A COMPARISON OF THE RESPONSES TO ENVIRONMENTAL STRESS OF THE GRAM-POSITIVE BACTERIUM *STAPHYLOCOCCUS XYLOSUS* AND THE GRAM-NEGATIVE BACTERIUM *HALOMONAS* HALO

BY

ABDULRAHMAN ABDULLAH AL-HUMIANY

(B.SC., MSC. THE UNIVERSITY OF KING ABDULAZIZ, JEDDAH, SAUDI ARABIA)

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE DEPARTMENT OF MOLECULAR BIOLOGY AND BIOTECHNOLOGY

UNIVERSITY OF SHEFFIELD

OCTOBER 1999

CONTENTS

SUMMARY.....viii

DEDICATION.....ix

ACKNOWLEDGEMENTS.....x

ABBREVIATIONS.....xi

CHAPTER 1: INTRODUCTION

1.1 THE HYPERSALINE ENVIRONMENT 1

1.1.1 THE GREAT SALT LAKE 2

1.1.2 THE DEAD SEA..... 2

1.1.3 HYPERSALINE LAKES IN ANTARCTICA 3

1.1.4 SABKHAS 3

1.1.5 ALKALINE LAKES 4

1.1.6 MARINE SALTERNS 4

1.1.7 HYPERSALINE SOILS 5

1.2 ORGANISMS WHICH INHABIT SALINE ENVIRONMENTS 5

1.2.1 SEA WATER TO 10% (*W/V*) SALINITIES 6

1.2.2 10-30% (*W/V*) SALINITIES 8

1.2.3 ABOVE 30% (*W/V*) SALINITIES 10

1.3 THE AVAILABILITY OF NUTRIENTS IN SALINE ENVIRONMENTS.... 11

1.4 THE TERMINOLOGY OF SALT ADAPTATION..... 13

1.4.1 WATER ACTIVITY (A_W)..... 13

1.4.2 WATER POTENTIAL 14

1.4.3 OSMOTIC PRESSURE 14

1.4.5 TURGOR PRESSURE 15

1.4.6 OSMOREGULATION 15

1.5 OSMOTIC STRESS AND THE ROLE OF COMPATIBLE SOLUTES 15

1.5.1 THE DIVERSITY OF COMPATIBLE SOLUTES 17

1.5.2 THE MODE OF ACTION OF COMPATIBLE SOLUTES 26

1.6 THE DIVERSITY OF HALOPHILIC AND HALOTOLERANT MICROORGANISMS 27

1.6.1 HETEROTROPHIC EUBACTERIA 28

1.6.2 ARCHAEA 33

1.6.3 CYANOBACTERIA 34

1.6.4 OTHER PHOTOTROPHIC EUBACTERIA 34

1.6.5 ALGAE 35

1.7 AIMS OF PROJECT 36

CHAPTER 2 : MATERIALS AND METHODS

2.1 ISOLATION AND SELECTION OF ORGANISMS 37

2.2 MEDIA AND GROWTH CONDITIONS 40

2.2.1 MEDIA 40

2.2.2 BATCH CULTURES 42

2.2.3 CONTINUOUS CULTURE 42

2.2.4 PURIFICATION AND MAINTENANCE OF THE ORGANISMS 43

2.2.5 EFFECT OF SODIUM CONCENTRATION ON GROWTH 44

2.2.6 EFFECT OF POTASSIUM CONCENTRATION ON GROWTH 44

2.2.8 EFFECT OF pH ON GROWTH 45

2.2.9 EFFECT OF INHIBITORS ON GROWTH 45

2.3 METHODS USED TO IDENTIFY JDP14 45

2.3.1 MORPHOLOGY 46

2.3.2 MOTILITY 47

2.3.3 CATALASE TEST 47

2.3.4 OXIDASE TEST 48

2.3.5 API STAPH KIT 48

2.4 MEASUREMENT OF RESPIRATION RATE 49

2.5 PROTEIN DETERMINATION 52

2.6 PROLINE DETERMINATION 53

2.7 GROWTH OF *HALOMONAS* HALO AND JDP14 ON CDM 53

2.8 EFFECT OF COMPATIBLE SOLUTES ON GROWTH AT 3 M NaCL ... 54

2.9 ^{14}C-GLYCINE BETAINE UPTAKE EXPERIMENTS 54

2.9.1 MANUFACTURE OF ^{14}C-GLYCINE BETAINE 54

2.9.2 MEASUREMENT OF BETAINE UPTAKE 55

2.9.3 EFFECT OF INHIBITORS ON ^{14}C-BETAINE TRANSPORT 56

2.10 SILICONE OIL TECHNIQUE 56

2.10.1 INTRACELLULAR VOLUME (ICV) DETERMINATION 56

2.10.2 MEASUREMENT OF MEMBRANE POTENTIAL ($\Delta\Psi$) 60

2.10.3 EFFECT OF EXTERNAL PH 61

2.10.4 EFFECT OF INHIBITORS..... 61

2.11 EFFECT OF TEMPERATURE AND SALT ON ENZYME ACTIVITIES OF *HALOMONAS* HALO AND JDP14 63

2.11.1 PREPARATION OF CELL FREE EXTRACT 63

2.11.2 GENERAL ASSAY CONDITIONS..... 64

2.11.3 FUMARASE (EC 4.2.1.2)..... 64

2.11.4 MALATE DEHYDROGENASE (EC 1.1.1.37)..... 65

2.11.5 ACID PHOSPHATASE..... 66

2.11.6 β-GALACTOSIDASE (EC 3.2.1.23) 67

CHAPTER 3 : SALT TOLERANCE OF *HALOMONAS* HALO AND *STAPHYLOCOCCUS XYLOSUS*

3.1 INTRODUCTION..... 70

3.2 RESULTS 73

3.2.1 ISOLATION AND SELECTION OF ORGANISMS 73

3.2.2 IDENTIFICATION OF JDP14..... 75

3.2.3 GROWTH OF *HALOMONAS* HALO AND *S. XYLOSUS* AT DIFFERENT SALINITIES..... 75

3.2.4 ELECTRON MICROGRAPHS OF *S. XYLOSUS* CELLS GROWN AT DIFFERENT SALINITIES..... 76

3.2.5 EFFECT OF KCL ON GROWTH OF *HALOMONAS* HALO AND *S. XYLOSUS*..... 97

3.2.6 EFFECT OF KCL AND NaCl ON GROWTH OF *HALOMONAS* HALO AND *S. XYLOSUS* 97

3.2.7 Na^+ REQUIREMENT FOR RESPIRATION IN HALOMONAS HALO AND S. XYLOSUS 102

3.2.8 EFFECT OF KCL ON THE RESPIRATION RATE OF *HALOMONAS* HALO AND *S. XYLOSUS* 102

3.2.9 EFFECT OF NACL AND KCL ON THE RESPIRATION RATES OF *HALOMONAS* HALO AND *S. XYLOSUS*..... 103

3.3 DISCUSSION..... 109

CHAPTER 4 : ROLE OF COMPATIBLE SOLUTES IN SALT TOLERANCE OF *HALOMONAS* AND *STAPHYLOCOCCUS XYLOSUS*

4.1 INTRODUCTION..... 115

4.2 RESULTS 118

4.2.1 GROWTH OF *S. XYLOSUS* AND *HALOMONAS* HALO ON CHEMICALLY DEFINED MEDIUM AT DIFFERENT SALINITIES. 118

4.2.2 EFFECT OF COMPATIBLE SOLUTES ON GROWTH OF *S. XYLOSUS* AND *HALOMONAS* HALO..... 121

4.2.3 BETAINE UPTAKE BY *HALOMONAS* HALO IN THE PRESENCE OR ABSENCE OF CCCP IN 0.5 M NaCl CHEMICALLY DEFINED MEDIUM. 124

4.2.4 BETAINE UPTAKE BY *HALOMONAS* HALO IN THE PRESENCE OR ABSENCE OF CCCP IN 3 M NaCl CHEMICALLY DEFINED MEDIUM. ... 127

