pH. For example, *D. hansenii* was able to grow in M9 minimal medium containing 1 M NaCl at pH 7, 5 and 3 (Figure 5-11), and tolerate 1.5 M NaCl at pH 7 and pH 5 in the same medium (Figure 5-12). In addition, *D. hansenii* shows a greater ability to grow at high acidity and high salinity when grown in YPD medium. For example, *D. hansenii* was able to thrive in YPD containing 2 M NaCl at pH 7, 5 and 3 (Figure 5-20), and tolerate 3 M of NaCl at pH 7 and pH5 in YPD medium (Figure 5-22).

On the other hand, *M .luteus* shows poor capability to thrive under many stresses when grown in low nutrient medium (M9 minimal medium) in particular at high acidity. *M. luteus* just thrives at all pH values (7, 5 and 3) when grown in normal M9 at 0.1 M NaCl (Figure 5-25), and barely grows in 0.4 M NaCl at pH 7 and pH 5 and no growth at pH 3 was observed (Figure 5-26). However, *M. luteus* showed a significantly greater ability to tolerate high acidity and high salinity when grown in LB medium. For example, *M. luteus* cells were able to tolerate 1 M NaCl at all pH values (7, 5 and 3) (Figure 5-31), tolerate 2 M NaCl at pH 5 and pH 7 (Figure 5-33), and tolerate 2.5 M NaCl at pH 7 (Figure 5-34).

To conclude, *D. hansenii* has range of optimum pH in low nutrient medium from 7 to 5 and the optimum salinity was 0.4 M NaCl at 25ºC (Figure 5-37), while the optimum pH in rich YPD medium was pH 5 and optimum salinity is 1.0 M NaCl at 25ºC (Figure 5-38). These results are consistent with the results of Norkrans (1966) who demonstrated that *D. hansenii* has the ability to tolerate high salt concentrations. The results also in agreement with the results of Prista and Madeira-Lopes (1995) and Thome-Oritz *et al.* (1998) who recorded that Na$^+$ was not toxic to *D. hansenii*. Moreover, the present results are consistent with the results of Prista *et al.* (1997) who reported that increasing salt concentrations improved *D. hansenii* growth under normal conditions, and also agree with the results of Almagro *et al.* (2000) who demonstrated that the presence of salt stimulated the growth of *D. hansenii* under stress conditions.

On the other hand, *M. luteus* optimum pH was 7 in both M9 and LB medium, and the optimum salinity was 0.1 M NaCl in M9 at 25ºC while 1.0 M NaCl was the optimum in LB medium at 25ºC (Figure 5-39). These results are consistent with the results of Chan and Leung (1979) who reported that *M. luteus* required 0.5 M NaCl for optimum growth in a chemically defined medium which contains different concentrations of salts, multi vitamins and some amino acids at 30ºC and required 0.2 M NaCl for optimum growth at 25ºC.

Respiration rates of *D. hansenii* and *M. luteus* were measured in order to determine the metabolic activity under salt stress at three different pH values (pH 7, 5 and 3). *D. hansenii* shows a higher respiration rate when the cells were grown in YPD medium at pH 3 and the lowest was at pH 7, the oxygen uptake slightly increased

when the salinity increased from 0.1 to 0.4 M NaCl and then the respiration rates fell with any further increase in NaCl concentration at all pH values studied (Figure 5-46). In contrast, *M. luteus* shows higher respiration rate when the cells were grown in LB medium at pH 7 while the lowest rate occurred at pH 3, the oxygen uptake goes up slightly when the salinity was increased from 0.1 to 0.4 M NaCl at pH 7 and then the respiration rate goes down with any further increase in salt concentration. For cells grown at pH 5 and pH 3, the respiration rate recast when NaCl concentration increased from 0.1 to 0.4 and to 1 M NaCl, after which the oxygen uptake fell with any further increase in salt (Figure 5-47).

In order to investigate the ability of both strains to alter the pH of the medium, the cells were grown in M9 minimal medium for both strains and YPD medium for *D. hansenii* and LB medium for *M. luteus* at different pH values and different salinity concentrations. For *D. hansenii* there was no change in the external pH when cells were grown at pH 7 and pH 3 in M9, but in pH 5 they tended to decrease the pH to 4 or less at all salt concentrations (Figure 5-41). When *D. hansenii* cells were grown in YPD medium at pH 7 the external pH was reduced when NaCl concentrations were more than 0.4 M, while no changes were recorded to the external pH when the cells grown at pH 3, but when cells were grown at pH 5 the external pH was raised to 6 in 0.4 M NaCl and then goes down to pH 4 when salinity reached 2 M NaCl. It appears that the changes in salinity are related to the rate of growth and less change takes place when growth is poor (Figure 5-42). There is no decrease in the external pH for *M. luteus* cultures at all conditions, the cells slightly increased all pH values in M9 minimal medium (Figure

5-43), and always tend to increase the external pH from acidity toward alkalinity in LB medium at all pH values (Figure 5-44).

The accumulation of compatible solutes for both strains was detected in M9 minimal medium (both strains), YPD medium (*D. hansenii*) and LB medium (*M. luteus*) by NMR spectroscopy. Glycerol was found in all spectra as a main compatible solute for *D. hansenii* and arabitol was the next most important compatible solute in both M9 and YPD medium (Figure 5-48 and Figure 5-51). The presence of betaine as a compatible solute in the eukaryotic *D. hansenii* cells (Figure 5-50 and Figure 5-53) was very interesting. On the other hand, as expected betaine was found in all spectra for *M. luteus* as the main compatible solute in both M9 and LB medium (Figure 5-56 and Figure 59). In addition, glutamate was found at 0.1 M NaCl in M9 minimal medium (Figure 5-55) and at 2 M NaCl in LB medium (Figure 5-60).

To conclude, *M. luteus* use betaine as a main compatible solute and glutamate as an additional compatible solute. These results are similar to the results of Imhoff and Rodriguez-Valera (1984) who reported that betaine plays the major role as compatible solute in halophilic bacteria such as *M. luteus*, and glutamate was also involved. In contrast *D. hansenii* use glycerol as a main compatible solute and another polyol (arabitol) an additional compatible solute. These results are consistent with the results obtained by Gustafsson and Norkrans (1970) and (1976) who suggested that glycerol is the compatible solute in *D. hansenii*. Also the present results agree with results of Adler *et al.* (1985) and Andre *et al.* (1988) who demonstrated that glycerol plays the main role in the high salt resistance of *D.*

hansenii. In addition, glycine betaine was also identified and to the best of our knowledge this is the first time that glycine betaine has been found in yeast cells.

It is well known that LB medium contains choline, which is a precursor for betaine synthesis (Cummings *et al.,* 1993). In the presence of choline, the bacteria preferentially, use betaine, but *M. luteus* also relied on betaine as a major compatible solute in the absent of choline in M9 minimal medium. So, the current data suggests that *M. luteus* is capable of synthesizing betaine without the presence of choline in the media, and also that glutamate is used as a second compatible solute particularly at high salt concentrations.

Clearly, these results demonstrated that *D. hansenii* has the ability to grow and adapt to hypersaline conditions in M9 minimal and YPD medium, while *M. luteus* has the ability to grow and adapt to high salt concentrations in LB medium but lacks the ability to adapt to high salinity (more than 0.4 M NaCl) when grown in M9 minimal medium. Therefore, compatible solutes synthesis depends on the type of medium (rich or poor in nutrients) and on the level of salinity in the medium.

D. hansenii and *M. luteus* were both able to adapt to grow in the presence of iron, sulphur and aluminum in M9 minimal medium at 25ºC (Figure 5-61 and Figure 5-62). However, it might be that the main reason for the low effect of those elements on the growth of *D. hansenii* and *M. luteus* is due to the availability of those elements at pH 7 and the low concentrations of iron, sulphur and aluminum used in this experiment. A previous study demonstrated that heavy metals such as copper, cobalt or zinc can prevent the production of riboflavin pigment by *D. hansenii* (this

pigment is produced by yeast cells when grown in the presence of iron-depletion or heavy metals) if extra iron was added to the growth medium (Gadd and Edwards, 1986). A previous study reported that *M. luteus* has the ability to grow on pyridine (Sims *et al.,* 1986). Leung *et al.* (2000) demonstrated that *M. luteus* also has the ability to remove significant amounts of heavy metals such as Pb and Cu when grown at pH 5.