4.2.5 BETAINE UPTAKE BY *S. XYLOSUS* IN THE PRESENCE OR ABSENCE OF CCCP IN 0.5 M NaCl CHEMICALLY DEFINED MEDIUM. 130

4.2.6 BETAINE UPTAKE BY *S. XYLOSUS* IN THE PRESENCE OR ABSENCE OF CCCP IN 3 M NaCl CHEMICALLY DEFINED MEDIUM 130

4.2.7 PROLINE CONTENT OF *S. XYLOSUS* 133

4.3 DISCUSSION 137

CHAPTER 5 : RESPONSE OF *HALOMONAS* HALO AND *STAPHYLOCOCCUS XYLOSUS* TO DIFFERENT pH LEVELS

5.1 INTRODUCTION 141

5.2 RESULTS 149

5.2.1 EFFECT OF DIFFERENT pH VALUES ON THE GROWTH OF *S. XYLOSUS* AND *HALOMONAS* HALO 149

5.2.2DETERMINATION OF THE ΔP AS A FUNCTION OF EXTERNAL pH IN *S. XYLOSUS* AND *HALOMONAS* HALO 149

5.2.3 EFFECT OF CCCP AND MONENSIN ON THE GROWTH OF *STAPHYLOCOCCUS XYLOSUS* AND *HALOMONAS* HALO 153

5.2.4 EFFECT OF CCCP AND MONENSIN ON ΔP OF *S. XYLOSUS* AND *HALOMONAS* HALO 157

5.2.5 EFFECT OF CCCP AND MONENSIN ON THE RESPIRATION RATE OF *HALOMONAS* HALO AND *S. XYLOSUS* 160

5.2.6 DETERMINATION OF ΔP AS A FUNCTION OF EXTERNAL pH IN *S. XYLOSUS* GROWN AT 1.5 M NaCl 165

5.2.7 DETERMINATION OF ΔP AS A FUNCTION OF EXTERNAL pH IN *S. XYLOSUS* GROWN IN CONTINUOUS CULTURE 165

5.3 DISCUSSION 170

CHAPTER 6 : EFFECT OF TEMPERATURE ON THE ACTIVITIES OF ENZYMES EXTRACTED FROM *STAPHYLOCOCCUS XYLOSUS* AND *HALOMONAS* HALO CELLS GROWN AT DIFFERENT SALINITIES

6.1 INTRODUCTION..... 177

6.2 RESULTS 179

6.2.1 FUMARASE (E.C 4.2.1.2)..... 179

6.2.2 MALATE DEHYDROGENASE (E.C 1.1.1.37)..... 182

6.2.3 ACID PHOSPHATASE..... 185

6.2.4 β-GALACTOSIDASE (EC 3.2.1.23) 188

6.2.5 DISCUSSION..... 190

CHAPTER 7 : GENERAL DISCUSSION 192

REFERENCE LIST 200

APPENDIX (A) STANDARD PROTEIN CONCENTRATION CURVE..... 223

APPENDIX (B) STANDARD PROLINE CONCENTRATION CURVE. 224

APPENDIX (C) STANDARD O-NITROPHENOL CONCENTRATION CURVE. 225

SUMMARY

Abdulrahman Al-Humiany (1999) **A Comparison of the Responses to Enviromental Stress of the Gram-Positive Bacterium *Staphylococcus xylosus* and the Gram-Negative Bacterium *Halomonas* Halo.** PhD Thesis, Department of Molecular Biology and Biotechnology, University of Sheffield.

Salt tolerance of the Gram-negative bacterium, *Halomonas* Halo, was compared with the salt tolerance of a newly isolated Gram-positive coccus *Staphylococcus xylosus*. Both organisms grew over a range of salinities from 0.1 - 3.0 M NaCl in both rich medium containing yeast extract and in minimal medium. In the absence of yeast extract, growth of *S. xylosus* was very slow at 3.0 M NaCl and its optimum salinity for growth was 0.1 M NaCl, whereas *Halomonas* Halo showed optimum growth at 0.5 M NaCl. Growth experiments replacing NaCl with KCl and the effect of Na^+ on the rate of respiration showed that *Halomonas* Halo had a greater requirement for Na^+ for growth than *S. xylosus*. When betaine was added to the minimal medium, it greatly increased the growth rate of both organisms at 3 M NaCl. The precursor of betaine, choline, was also effective in increasing the growth rate of *Halomonas* Halo, but was much less effective for *S. xylosus*. Both organisms transported betaine into the cells by an energy dependent transport system; transport rates were broadly similar, but it appeared that the halotolerant *S. xylosus* took up betaine more efficiently than *Halomonas* Halo.

Halomonas Halo and *S. xylosus* were shown to grow across a pH range from 5.5 - 8.5, but *S. xylosus* showed optimum growth across the full range whereas *Halomonas* Halo showed a distinct optimum at pH 7.0. The proton motive force (Δp) was found to be low in both organisms and at pH 8.5, it fell below the theoretical minimum (150 mV) which is required for ATP synthesis. Δp was significantly reduced by the inhibitor carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and to a much lesser extent by monensin. Both inhibitors completely stopped the growth of both organisms at pH 7.0. The possibility that compatible solutes may protect enzymes from thermal denaturation was examined, but the results were inconclusive.

DEDICATION

This thesis is dedicated to my parents, who are waiting in pain to see me get through my research work and return back home to be with them, to my wife, to my brothers and sisters and my seven children Hisham, Hashim, Fatin, Faisal, Abdullah, Sarah and Fatimah who have shared my stress and difficulties, offering me happiness and encouragement throughout my research work.

Acknowledgements

Many thanks to Almighty Allah, who blessed me with health, courage, enthusiasm and determination which enabled me to carry out and conclude this research.

I would like to express my gratitude and appreciation to the ministry of education for giving me the opportunity to continue my higher education.

I would like to express my appreciation to Dr. D. J. Gilmour, my supervisor, for his consistent guidance, encouragement, valuable supervision and what is more important, a very sincere friendship.

I am most grateful to my mother, father, brothers and sisters for their great encouragement, support and providing every possible assistance. Special thanks to my mother and father who sacrificed much for bringing up and the educating their sons and daughters.

I am grateful to my family members – my wife, my sons and my daughters who sacrificed their personal comfort and career to the accomplishment of my goals and for their unlimited support, patience and tolerances during the ups and downs of conducting this reseach.

I also would like to thank my friends in Saudi Arabia and my friend in Sheffield for their support over the years.

Finally, I would like to thank the research students, staff and technicians of the department who have been a pleasure to work with.

ABBREVIATIONS

a_w : water activity.

BSA : bovine serum albumin.

CCCP: carbonyl cyanide-m-chlorophenyl hydrazone.

CDM : chemically defined medium.

ECV : extracellular volume.

HDM : *Halomonas* defined medium.

ICV : intracellular volume.

kBq : kilo Becquerel

LB : L-broth.

OD : optical density.

ONPG: *o*-nitrophenyl-β-D-galactopyranoside.

NAD : nicotinamide-adenine dinucleotide.

NADH: reduced nicotinamide-adenine dinucleotide.

pH_i : internal pH.

pH_o : external pH.

TCA : trichloroacetic acid.

TDS : total dissolved salts.

TLC : thin layer chromatography.

TPP^+ : tetraphenyl-phosphonium cation.

Tris : tris (hydroxymethyl) methylamine.

Δp : electrochemical proton gradient or proton motive force.

ΔpH : transmembrane pH gradient.

$\Delta\Psi$: transmembrane electrical potential.

CHAPTER 1

INTRODUCTION

1.1 The Hypersaline Environment

Hypersaline environments are a collection of biotopes in which the diversity of life forms present is limited due to the availability of water. It is an extreme environment requiring organisms which grow in it to possess special adaptations to overcome the limitations placed upon them by the low availability of water (Brown, 1990).

Hypersaline environments can be divided into water and soil environments. Of these two habitats hypersaline waters have been most extensively studied. These types of environment can be subdivided on the basis of their chemical make-up (reviewed by Rodriguez-Valera, 1988). The first type of hypersaline water has proportions of inorganic ions that are the same as those observed in seawater, even though the net amounts are much greater. These are thalassohaline waters and are usually derived from the evaporation of seawater. The second type of hypersaline water has proportions of inorganic ions markedly different to those seen in sea water, these are known as athalassohaline and are derived from mineral deposits dissolved in bodies of water (Grant and Ross, 1986). There are examples of hypersaline waters on every continent on Earth. To illustrate the range of

such habitats a brief overview is given below starting with the most famous examples, the Great Salt Lake and the Dead Sea.

1.1.1 The Great Salt Lake

The Great Salt Lake is situated in the state of Utah in the west of the United States. It sits in the Great Basin, an arid region in the rain shadow of the Sierra Nevada-Cascade mountain range which are 800 km further westward (Post, 1977). It is a thalassohaline lake, the major solute being NaCl. It was divided in 1957 by a railway causeway into northern and southern basins. This has led to the interesting phenomenon of the southern basin having its salinity drastically reduced as 95% of watershed streams flow into this half of the lake, such that the salt concentration is rapidly approaching that of seawater. In contrast evaporation in the shallow northern basin has led to a virtually saturated brine. The temperature range is -5 to 40 °C and the pH is around 7.7 (Larsen, 1980).

1.1.2 The Dead Sea

The Dead Sea occupies a deep depression of the East African Rift Valley which lies 400 m below sea level. Fresh water flows into the sea from the river Jordan but there is no outlet. The Dead Sea has a similar overall salinity to the Great Salt Lake but it is of an athalassohaline composition, with Mg^{2+} and Ca^{2+} present in much higher proportions than is observed in seawater. There is also much less SO_4^{2-}. This body of water is of greater depth (320 m) than the Great Salt Lake. The temperature is very stable ranging from 21 to 23 °C and the pH is 5.8 to 6.4 (Larsen, 1980).

1.1.3 Hypersaline Lakes in Antarctica

The distribution of hypersaline lakes is not confined to regions which experience hot temperature, the Vestfold Hills are a coastal area of east Antarctica which is ice free and geologically young, formed after an ice retreat about 7000 years ago. The area has hypersaline lakes which have developed from seawater trapped in natural depressions in the terrain (Franzmann, 1991). They range in composition from Deep Lake which has 21 to 28 % (w/v) salinity and so never freezes, to Organic Lake (0.8 to 21 % (w/v) salinity) which is ice bound nine months a year (Ashbolt, 1991).

1.1.4 Sabkhas

Sabkhas are fairly common in arid hot climates and consist of two types. Firstly, the continental sabkhas which from by airborne salts concentrating in geographical basins with internal drainage and a high water table. The second type is the coastal sabkha which can be in the form of an open supratidal sabkha where the brines form a saline groundwater table and permeate the sediment by subsurface leakage and an occasional flood. There are also sabkhas formed by evaporitic deposits on the sea margin with a restricted and uneven sea water supply (Gavish, 1980). As such they are usually thalassohaline environments, but often appear dry with the water below the surface, although periodically there may be standing water which dries out seasonally as is the case with the Gavish Sabkha in Egypt (Purser, 1985).

1.1.5 Alkaline Lakes

The alkaline saline lakes are an interesting example of a saline habitat in that there is an additional stress of high pH (9-11) for organisms to contend with. In a recent review, Tindall (1988) described the microflora of several alkaline saline environments. Examples of this type of habitat are found in the Kenyan Rift Valley where there are many alkaline lakes. The least saline of these (1-2% w/v) contain a variety of invertebrates, algae and cyanobacteria, even fish (*Tilapia grahami*) may be present (Grant and Tindall, 1986). The predominant organisms at higher salinities are phototrophic microorganisms which include in addition to the cyanobacteria, members of the genera *Ectothiorhodospira, Rhodopseudomonas* and *Chromatium* (Tindall, 1988).

1.1.6 Marine Salterns

Not all hypersaline environments are naturally occurring. The commercial production of salt from seawater is performed using a marine saltern. This is a series of interconnected shallow ponds. Seawater is let into the first and retained while solar evaporation occurs. This process gives a series of increasingly concentrated brines in each of the pools. Fractional precipitation of the salts from the seawater occurs with the least soluble, calcium carbonate and calcium sulphate, precipitating first. These are followed by sodium chloride, which is then harvested, washed and dried in the sun (Larsen, 1980).

1.1.7 Hypersaline Soils

Hypersaline soils are distributed world-wide and are derived from mineral deposits and the evaporation to dryness of hypersaline bodies of water. However, there has been little work done on the distribution and constitution of hypersaline soils. They are also likely to increase as a burgeoning population demands greater food production which in many cases leads to excessive and poorly managed irrigation. The consequence of which is an increase in the salinity of soil which in turn inhibits microbial processes such as cellulolytic activity and nitrogen fixation (Ramos-Cormenzana, 1991). The effect of the latter is an inevitable decline in productivity of the vegetation.

1.2 Organisms Which Inhabit Saline Environments

The saline environment is a broad range of habitats which at one extreme include any biotope in which NaCl is present at a significant level, even if the concentrations are not sufficient to severely tax those organisms within it, to concentrated brines which support only the most specialised halophilic life-forms. As the salinity of a biotope increases it becomes an extreme environment, which has been defined as one in which the species diversity is reduced (Brock, 1979). Therefore, saline environments can range from those which have a rich diversity of species such as the marine habitat to those which may be virtual monocultures of extreme halophiles.