CHAPTER SIX

6 <u>Bioenergetics of Growth of *Debaryomyces hansenii*</u>

6.1 Introduction

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

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

Mitchell (1961) described the chemiosmotic theory which explains the key role that membranes play in the generation of metabolic energy in the form of ATP (Albers *et al.,*

2001). Electron transport in the cytoplasmic membrane of bacteria induces a difference in electrochemical potential of H^+ across the membrane (proton motive force, PMF, Δp), which is the sum of both membrane potential (transmembrane electrical potential, $\Delta\Psi$) and transmembrane pH gradient (ΔpH), and is required for production of ATP by H^+-ATPase. The proton motive force is calculated using following equation:

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

$$\Delta pH = pH_i - pH_o$$

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

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

The respiratory chain pumps out H^+ from the inside to the outside of the membrane associated with the flow of electrons from NADH to O_2. ATPase produces ATP by translocating H^+ from the outside to the inside of the cell membrane of bacteria. Moreover, the cell membrane of bacteria possesses a number of transporter systems for obtaining substrates from the outside of the membrane, which work by using the electrochemical potential of H^+ across the cell membrane (Δp) as their driving force.

In fact, the survival of eukaryotic acidophiles microorganism in acidic environments is a complex process and differs from the mechanisms used by prokaryotes. For example, eukaryotes must cope with acid conditions on the surface of the plasma membrane, but

their internal organelle membranes are not in direct contact with the external acidity. Ion channels and transporters are in contact with the low pH of the external environment, which would minimally require molecular modifications compared to the equivalent proteins of neutralophilic organisms (Messerli *et al.,* 2005). Nevertheless, several yeast, multicellular fungal and algal species thrive in acidic environments, often playing key roles in the microbial communities present in these environments, particularly when biofilms are present (Aguilera *et al.,* 2007, Baker *et al.,* 2009).

In the present chapter, the bioenergetics of the yeast *Debaryomyces hansenii* cells adapted to grow at different pH values in M9 minimal medium were examined using the silicone oil technique. Intracellular volumes of *D. hansenii* were investigated at pH 7 and pH 3 (section 6.2.1). Furthermore, in section 6.2.2 membrane potentials ($\Delta\Psi$) were also measured at pH 7 and pH 3. Internal pH (pH_i) of *D. hansenii* was also determined at pH 7 and pH 3 in M9 minimal medium (section 6.2.3).

6.2   Results and Discussion

6.2.1   Determination of Intracellular Volume of *D. hansenii* at pH 3 and pH 7

In order to calculate the concentration ratios of probes across the cytoplasmic membrane and hence membrane potential ($\Delta\Psi$) and internal pH (pH_i) the intracellular volume (ICV) was determined. The intracellular volume of *D. hansenii* cells was investigated by centrifugation through silicone oil as described in section 2.20 following the protocol described by Gimmler *et al.* (1978). Determination of both pellet volume (PV) using tritiated water (^3H_2O) and extracellular volume (ECV) using ^{14}C-dextran for all cells

grown over a wide pH range of culture media enables us to calculate the intracellular volume by the equations given in Hard and Gilmour (1996).

The tritiated water (^3H_2O) can diffuse into cells. In contrast, ^{14}C-dextran is not taken into cells due to the complex structure of this high molecular weight polymer preventing diffusion of ^{14}C-dextran across the cell membrane. Figure 2.5 (Chapter 2) shows the ration of ^3H_2O between the supernatant and pellet fractions corresponds to the total volume of the pellet. A percentage of this volume is due to fluid packed between the cells. The ratio of ^{14}C (also between pellet and supernatant) corresponds to this volume and thus the subtraction of the ^{14}C volume or extracellular volume from the ^3H total pellet volume (including excess liquid trapped between the cells) results in the estimation of the intracellular volume alone.

Time course experiments were carried out at pH 7 and pH 3 using ^{14}C-Dextran and ^3H_2O in order to ensure there was no active uptake or efflux of the isotopes after the initial distribution between cells and medium i.e. the uptake was rapid and levelled off after a short time. The results indicated that 5 minute incubation periods were suitable for determining pellet volume (PV), extracellular volume (ECV) and the subsequent intracellular volume determination (ICV) (Figures 6-1, 6-2, 6-3 and 6-4).
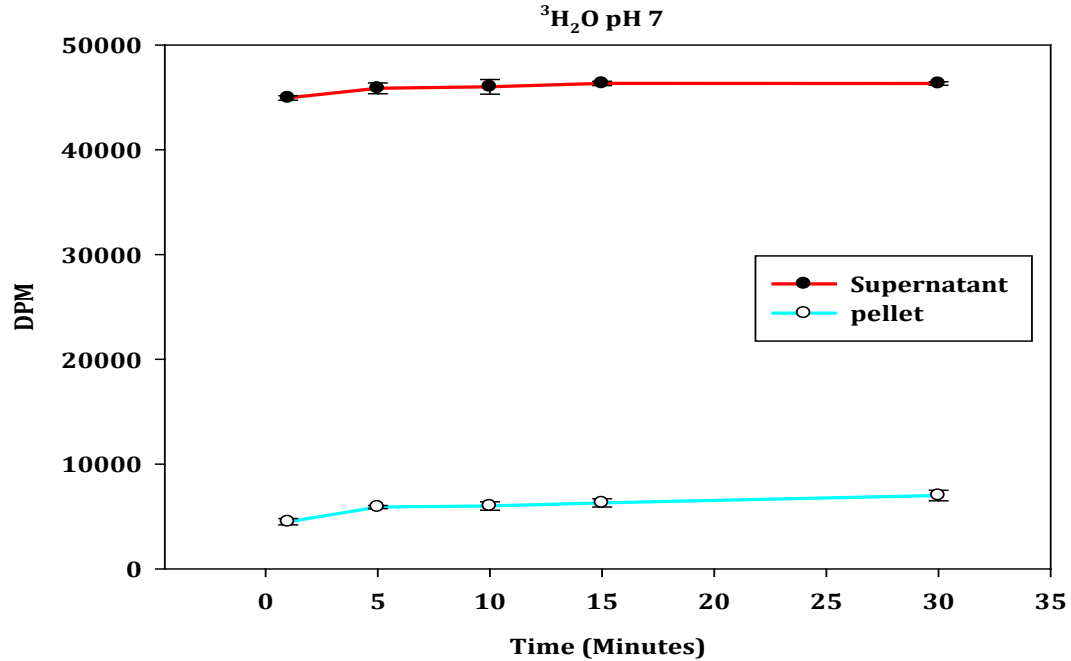
Figure 6-1: Time course of ^3H_2O uptake by *D. hansenii*. DPM in supernatants was measured during the 30 minute incubation period. Cells were grown at pH 7 in M9 minimal medium overnight at 25ºC. Means of three replicates were used.
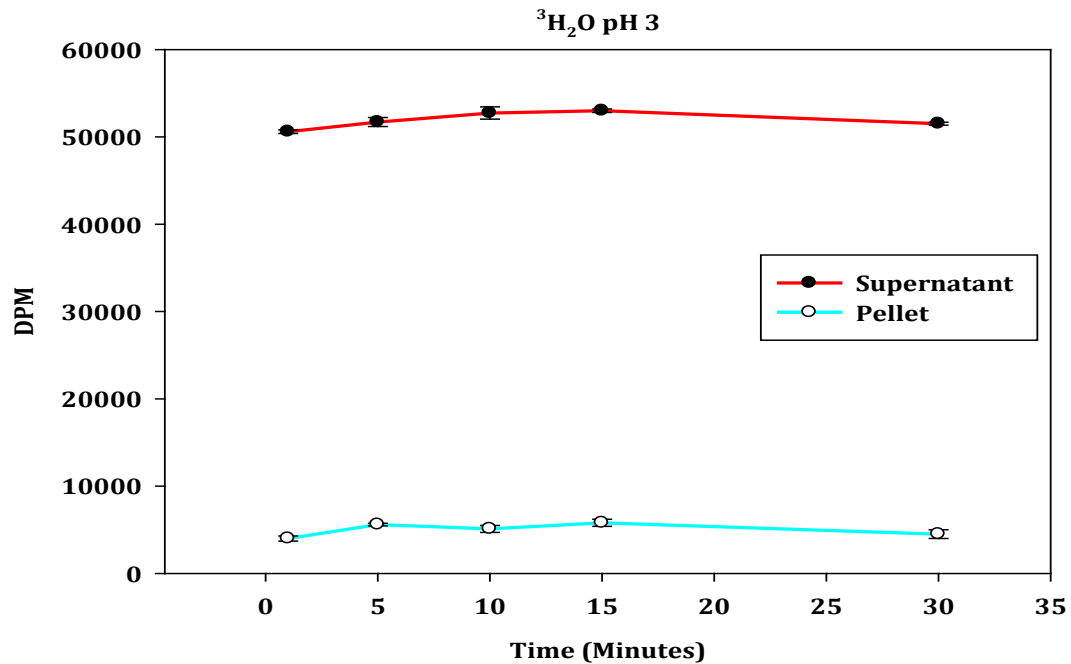
Figure 6-2: Time course of ^3H_2O uptake by *D. hansenii*. DPM in supernatants was measured during the 30 minute incubation period. Cells were grown at pH 3 in M9 minimal medium overnight at 25ºC. Means of three replicates were used.

Figure 6-3: Time course of ^{14}C-Dextran uptake by *D. hansenii*. DPM in supernatants was measured during the 30 minute incubation period. Cells were grown at pH 7 in M9 minimal medium overnight at 25°C. Means of three replicates were used.