The salinity range which will be considered in this section is 2.5 to 30 % (w/v) salt i.e. 0.4 – 5.1 M NaCl. The types of organisms discussed will also be related to the specific examples of saline habitats outlined in section 1.1. The

concentration of salt in the habitat is described as a % w/v rather than molarity of NaCl as the former term encompasses all those salts which contribute to the water activity of the environment and not just specifically NaCl. This consideration is particularly important in athalassohaline environments where NaCl may not be the major salt present.

1.2.1 Sea Water to 10% (w/v) Salinities

A number of microorganisms can survive in seawater and the group of enteric bacteria have been particularly well studied.

Survival of enteric bacteria in seawater largely depends on their ability to overcome osmotic shock when entering the sea and to restore homeostasis at high osmolarity (Gauthier *et al.* 1987; Munro *et al.* 1987, 1989). When bacterial cells are exposed to high external osmolarity, they respond by increasing the cytoplasmic concentration of a limited number of solutes (Csonka, 1989).

In addition, coliforms and enteric pathogens can undergo morphological and physiological changes when starved in sea water and rapidly evolve toward a viable nonculturable state; they remain alive for some time and, at least in some cases, are capable of producing pathological processes when introduced into test animals (Roszak *et al*, 1984; Tamplin and Colwell, 1986).

Sediments can play an important role in survival of enteric bacteria in the marine environment. The presence of sediments increased *E. coli* survival, organic matter in the sediments provided not only nutrient sources, but also

solutes for osmoprotection (Gerba and McLeod, 1976). The same result observed with autoclaved sediments was attributed to the fact that cells took up significant amounts of glycine betaine (Ghoul *et al*, 1990).

The ability of enteric bacteria to adapt to fluctuations in the ambient osmolarity is of fundamental importance for their survival. In order to grow, bacterial cells must maintain a positive turgor, i.e., an outwardly directed pressure that is derived from maintaining cytoplasmic osmotic pressure at a point higher than that of the environment (Munro *et al*, 1989).

As salinity increases to a level greater than that seen in sea water (2.5%), there is a gradual reduction in the diversity of organisms inhabiting the biotope. In marine salterns the first pond in the series of increasing salt concentrations usually has a very high productivity. The primary producers being the green alga *Dunaliella viridis* in the plankton and cyanobacteria such as *Aphanothece halophytica*, *Spirulina* and *Oscillatoria* forming mats at the bottom of the ponds (Larson, 1980). The ditch weed *Rupia* and the macroscopic green alga *Enteromorpha* may also be present (Javor, 1983). Higher animals such as fish can still survive in these salinities but the number of species rapidly declines, and none are found in salinities greater than 13.5% (Hildebrand, 1958).

There are many other macroorganisms present at salinities between 3.5 and 10 %, including brine shrimp (*Artemia salina*), and the brine ciliate *Fabrea salina* (Larsen, 1980). Diatoms of genus *Navicula* are abundant in the mat

communities, and eubacteria of marine origin are common in such pools (Rodriguez-Valera, 1988). Ground water sources containing 8 % salt have also been investigated and shown to contain a variety of halotolerant bacteria belonging to the genus *Halomonas* (Vreeland and Huval, 1991).

1.2.2 10-30% (w/v) Salinities

As the salinity increases to above 10 %, there is a change in the types of organism, which are most commonly found, with genuinely halophilic organisms replacing those characteristic of less saline waters. The best example of this type of environment is the southern basin of the Great Salt Lake which has a salinity approaching 12%. In the Great Salt Lake the only macroorganisms found, are the brine fly (*Ephydra* spp.), and the brine shrimp (*Artemia salina*). This latter organism was once harvested commercially from the lake, but has declined in numbers in recent years, as the salinity in the southern basin has fallen, despite the fact that the shrimp can flourish in salinities lower than those presently found in the lake (Crogham, 1958). The primary producers in the southern basin of the lake are the algae *Dunaliella viridis* and *Coccochloris elebans,* the cyanobacterium *Oscillatoria* is also found on reef-like calcium carbonate deposits on the lake bed (Post, 1977).

In saltern ponds of a similar salinity, it was found that *Dunaliella spp* were the primary producers, and the mat communities of less saline ponds disappeared (Rodriguez-Valera, 1988). This is contradicted by Javor and Porta (1991), who describe microbial mats of very similar composition to those mentioned above in saltern ponds of 17.8 % salinity. These mats also contained bacteria such as the filamentous *Beggiatoa*, and *Achromatium*. In

the Gavish Sabkha, cyanobacterial mats are also found, these mats were comprised of a variety of species (Erlich and Dor, 1985), the composition of which displayed zonation depending on the salinity which varies widely from 12 to 30 % in the sabkha (Gerdes *et. al.*, 1985a). The predominant heterotrophic bacteria in these salinities are moderately halophilic species belonging to the genera *Vibrio, Pseudomonas, Flavobacterium* and Gram positive cocci (Rodriguez-Valera *et. al.*, 1985). In the case of the Antarctic lakes of this salinity species of psychrotrophic *Halomonas* and *Flavobacterium* dominate (Franzmann 1991; Dobson *et. al.*, 1991).

When the salinity reaches a concentration of over 20 % there is an observable change in the composition of the saltern ponds, which change from a green colour to one of orange-red of increasing intensity and turbidity (Rodriguez-Valera, 1988). There is a sharp decrease in the number of species present, *Dunaliella salina* is the sole eukaryote present, at the bottom of the ponds a gypsum crust forms which contains and overlies stratified bands of photosynthetic bacteria such as *Ectothiorhodospira*, *Rhodospirillum* and the cyanobacterium *Spirulina* (Rodriguez-Valera *et. al.*, 1985). Beneath this crust there is also anaerobic life of unknown provenance.

The eubacteria are replaced at about 25 % salinity by increasing numbers of halophiles belonging to the archaea (formerly archaebacteria) (Rodriguez-Valera, 1986), but halophilic eubacteria such as *Halomonas elongata* can still be isolated (Vreeland and Martin, 1980). However in most cases archaea dominate, for example the Antarctic Deep Lake (salinity 25 %) has few

species present the most abundant being *Halobacterium* (now *Halorubrum*) *lacusprofundi*, the only species of *Halobacteriaceae* to grow below 10 °C (Franzmann, 1991; Kamekura, 1998).

1.2.3 Above 30% (w/v) Salinities

In waters of salinities in excess of 30 %, such as the Dead Sea, the northern basin of the Great Salt Lake, and the most concentrated ponds in salterns, there are very few species, although the biomass can be quite high. The northern basin of the Great Salt Lake has two primary producers, the major one is the alga *Dunaliella salina* which is planktonic, *Dunaliella viridis* is also present but usually attached to a substrate (Larson, 1980). There are also cyanobacteria present of unspecified species, however, their contribution to the lake productivity can only be minimal (Post, 1977). The abundance of *Halobacterium salinarium*, and *Halococcus* species produces a red colour in the brine, sulphate reducing bacteria have also been found (Larson, 1980). The other organisms which have been found in this habitat are protozoa, brine shrimp, brine flies and bacteriophages specific to halobacteria (Post, 1977).