Figure 6-4: Time course of ^{14}C-Dextran uptake by *D. hansenii*. DPM in supernatant was measured during the 30 minute incubation period. Cells were grown at pH 3 in M9 minimal medium overnight at 25°C. Means of three replicates were used.

Table 6-1: Intracellular volume (ICV) of *D. hansenii* as a function of external pH. 10 ml of cells adapted to grow at pH 7 or pH 3 in M9 minimal medium and grown overnight at 25°C were transferred to 50 ml Falcon tubes and harvested and then the pellet was resuspended in 8 ml of fresh M9 minimal medium of the same pH (section 2.20.1). Means and standard deviations for three replicates are shown.

Growth pH	Pellet Volume (PV)	Extracellular Volume (ECV)	ICV (μl mg^{-1} protein)
pH 7	8.6 ± 0.6	3.5 ± 0.1	5.1 ± 0.8
pH 3	5.6 ± 0.2	3.9 ± 0.1	1.7 ± 0.3

Table 6-1 shows the intracellular volume measurements of *D. hansenii* cells adapted to grow in M9 minimal medium at pH 3 and pH 7. It was clear that *D. hansenii* cells showed different volumes when grown in different pH media. This was interpreted as a direct result of pH stress on the *D. hansenii* cells.

The intracellular volume of *D. hansenii* cells was 5.1 µl mg^{-1} soluble protein when they were grown at pH 7 in M9 minimal medium, but when the cells grown at pH 3 the intracellular volume of the cells fell to 1.7 µl mg^{-1} soluble protein (Table 6-1). The decrease of cell volume of *D. hansenii* cells may be considered to be a direct response and potential defence mechanism against extreme acidity stress.

6.2.2 Determination of Membrane Potential (ΔΨ) of *D. hansenii*

The definition of membrane potential is the electrical potential difference that would be measured between two identical reference electrodes positioned on opposite sides of a cell membrane. It is a property of the whole system and is independent of the spatial location of the electrodes (Rottenberg, 1979). So, it is necessary for microorganism cells when exposed to an external stress to keep a stable internal pH balance. Thereby, it is essential to determine the membrane potential and internal pH of cells in relation to changes in external pH.

Section 2.20.3 illustrated the equation which enables us to calculate the proton electrochemical potential difference or proton motive force (Δp) after the determination of both (ΔΨ) and pH gradient (ΔpH) across a membrane. Rottenberg in 1979 reported that the measurements of Δp for cells growing or surviving at different external pH values are very important for an experimental evaluation of the chemiosmotic hypothesis and are

also an indicator of the coupling in energy conversion membrane systems (Rottenberg, 1979).

In the present study ^{14}C-TPP$^+$ (tetraphenylphosphonium) was used as a probe for $\Delta\Psi$ determination, using the silicone oil centrifugation technique as described by Rottenberg (1979 and 1989). TPP$^+$ a positively charged ion is distributed across the membrane according to the membrane potential.

In order to measure membrane potential based on the distribution of isotopes, the time course of uptake for ^{14}C-TPP$^+$ probe was carried out using of *D. hansenii* cells to ensure there was no active uptake or efflux of the isotope after the initial distribution between cells and medium, and that the uptake was rapid and levelled off after a short time. This indicated that ^{14}C-TPP$^+$ probe was suitable for membrane potential determination, if 5 minutes were used as an incubation period (Figure 6-5 and Figure 6-6).
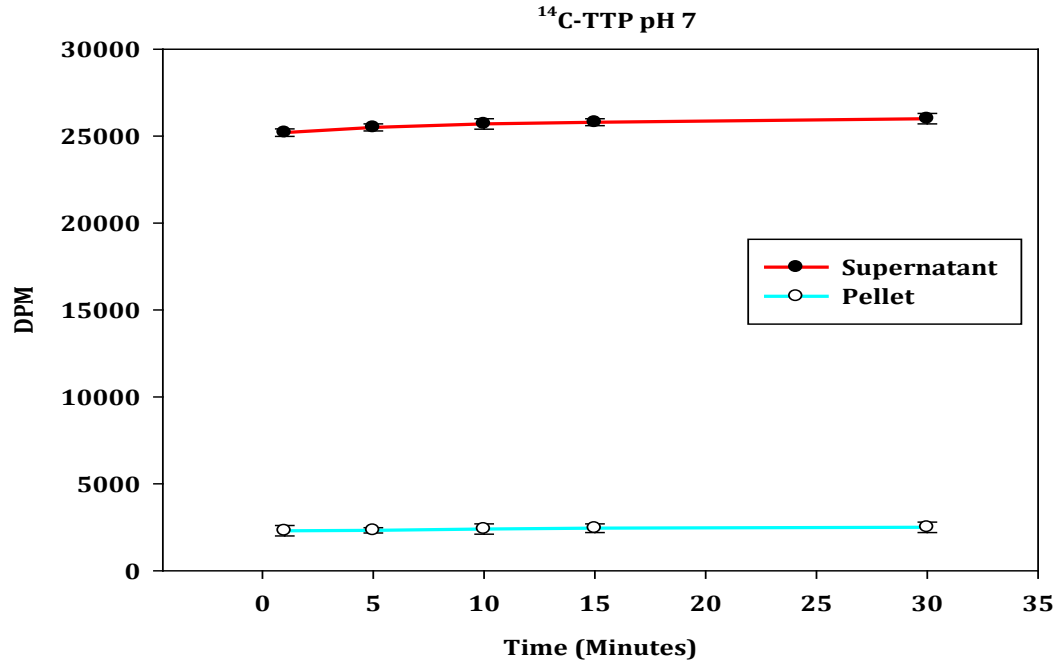
Figure 6-5: Time course of ^{14}C-TTP[+] uptake by *D. hansenii*. DPM in supernatants was measured during the 30 minute incubation period. Cells were grown at pH 7 in M9 minimal medium overnight at 25°C. Means of three replicates were used.
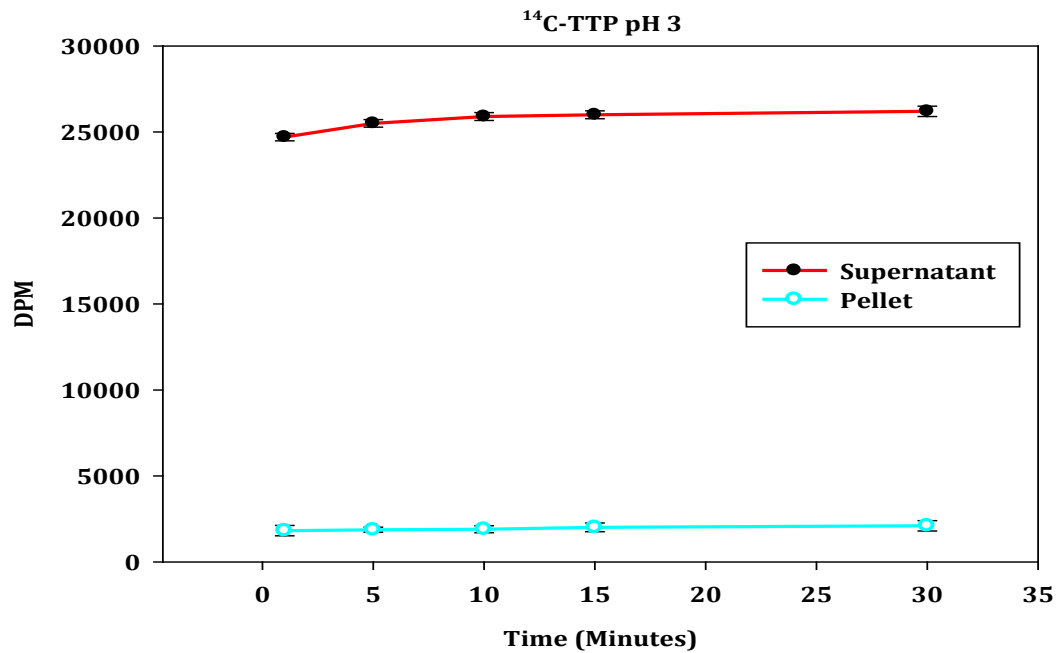
Figure 6-6: Time course of ^{14}C-TTP[+] uptake by *D. hansenii*. DPM in supernatants was measured during the 30 minute incubation period. Cells were grown at pH 3 in M9 minimal medium overnight at 25°C. Means of three replicates were used.

Table 6-2: Membrane potential (ΔΨ) of *D. hansenii* as a function of external pH. 10 ml of cells adapted to grow at pH 7 and pH 3 in M9 minimal medium and grown overnight at 25°C were transferred to 50 ml Falcon tubes and harvested and then the pellet was resuspended in 8 ml of fresh M9 minimal medium of the same pH (blue figures) of subjected to a pH shock (red figures)(Section 2.20.2). Means and standard deviations for three replicates are shown.

Incubation pH	ΔΨ (mV)	shock pH	ΔΨ (mV) after pH shock
pH 7	-30.3 ± 2.96	pH 3	-19.7 ± 0.33
pH 3	-73 ± 2.88	pH 7	-90.7 ± 3.17

Membrane potential was investigated in two different ways, the first was a direct measurement using same external pH value, whereas the second was a shock measurement in which the cells were resuspended in the opposite pH to their incubation pH. The cells in the shock experiment were incubated for 30 minutes in the other pH value before measuring $\Delta\Psi$. For example, if the cells incubated over course in M9 minimal medium at pH 7, the cells pellet and re-suspend in M9 minimal medium at pH 3 for 30 minutes before starting the determination of membrane potential and carried out using ^{14}C-TPP$^+$ probe, and just the opposite was done with pH 3.

Table 6-2 shows the measurements of $\Delta\Psi$ as a function of the external pH for *D. hansenii* cells grown at external pH 7 and pH 3 in M9 minimal medium. It was obvious from the results that *D. hansenii* cells produced different membrane potential values at pH 7 and pH 3. The cells generated a significant higher $\Delta\Psi$ at pH 3 (-73 mV) than at pH 7 (-30.3 mV). It is clear that the membrane potential of *D. hansenii* is increasing with decreasing external pH (Table 6-2).

However, when the cells were incubated at external pH 7 and shocked by external pH 3 the membrane potential decreased from -30.3 mV to -19.7 mV, while for cells grown at pH 3 medium and shocked using external pH 7 medium, the $\Delta\Psi$ increased from -73 mV to -90.7 mV (Table 6-2).

6.2.3 Determination of Internal pH (pH$_i$) and ΔpH of *D. hansenii*

The silicone oil centrifugation method as described by Rottenberg, (1979) was carried out in order to determine the cytoplasmic pH of *D. hansenii* cells. It was again essential to completely separate cell pellets from their external media to enable the measurement of both intracellular and extracellular volumes of cells (section 2.20). The measurement of cytoplasmic pH of cells adapted to grow and survive at different external pH values is very important for an experimental evaluation of the limits of acidic pH homeostasis.

The controlled pH (pre-set external pH) and the pH gradient (ΔpH) across the cytoplasmic membrane of the cell have usually been used to calculate the cytoplasmic pH (Padan *et al.,* 2005). In the current study in order to measure the cytoplasmic pH of *D. hansenii* cells grown at external pH 7 and pH 3 in M9 minimal medium the radioisotope probe ^{14}C-Benzoic acid, with a pKa of 4.2, was used as described in section 2.20.3.

In order to ensure there was no active uptake or efflux of the isotope after the initial distribution between cells and medium the time course of uptake for ^{14}C-Benzoic acid probe was performed using *D. hansenii* cells. The uptake was rapid and levelled off after a short time. The results indicated that a 5 minute incubation period was suitable for cytoplasmic pH determination (Figure 6-7 and Figure 6-8).
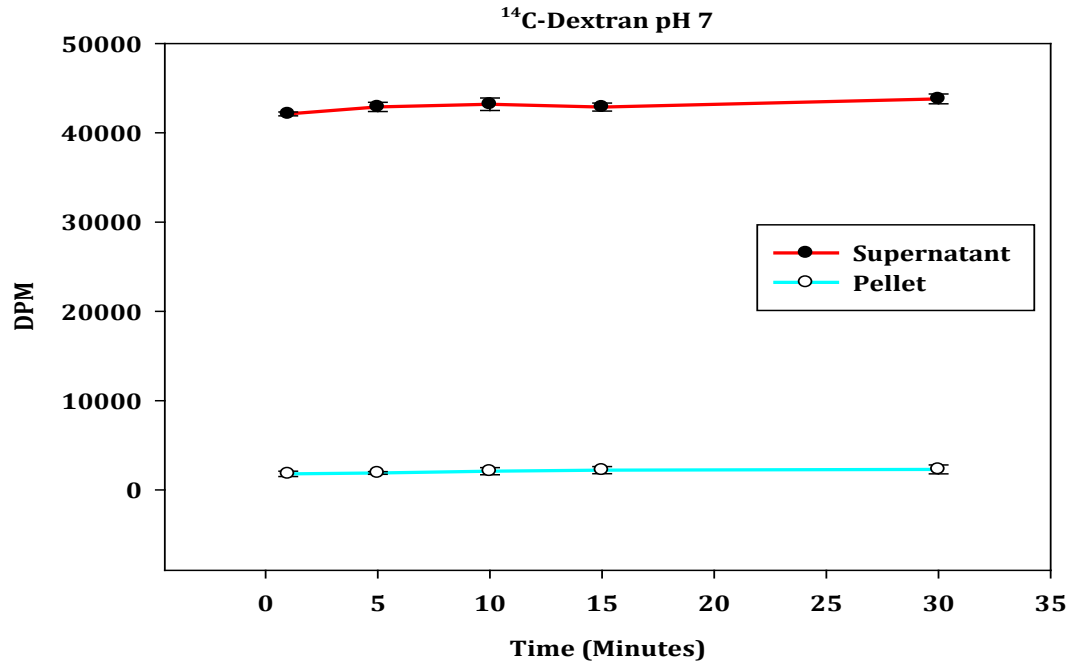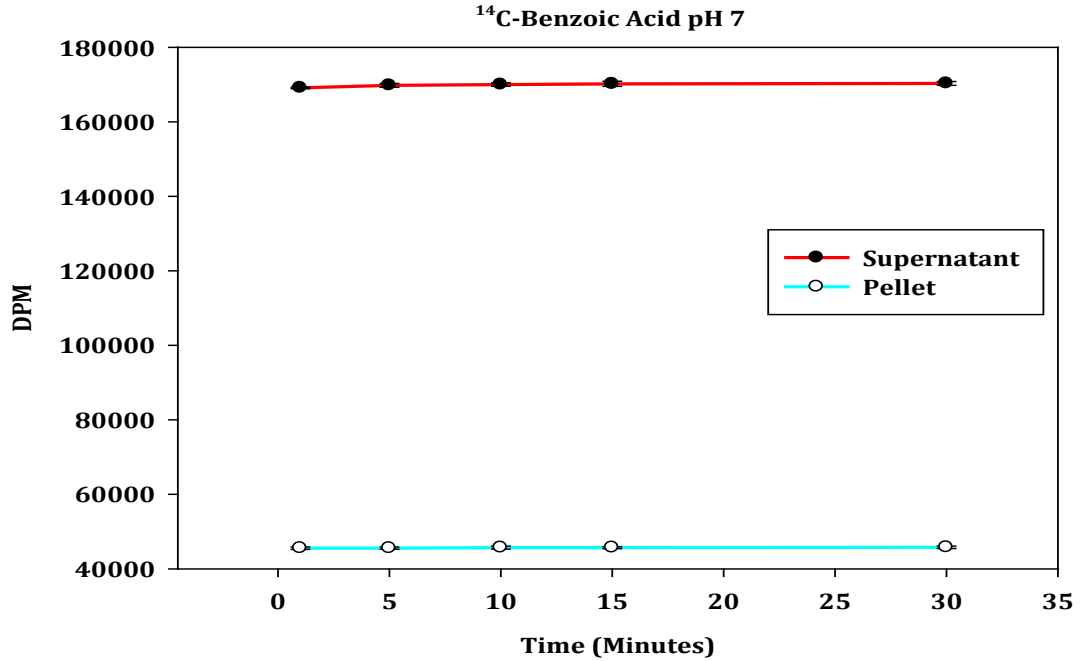
Figure 6-7: Time course of ^{14}C-Benzoic acid uptake by *D. hansenii*. DPM in supernatant was measured during the 30 minute incubation period. Cells were grown at pH 7 in M9 minimal medium overnight at 25ºC. Means of three replicates were used.
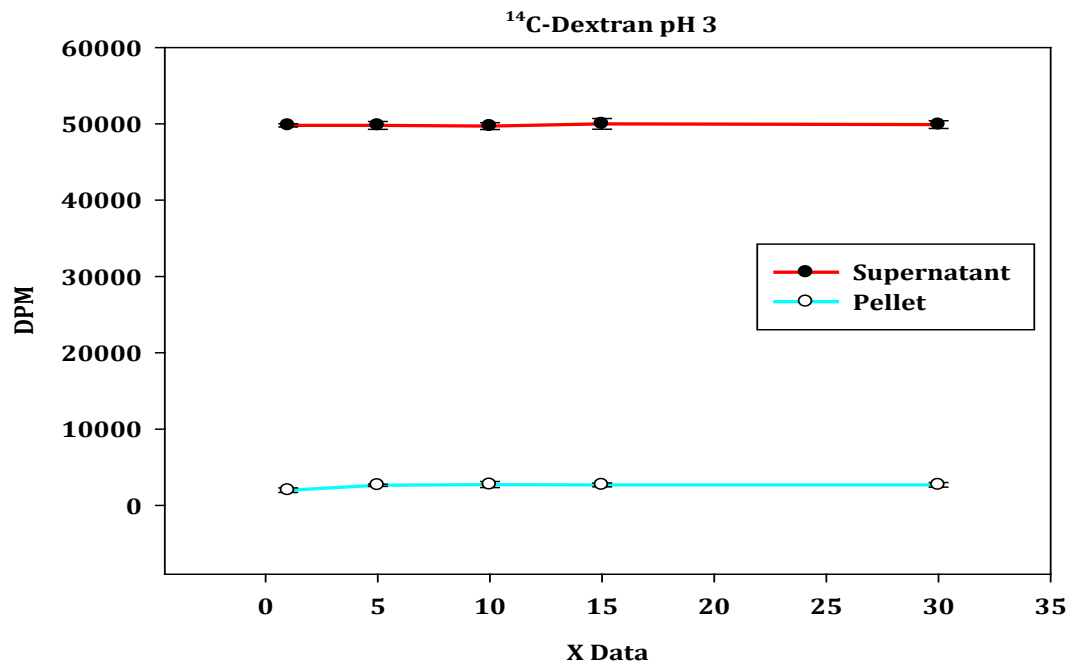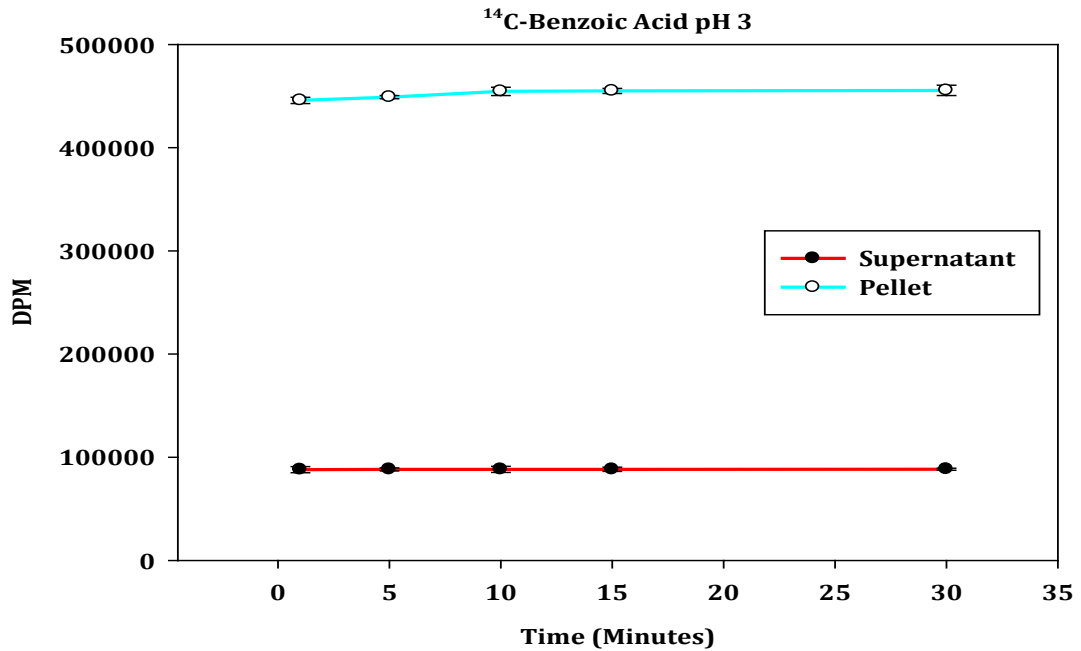