The Gavish Sabkha also contains a diverse range of halobacteria including *Halobacterium halobium* in its most saline areas (Stoeckenius *et. al.*, 1985). There are also present the so called square bacteria (Walsby, 1980), and stalked bacteria of the genus *Pseudomicrobium* (Kessel *et. al.*, 1985). The Dead Sea has a very similar flora, the upper 80 m of the lake are aerobic and *Dunaliella viridis* is the dominating alga (Larsen, 1980), *Dunaliella parva* is also present and occasionally produces blooms when a spate of the river

Jordan gives rise to a layer of less saline brine on the surface of the sea (Oren and Shilo, 1982). *Halococcus, Halobacterium marismortuii* and *Haloferax volcanii* are also present, the latter two species being most common, however halobacterial counts show only a tenth of the biomass that is observed in the Great Salt Lake (Larsen, 1980). In the lower anoxic region of the Dead Sea, sulphate reduction is apparent which suggests the presence of sulphate reducing bacteria.

1.3 The Availability of Nutrients In Saline Environments

Saline environments have a very high productivity which can be noticed by the pink colour found in waters caused by the blooms of algae and bacteria. Large amounts of nutrients must be present to support such prolific growth, although under certain circumstances some inorganic ions do become limiting. Precipitation of Mg^{2+} and Ca^{2+} salts out of solution is found in extremely high salinities, which reduces the population size of certain organisms.

100 mg l^{-1} of soluble organic matter has been found in the Great Salt Lake, much of this was found in the form of brine shrimp pupal cases, dead shrimps and egg masses. The main primary producers are algae, which provide food for the bacteria in the form of excretion products and autolysates. The brine shrimps graze directly on the algae. Nitrogen seems to be the limiting element in this ecosystem. Ammonia, which is utilised directly by the algae and then reconverted to ammonia by organotrophs in a very simple nitrogen cycle, is the main source of inorganic nitrogen. Since no nitrogen fixation, nitrification or denitrification has been detected, the route

considered above is the most likely. Sulphate is abundant in the lake (Larsen, 1980).

Regarding the Dead Sea, much lower levels of dissolved organic matter are found, around 10 mg l^{-1}. As mentioned before, algae are the main producers with their excretion products supplying the halobacteria with organic carbon, in addition to that brought by the river Jordan. The low phosphate level is most likely the main limiting factor for growth, ammonia and organic nitrogen are abundant (Larsen, 1980). Among the alkaline saline lakes there is the Wadi Natrun which has algae and cyanobacteria as the primary producers, such lakes often have a very high primary productivity due to the presence and predominance of phototrophic organisms. Decay of waterside vegetation can also provide organic matter (Larsen, 1980). Organic matter in marine salterns is very high in lower salinity pools and is provided by primary producers, and influx from sea water. The nutrients for growth in the higher salinity pools, where precipitation of calcium may be a limiting factor, are provided by the autolysis of the organisms (Larson, 1980). The seawater which is trapped by the biological community is the main source of nutrients for the Gavish Sabkha. Other sources for nutrients are the primary producers predominantly cyanobacteria and diatoms. Plant litter and camel dung also contribute to the total nutrient pool, as does detritus from dead organisms of the community (Gerdes *et. al.*, 1985b).

There is a limitation to the availability of nutrients caused by the stratification of saline waters as observed in the Dead Sea, where recovery of nutrients

from the lower level hypolimnion is very slow (Steinhorn and Assaf, 1980). However, this problem is alleviated in shallower lakes by the seasonal turnover of the water column.

1.4 The Terminology of Salt Adaptation

It is appropriate at this time to define the terms commonly used to describe the effects of high solute concentrations in an aqueous medium. A review of the literature reveals a variety of terms employed to describe the physical characteristics of highly saline water. These are often used merely as qualitative terms which has led to confusion and inaccuracy when strict definitions of such terminology are subsequently applied (see Brown, 1990). The terminology used in this work will be defined to clarify how the author employs them.

1.4.1 Water Activity (a_w)

A measure of the effective proportion of water in a solution is called water activity (Brown, 1990). It can be defined by the following equation:

$$\frac{P}{P_0} = \gamma_W N_W = a_W$$

where,

P = Vapour pressure of solution,

P_0 = Vapour pressure of pure solvent,

γ_w = Activity coefficient to compensate for deviation from ideality,

N_w = Moles of water.

In an ideal solvent the activity is 1, addition of solutes decrease the water activity. The advantages of using a_w to denote the status of aqueous systems are (1) its conceptual simplicity and lack of ambiguity; (2) its ease of manipulation both mathematically and experimentally. The disadvantages of a_w are its very limited application to a complex, physically heterogeneous environment, and its lack of predictive value for the direction of flow of water.

1.4.2 Water Potential

Water potential is a measure of the energy of water (Brown, 1990). Total potential can be defined by the formula:

$$\psi = \psi_s + \psi_p + \psi_m$$

where,

ψ_s = Water potential attributable to solutes in solution,

ψ_p = Water potential due to hydrostatic pressure,

ψ_m = Water potential due to capillarity of porous structures such as soil.

ψ_s is due to water activity of solution and related to it by the equation:

$\psi_s=(RT/V_w)\ln a_w$ (Csonka, 1989)

where,

R is the gas constant,

T is absolute temperature,

V_w is partial molar volume of solvent.

1.4.3 Osmotic Pressure

The osmotic pressure is equal to the hydrostatic pressure that must be applied to prevent the flow of water from pure solvent into a solution through

a perfect semi-permeable membrane (Brown, 1990). Osmotic pressure is defined by the equation:

$$\pi = \frac{-RT \ln a_w}{V_w}$$

1.4.4 Osmolality

Solutions can also be specified by their solute status which, in practice, means the effective concentrations of all solutes. The measure of concentration commonly used for this purpose is osmolality (Brown, 1990).

1.4.5 Turgor Pressure

Turgor pressure (P) is equal to the difference between the external and internal osmotic potential:

$\psi_e = \psi_\pi + P$

$P = \psi_e - \psi_\pi$

Where,

ψ_e is the water potential of extracellular fluid,

ψ_π is the osmotic potential (Brown, 1990)

1.4.6 Osmoregulation

Osmoregulation can be defined as the maintenance of turgor pressure and/or cell volume within limits necessary for growth and multiplication of an organism (Brown *et. al.*, 1986).

1.5 Osmotic Stress and the Role of Compatible Solutes

Most living cells have to adapt, within limits, to fluctuation in the osmotic strength of their milieu, their ability to proliferate in a given habitat is strictly

dependent on their ability to maintain an internal osmotic pressure higher than that of the surrounding medium (Jebbar *et. al.*, 1992). In order for an organism to survive and proliferate in conditions of low water potential (low a_w values) it must adopt one of two physiological strategies. Either, adaptation of the cells protein chemistry to high internal concentrations of salt (Foissy, 1974), or the adjustment of the intracellular osmotic potential using small molecular weight organic compounds (compatible solutes) which are not deleterious to cell function (Gilmour, 1990; Frings *et al.*, 1993).