Figure 6-8: Time course of ^{14}C-Benzoic acid uptake by *D. hansenii*. DPM in supernatant was measured during the 30 minute incubation period. Cells were grown at pH 3 in M9 minimal medium overnight at 25ºC. Means of three replicates were used.

The internal pH (pH_i) was measured under two different conditions; the first was a direct measurement using same external pH value, whereas the second was a shock measurement in which the cells were resuspended in the opposite pH to the growth pH as was the case for membrane potential measurements (section 6.2.2).

Table 6-3 shows that *D. hansenii* cells tend to maintain their internal pH (pH_i) close to natural at both pH 7 and pH 3. However, the cells keep their internal pH at 7.1 when they were grown at pH 7 compared to a pH_i of 6.7 when grown at pH 3. The proton motive force (Δp) value for cells of *D. hansenii* was calculated to be -36.3 mV at external pH 7 while the Δp value was much higher at external pH 3 i.e -295.2 (Table 6-4).

On the other hand, when the cells were shocked noticeably different responses were seen (Table 6-3). The results indicated that when the cells were grown at pH 7 and shocked by external pH 3 medium their pH_i increased from 7 to 9.5, while when the cells grown at pH 3 were shocked by pH 7 the pH_i increased from 3 to 4.2. The total proton motive force (Δp) value was found to be similar (i.e. -169.8 and -162.8) when the cells were shocked by pH 3 and pH 7 respectively (Table 6-5).

Table 6-3: Internal pH (pH$_i$) of *D. hansenii* as a function of external pH. 10 ml of cells adapted to grow at pH 7 or pH 3 in M9 minimal medium overnight at 25°C were transferred to 50 ml Falcon tubes and harvested and then the pellet was resuspended in 8 ml of fresh M9 minimal medium of the same pH or different pH when shock experiment was run (section 2.20.3). Means and standard deviations for three replicates are shown.

Incubation pH	pH$_i$	shock pH	pH$_i$ after pH shock
pH 7	7.1 ± 0.07	pH 3	9.5 ±0.06
pH 3	6.7 ± 0.03	pH 7	4.2 ±0.09

Table 6-4: Internal pH (pH_i), ΔpH generation and proton motive force (Δp) measurements from cells of *D. hansenii* as a function of external pH. 10 ml of cells adapted to grow at pH 7 or pH 3 in M9 minimal medium were grown overnight at 25°C, transferred to 50 ml Falcon tubes and harvested. The pellet was resuspended in 8 ml of fresh M9 minimal medium of the same pH (section 2.20.3). Means and standard deviations for three replicates are shown. The Δp figures take into account the $\Delta\Psi$ measured under the same conditions (see Table 6.2).

Incubation pH	pH_i	ΔpH	Δp (mV)
pH 7	7.1 ± 0.07	0.1	-36.3
pH 3	6.7 ± 0.03	3.7	-295.2

Table 6-5: Internal pH (pH$_i$), ΔpH generation and proton motive force (Δp) measurements from cells of *D. hansenii* as a function of external pH. 10 ml of cells adapted to grow at pH 7 or pH 3 in M9 minimal medium were grown overnight at 25℃ , transferred to 50 ml Falcon tubes and harvested. The pellet was resuspended in 8 ml of fresh M9 minimal medium of the different pH (section 2.20.3). Means and standard deviations for three replicates are shown. The Δp figures take into account the ΔΨ measured under the same conditions (see Table 6.2).

pH		pH$_i$	ΔpH	Δp (mV)
Incubation	Shock			
pH 7	pH 3	9.5 ± 0.06	2.5	-169.8
pH 3	pH 7	4.2 ± 0.09	1.2	-162.8

To conclude, *D. hansenii* cells maintained their pH_i close to neutral when grown at pH 7 (pH_i was maintained to 7.1) and pH 3 (pH_i was maintained to 6.7), but the cells showed an inability to balance their pH_i 30 minutes after being resuspended in a different pH. The cells increased their pH_i to 9.5 when they shocked by external pH 3 medium, and but they only slightly increased pH_i to 4.2 when cells grown at pH 3 were shocked by external pH 7 medium.

The results suggest that *D. hansenii* was able to grow at low external pH levels despite their pH_i becoming more acidic or more alkaline. Nevertheless, the pH_i levels are always higher (more alkaline) than the external pH under all conditions tested.

6.3 Conclusions

To study the effect of the pH of the culture medium on the bioenergetics of growth of *D. hansenii*, the intracellular volume (ICV), membrane potential ($\Delta\Psi$) and cytoplasmic pH (pH_i) of cells were all examined. The use of radiolabelled isotopes in the silicone oil experiments described in the present study produced consistent results. This method allows the calculation of a volume for a pellet of cells through the ratio of two isotope species; tritiated water (^3H_2O) and ^{14}C-dextran.

The cell volume of *D. hansenii* was determined in cells adapted to grow at different external pH values of 7 and 3. It can be seen from the current chapter that there was a change in the ICV of *D. hansenii* cells when they were grown at different pH values. The ICV decreased with decreasing external pH, it was 5.7 μl mg^{-1} soluble protein when the cells were grown at pH 7 but fell to 1.7 μl mg^{-1} soluble protein when the external pH decreased to 3. This decrease in cell volume may be considered to be a self defence mechanism of this yeast to the extreme pH of the culture medium. Berner and Gervais in 1994 demonstrated that the cell volume of yeast decreased to a minimum with increasing salt concentrations (Berner and Gervais, 1994). In 2006 Mortensen *et al.,* recorded cell shrinkages of *D. hansenii* cells of approximately 30% to 35% under NaCl stress (Mortensen *et al.,* 2006).

^{14}C -TPP^+ (tetraphenylphosphonium) was used for membrane potential determination using the silicone oil centrifugation technique at a range of external pH values. Membrane potential ($\Delta\Psi$) of *D. hansenii* cells was found to be highest (most negative) at low external pH, the $\Delta\Psi$ was increased from -30.3 mV at external pH 7 to -73 mV at

external pH 3. The difference in membrane potential was more pronounced when they were subjected to pH shocks. The $\Delta\Psi$ was about -19.7 mV when the cells grown at pH 7 were shocked by external pH 3 and it was -90.7 mV when cells grown at external pH 3 were shocked by external pH 7. In 1998 Thome-Ortiz *et al.,* reported that K^+ stimulated proton pumping decreased the $\Delta\Psi$ of *D. hansenii* and they also found evidence for a K^+/H^+ exchange system that can control pH (Thome-Oritz *et al.,* 1998).

Additionally, the internal pH (pH_i) of *D. hansenii* cells was measured by the distribution of ^{14}C-Benzoic acid between the cell and the surrounding medium at different external pH values. The results show that *D. hansenii* cells are able to maintain their cytoplasmic pH close to neutral when they are grown in M9 minimal medium at pH 3 (pH_i was 6.7) and pH 7 (pH_i was 7.1). Moreover, shock experiment was designed to investigate *D. hansenii* cells behaviour under rapid changes of external pH values. However, when the cells were grown at pH 7 and shocked by external pH 3 the pH_i increased to 9.5 (alkaline), while when the cells were grown at pH 3 and shocked by external pH 7 medium the pH_i was maintained at 4.2 (acidic).

These results suggest that *D. hansenii* cells have a problem in maintaining their internal pH against external pH stress in M9 minimal medium when they face rapid change in the external pH value (measurements made 30 minutes after pH shock). Previous study of two *D. hansenii* strains showed that a more NaCl-tolerant strain was able to maintain pH_i homeostasis, whereas intracellular acidification occurred in the less NaCl-tolerant strain (Mortensen *et al.,* 2006). This suggests that the NaCl tolerant *D. hansenii* strain had a greater intracellular buffering capacity. Another study done by (Mortensen *et al.,* 2008) showed that the intracellular acidification of *D. hansenii* cells was induced by 200 µg/ml

of nitrite at external pH 4.5, while the cells show a good homeostasis maintenance of pH_i in the presence of nitrite at external pH 5.5. Parallel studies with *Candida zeylanoides* showed that this yeast was acidified at external pH 5.5 in the presence of nitrite. This suggests that *D. hansenii* is better able to control pH_i than less acid tolerant yeast species.

CHAPTER SEVEN

7 <u>General Conclusions and Future Work</u>

7.1 Conclusions

The major initial aim of this research project was to isolate acidophilic or acidotolerant microorganisms from sites in Sheffield (Rivelin and Limb valleys), which were known to have been polluted by industrial activity dating back several hundred years. In the event, four microorganisms (*Bacillus cereus*, *Micrococcus luteus*, *Aureobasidium pullulans* and *Debaryomyces hansenii*) were isolated as pure cultures and shown to be capable of growth over the pH range from 3 to 7 (Chapters 3 and 4). After initial characterisation and identification by 16S or 18S rDNA sequencing, it was decided to concentrate work on one bacterial species (*M. luteus*) and one eukaryotic species (*D. hansenii*). The major reason for selecting *M. luteus* and *D. hansenii* for further characterisation was the ability of these two strains to grow well at high salinities (up to 2.5 M NaCl) at pH 3 (Figure 3.17) (i.e. they show potential to be polyextremophiles) (Gomes and Steiner, 2004).

This potential was realised for *D. hansenii,* which was able to grow well at high salt concentrations and low pH values in minimal M9 medium. *D. hansensii* showed a greater ability to grow at high acidity and high salinity when grown in rich YPD medium. The optimum pH for *D. hansenii* in M9 medium was from 7 to 5 and the optimum salinity was 0.4 M NaCl at 25ºC. In YPD medium, the optimum pH was 5 and optimum salinity was 1.0 M NaCl at 25ºC. This clearly illustrates the

importance of rich medium in extending the range for optimal growth under stress conditions (Cummings *et al.,* 1993).

On the other hand, *M .luteus* shows poor ability to thrive under salt and acid stresses when grown in low nutrient M9 medium. However, *M. luteus* showed a significantly greater ability to tolerate high acidity and high salinity when grown in rich LB medium. It was found that pH 7 was the optimum pH for *M. luteus* in both M9 and LB medium, and 0.1 M NaCl was the optimum salinity in M9 at 25ºC while 1.0 M NaCl was the optimum in LB medium at 25ºC. Therefore, in general *M. luteus* favours non-extreme conditions, but it can be classified as a slight halophile when grown in rich medium (Gilmour, 1990).

The ability of microorganisms to adapt to high external salt concentrations depends on their efficiency at accumulating osmotically active compounds intracellularly. These compounds are known as compatible solutes to denote their compatibility with cell function (Brown, 1976). *D. hansenii* cells accumulate glycerol as the main compatible solute and another polyol (arabitol) as an additional compatible solute. The presence of polyols in *D. hansenii* has been found previously (Norkrans, 1966), but the identification of glycine betaine as a compatible solute in *D. hansenii* (Figures 5-48 to 5-54) is a new observation to the best of our knowledge. Even more surprising is the presence of glycine betaine in M9 minimal medium cultures, which suggests that *D. hansenii* is synthesising this compatible solute *de novo* and not just taking it up (or its precursor choline) from rich YPD medium (Cummings *et al.*, 1993). The presence of glycine betaine in cell extracts of *M. luteus* was to be expected based on previous work with related bacteria (Ventosa *et al.*, 1998) and *M.*

luteus was shown to use betaine as the main compatible solute and glutamate as an additional compatible solute (Figures 5-55 to 5-60).

D. hansenii and *M. luteus* were both able to adapt to grow in M9 minimal medium containing iron, sulphur and aluminum at 25ºC. However, it might be that the main reason for the low effect of those elements on the growth of *D. hansenii* and *M. luteus* is due to the availability of those elements at pH 7 and the relatively low concentrations of iron, sulphur and aluminum used in this experiment.

The intracellular volume (ICV), membrane potential ($\Delta\Psi$) and cytoplasmic pH (pH_i) of *D. hansenii* cells were measured in M9 minimal medium at pH 7 and pH 3. The ICV of *D. hansenii* decreased with decreasing external pH from 5.7 µl mg^{-1} soluble protein when the cells were grown at pH 7 to 1.7 µl mg^{-1} soluble protein at pH 3. This indicates that cells grown at pH 3 were significantly smaller than cells grown at neutral pH, a finding that was confirmed by electron micrographs of *D. hansenii* (Figure 5.1). The $\Delta\Psi$ of *D. hansenii* cells was found to be highest (most negative) at pH 3 (-73 mV) compared with -30.3 mV at pH 7. Furthermore, the difference in membrane potential was more obvious when they were subjected to pH shocks, the $\Delta\Psi$ was -90.7 mV when cells were grown at pH 3 and shocked by resuspension in pH 7. The $\Delta\Psi$ was -19.7 mV when the cells grown at pH 7 were shocked by resuspension in pH 3.

D. hansenii cells are able to maintain their cytoplasmic pH close to neutral when they are grown in M9 minimal medium at pH 7 (pH_i was 7.1) or pH 3 (pH_i was 6.7). This means that the pH gradient between the inside and outside of the cells is extremely large in cells grown at pH 3. In bacterial cells, where the ΔpH is very large, the $\Delta\Psi$ is reversed (inside

positive), but as noted in the paragraph above this does not happen with *D. hansenii* cells. The most likely explanation is that the eukaryotic yeast cell does not use its cytoplasmic membrane as the site of ATP generation and therefore controlling the overall level of Δp may not be crucial.

When *D. hansenii* cells were grown at pH 7 and shocked by resuspension in pH 3 the pH_i jumped to 9.5 (alkaline), while when the cells were grown at pH 3 and shocked by resuspension in pH 7 the pH_i decreased to 4.2 (became more acidic). These results suggest that *D. hansenii* cells have a problem in maintaining their internal pH against external pH stress in M9 minimal medium when they face a rapid change in the external pH value (measurements made 30 minutes after pH shock).

7.2 Future Work

Despite the success of isolating four microorganisms (two bacteria and two yeasts) in the present work from Rivelin and Limb Valleys in Sheffield, UK using traditional culturing methods, it was perhaps surprisingly not to isolate more novel strains or at least a greater number than just four microorganisms. As noted in Chapter 3, several different culture media were used, but future work could include the use of a larger variety of selective media to try to isolate additional microorganisms from these natural habitats.