Halobacteria of the domain Archaea (Lanyi, 1974) and anaerobic halophilic eubacteria (Rengpipat *et. al.*, 1988) accumulate inorganic ions like Na^+ or K^+. Most halophilic eubacteria (Severin *et. al.*, 1992) and some methanogenic archaea (Robertson *et. al.*, 1990) are able to synthesize and/or accumulate organic compatible solutes (e.g. glycine betaine and ectoine) in response to osmotic stress. The most extremely halophilic methanogens e.g. *Methanohalophilus* Z7302, accumulate molar concentrations of both K^+ and glycine betaine, and therefore appear to use both strategies (da Costa *et. al.*, 1998).

Compatible solutes are best described as organic osmolytes responsible for osmotic balance and at the same time compatible with cell metabolism (Galinski, 1993). The ability of bacteria to adapt to hyperosmotic conditions allows them to survive in a variety of hostile environments such as relatively dry surfaces or saline fluids (D'souza-Ault, *et. al.*, 1993). Adaptation to high

osmolality (osmoregulation) is generally accomplished by the intracellular accumulation of compatible solutes (Yancey *et. al.*, 1982).

1.5.1 The Diversity of Compatible Solutes

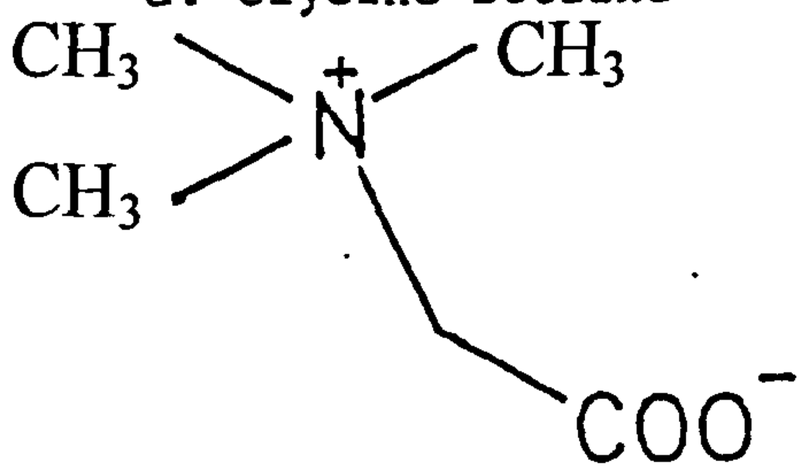
The most important compatible solutes found in bacteria can be defined as those that are accumulated to intracellular concentrations well above 0.5 M. These compatible solutes are further characterized below: (Figure 1.1)

a) KCl:

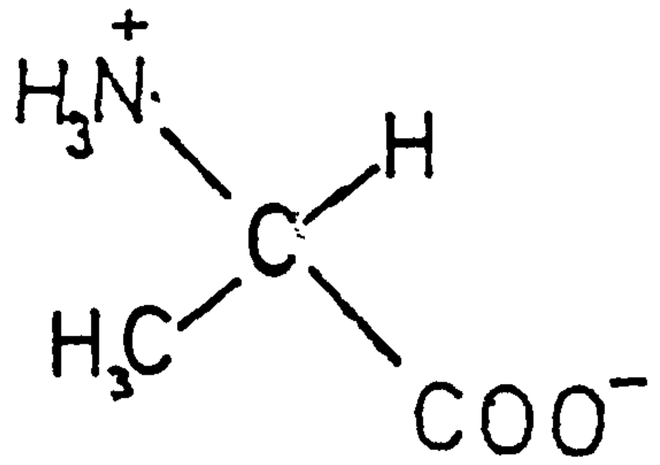
In many enteric and related bacteria, which are not halophilic, K^+ accumulation allows growth in salinities up to 0.5 M NaCl (Epstein, 1986). In enteric bacteria, the excess positive charge which results from the accumulation of K^+ is balanced by glutamate and derivatives (da Costa *et. al.*, 1998). The upper limit for cytoplasmic glutamate concentration appears to be about 0.5 M (Welsh *et. al.*, 1991).

In contrast, the halophilic *archaea* belonging to the family *Halobacteriaceae* use Cl^- ions to counterbalance the massive accumulation of K^+ which allows them to grow at salinities of 5 M NaCl (Gilmour, 1990). Typical internal salt concentrations for *Halobacterium* spp. growing at 5 M NaCl are 4 M KCl plus 0.7 M NaCl (Ginzburg *et. al.*, 1971). To cope with this level of intracellular salt, the internal components of the *Halobacteriaceae* have become dependent on high salinity (Gilmour, 1990).