The use of electron microscopy (SEM and TEM) allowed the observation of some stress-induced changes in the cell structure of *D. hansenii* and *M. luteus* cells (Figures 5-1 to 5-4). However, it is highly recommended to use Environmental

Scanning Electron Microscope (ESEM) in future work which may lead to the discovery of further significant changes happening to microbial cells when grown under stress.

The present experiments on the bioenergetics of growth of *D. hansenii* gave promising results, but it would be very interesting to carry out further research looking at the uptake of compatible solutes (or their precursors) from rich media. In addition, extending the pH shock experiments to times beyond 30 minutes after the pH change would allow the determination of the time taken for the cells to recover and return their ΔpH and $\Delta\Psi$ to normal values.

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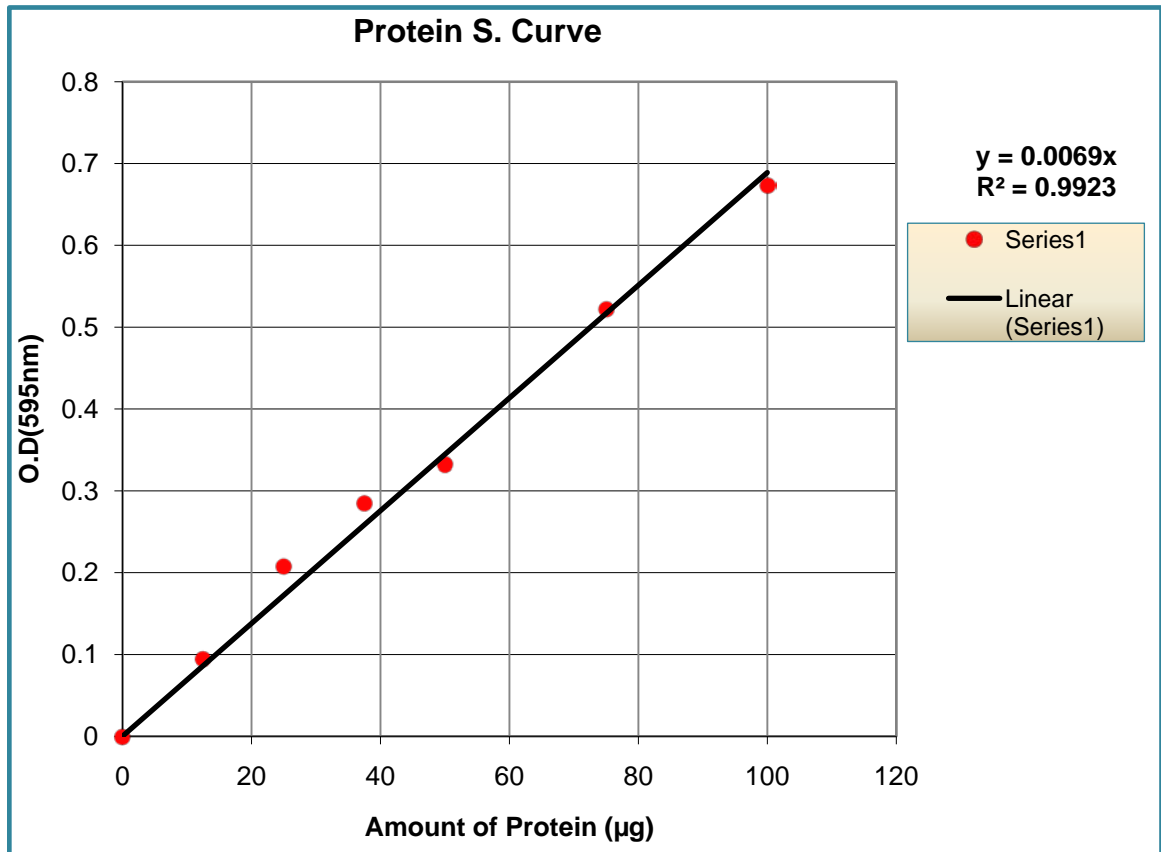
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APPENDICES

Appendix A:

Protein Standard Curve:

Protein S. Curve

y = 0.0069x
R² = 0.9923

Standard curve of Bovine Serum Albumin (BSA) 5 mg ml^{-1}.

Appendix B:

List of Solution Used:

1. CTAB buffer

2% CTAB (hexadecyltrimethylammonium bromide)

100 mM TrisHCl [pH=8]

20 mM EDTA,

1.4 M NaCl

2% β-mercaptoethanol [added just before use]

2. 50× TAE buffer

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

3. Buffers used to provide a range of pH in M9 minimal, ½ LB, LB and YPD medium

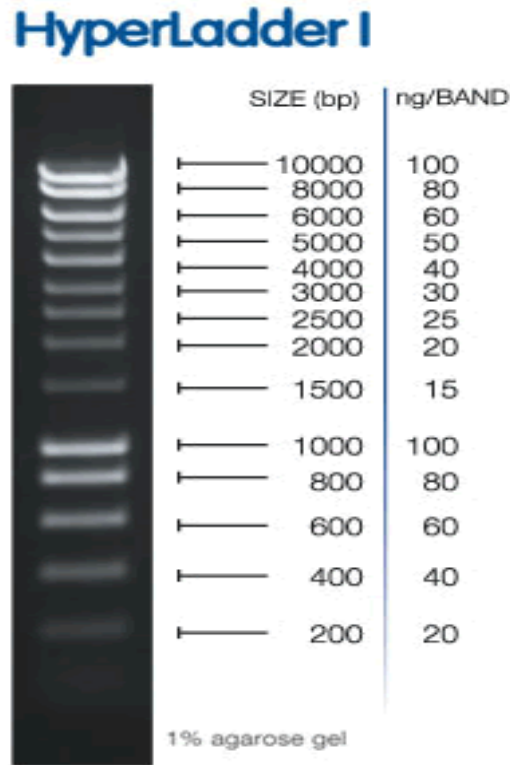|		50 mM Buffers		
Common name	Molecular formula	Useful pH range	Working pH range	g l^{-1}
Mops	$C_7H_{15}NO_4S$	2.5 - 4.0	3.5 - 4.5	10.46
Mes	$C_6H_{13}NO_4S$	5.0 – 6.7	5.0 - 6.5	9.76
Trizma	$C_4H_{11}NO_3$	7.0 – 9.0	7.5 - 8.5	6.05

4. SOC medium

The following reagents are added to 900 ml dH2O:

20 g Bacto Tryptone, 5 g Bacto Yeast Extract, 2 ml of 5 M NaCl, 2.5 ml of 1 M KCl, 10 ml of 1 M MgCl2, 10 ml of 1 M MgSO4 and 20 ml of 1 M glucose before adjusting the final volume to 1 litre prior to autoclaving at 121 oC and 15 psi for 15 minutes.

5. Standard Hyperladder

HyperLadder I

SIZE (bp)	ng/BAND
10000	100
8000	80
6000	60
5000	50
4000	40
3000	30
2500	25
2000	20
1500	15
1000	100
800	80
600	60
400	40
200	20

1% agarose gel

- Higher intensity bands: **1000bp and 10000bp**
- Supplied in a ready-to-use format
- Each lane (5µl) provides 720ng of DNA

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

## 6. <u>Plasmid pCR 2.1 TOPO (3.9 kb)</u>
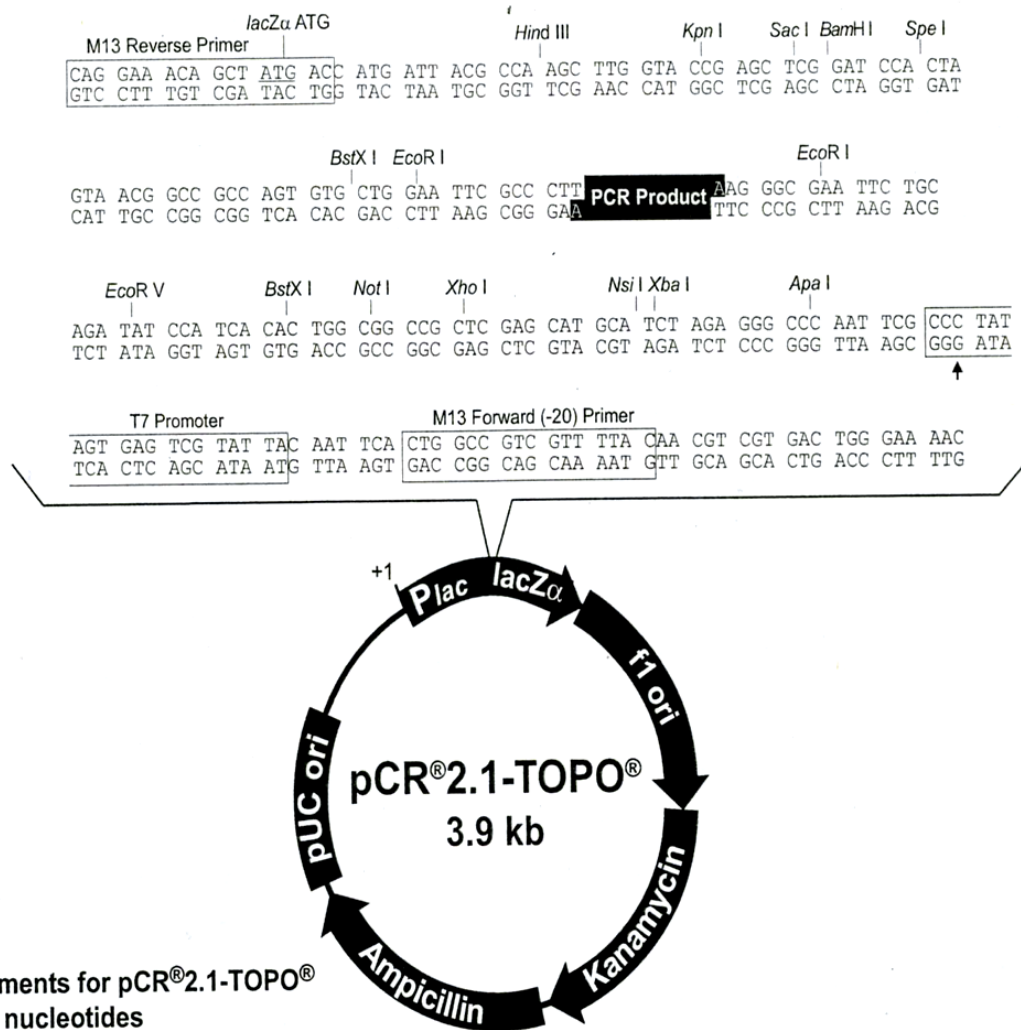
Schematic illustration of structure plasmid pCR 2.1 TOPO (3.9 kb) used as coloning vector. Vector data for pCR 2.1 indicating the ligation site for the TA cloning system and the available restriction sites. Information extracted from the Invitrogen TA cloning kit manual.

Appendix C:

1. LV$_1$ Strain 16S rDNA Foreword Sequences Applied in FinchTV Program

2. LV$_1$ Strain 16S rDNA Reverse Sequences Applied in FinchTV Program

3. RV₃ Strain 16S rDNA Foreword Sequences Applied in FinchTV Program
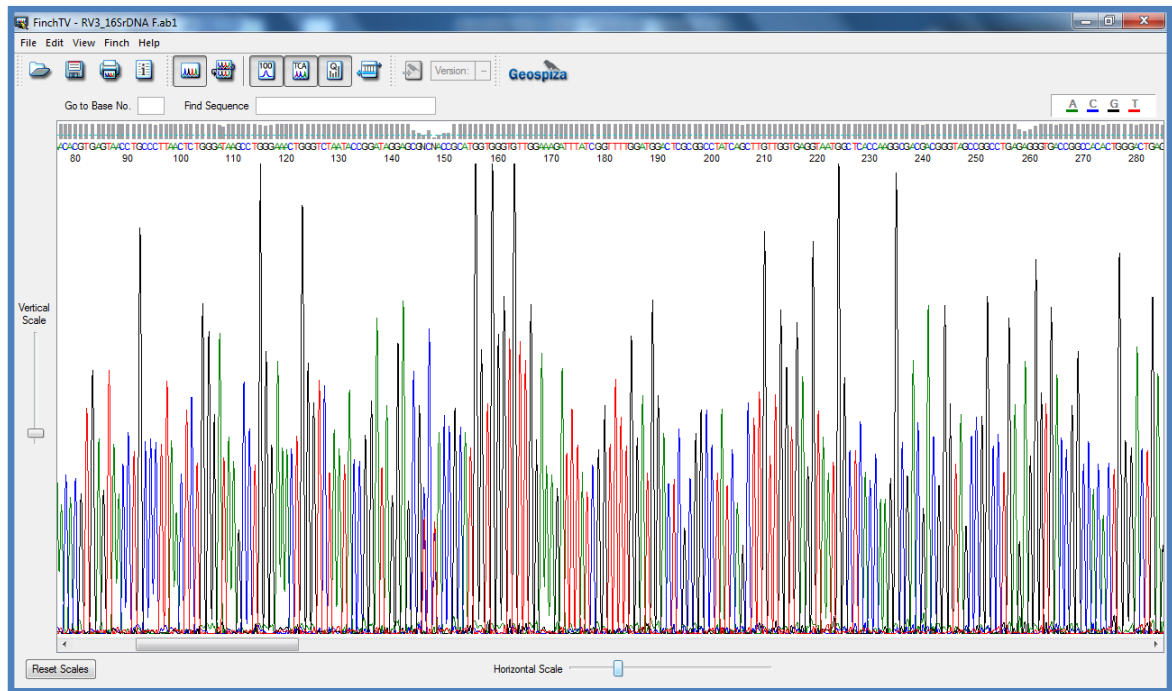
4. RV₃ Strain 16S rDNA Foreword Sequences Applied in FinchTV Program
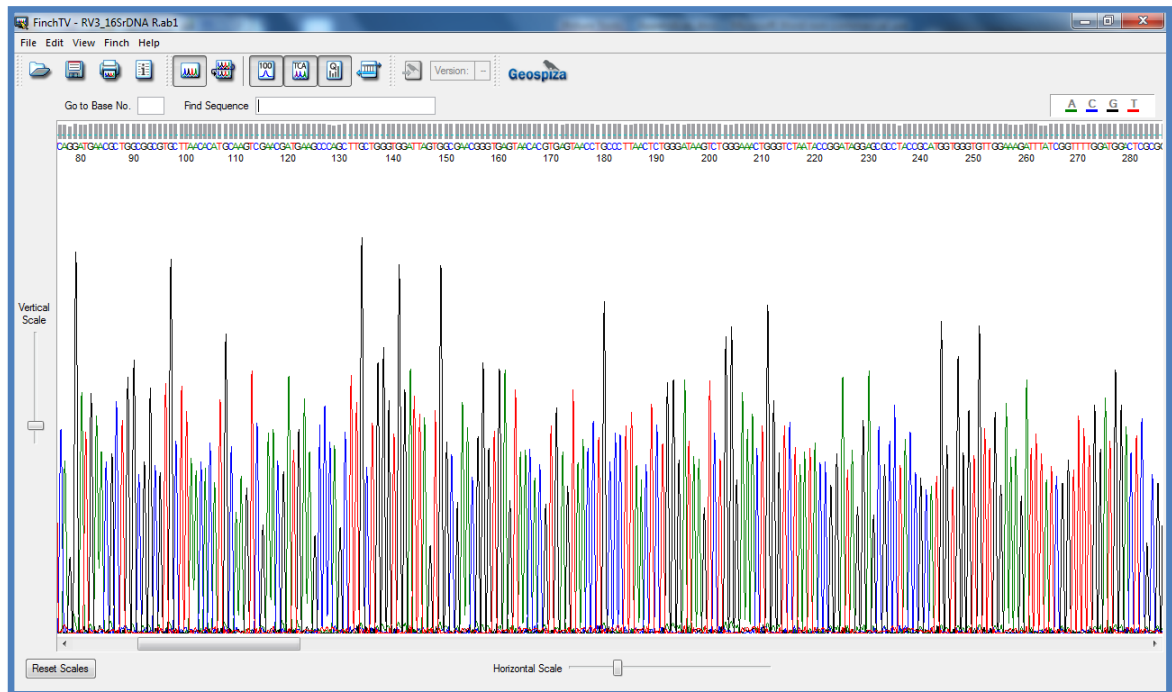
Appendix D:

1. DR. Gilmour Lab Facility:

A. Oxygen Electrode:

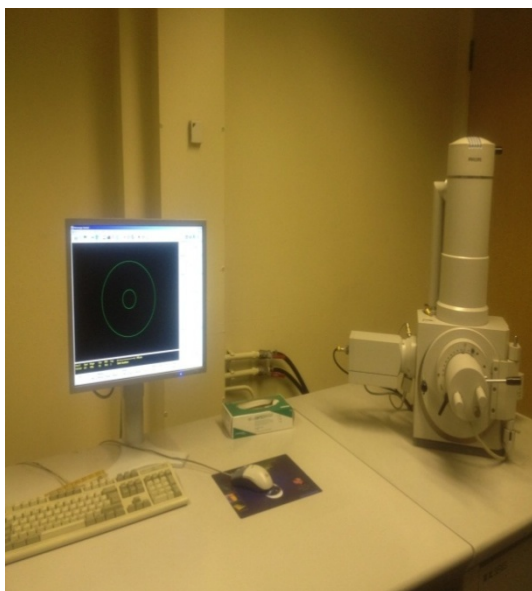
B. PCR Equipments:

C. Other Equipments:

2. <u>Electron Microscope Facility:</u>

SEM

TEM

3. <u>NMR Facility:</u>