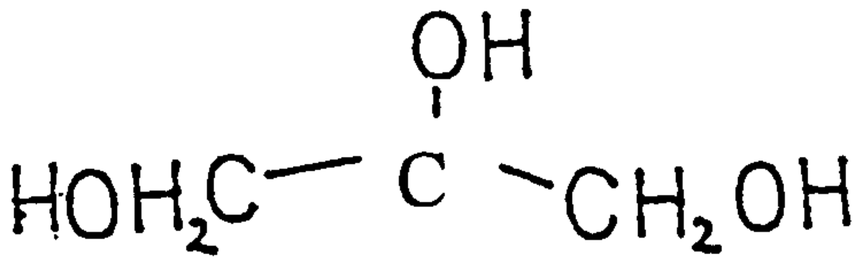
a. Glycine Betaine



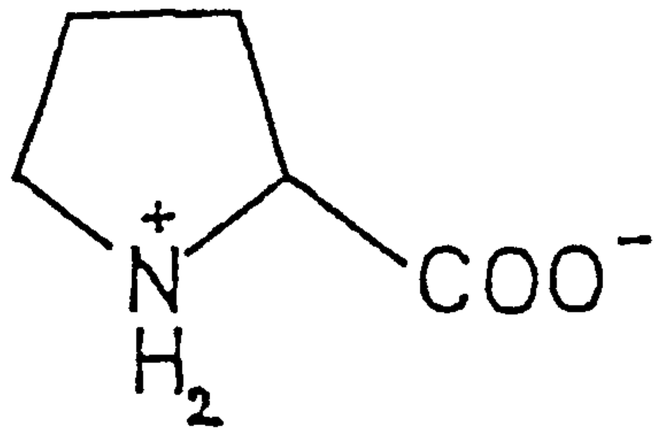
b. Alanine



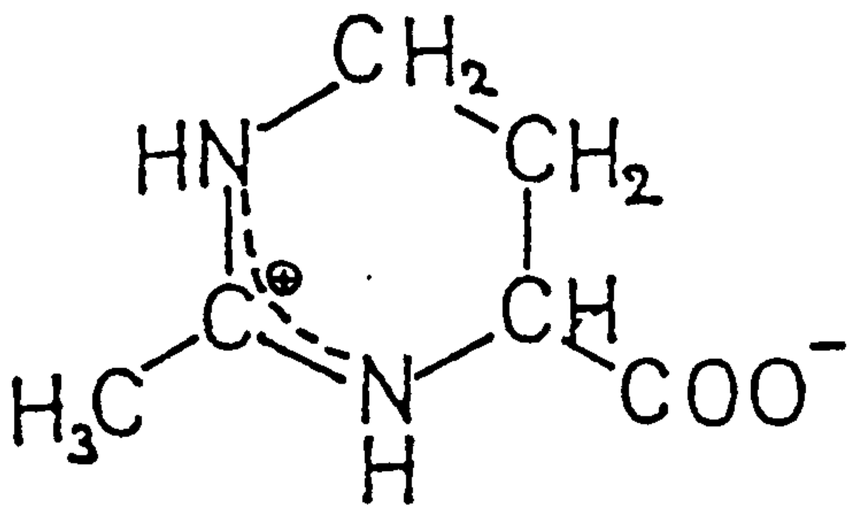
c. Glycerol



d. Proline



e. Ectoine



f. Glutamate

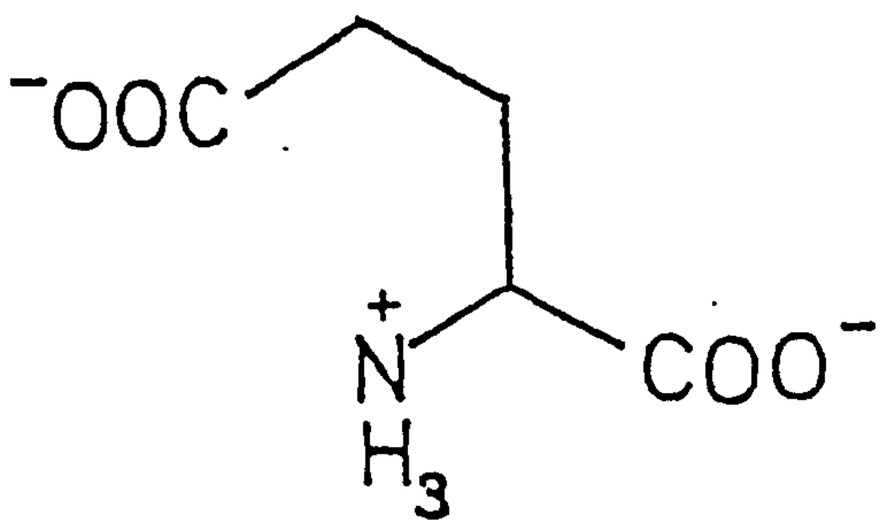
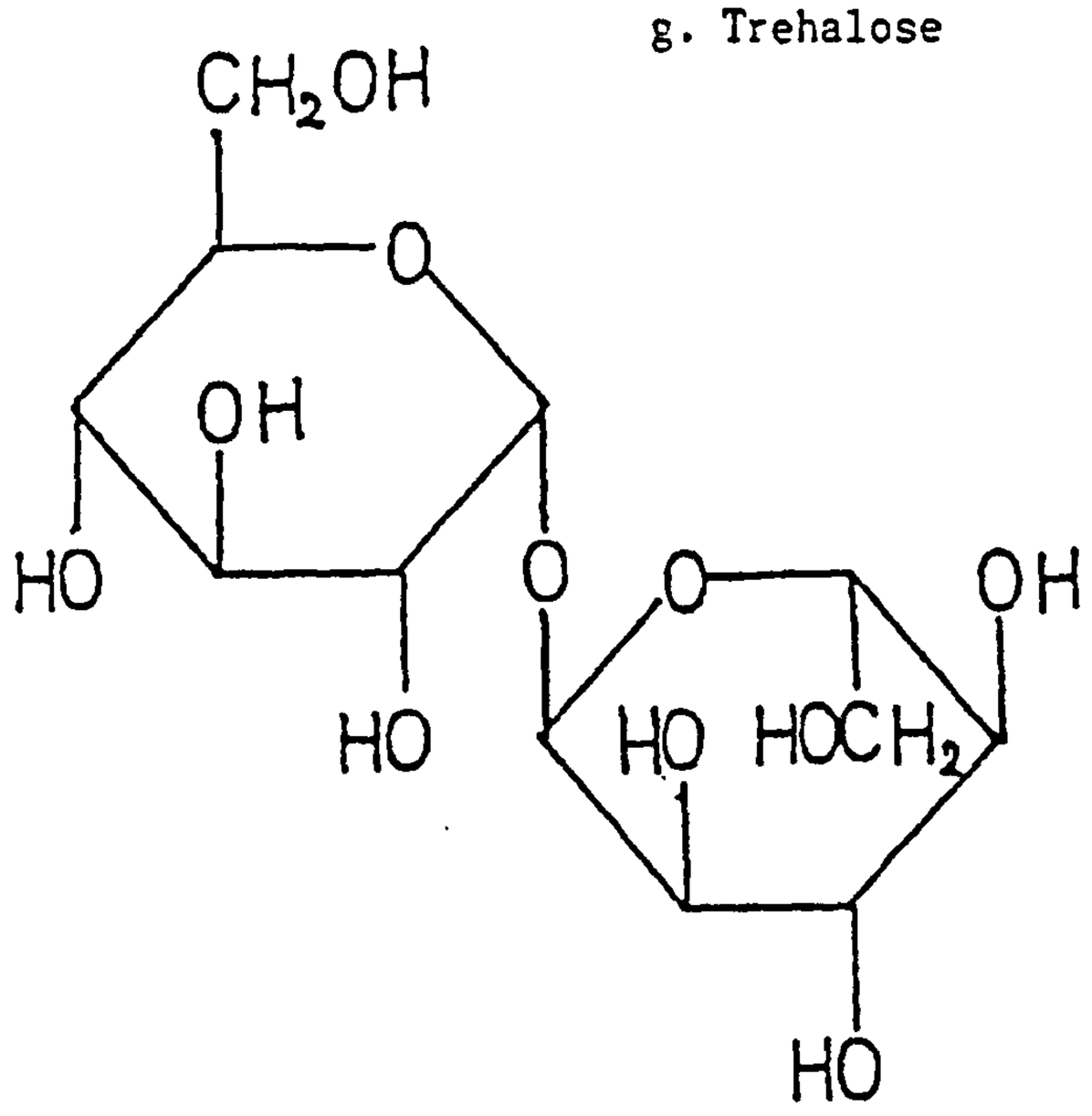
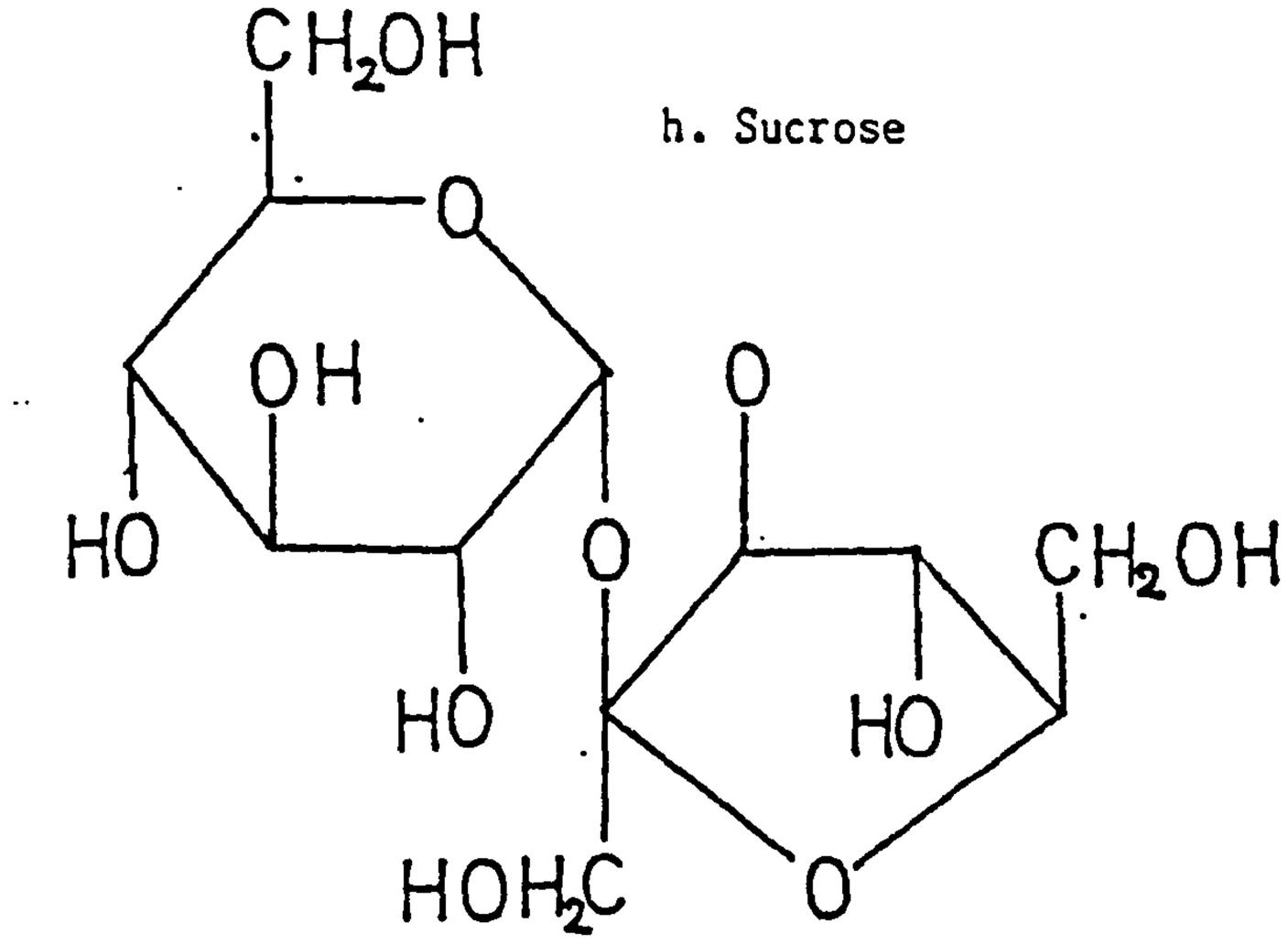


Fig (1.1) The diversity of compatible solutes.

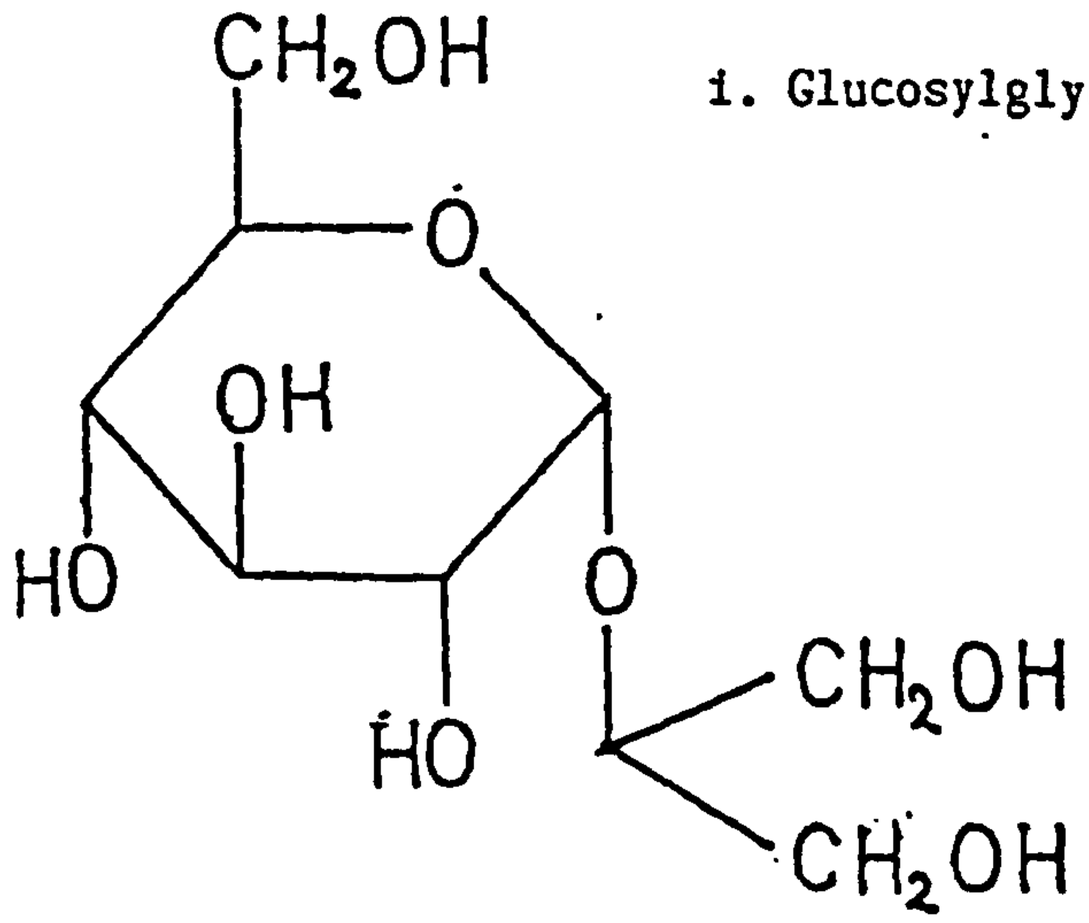
g. Trehalose



h. Sucrose



i. Glucosylglycerol



b) Glycine betaine:

Glycine betaine (normally called betaine) was recognized in the early 1980s as the most important organic osmotic solute in photosynthetic purple bacteria and halophilic cyanobacteria (Ventosa *et. al.*, 1998). Glycine betaine is the typical product of phototrophic eubacteria, especially those displaying a high salt tolerance (Imhoff, 1986) and it is also found as a primary product in halophilic archaebacterial methanogens (Robertson *et. al.*, 1990; Lai *et. al.*, 1991). However, the ability to synthesize betaine *de novo* is rare among aerobic heterotrophic eubacteria (Galinski, 1993). With the possible exception of the actinomycete *Actinopolyspora halophila*, none of the halophilic or halotolerant heterotrophic bacteria seem to be able to synthesize the compound *de novo* (Galinski, 1993). However, if choline is provided in the medium, this can be transported into the cell and converted to glycine betaine by the pathway shown in Figure 1.2. Certain halophilic bacteria can use glycine betaine not only as an osmotic stabilizer but also as a carbon and energy source (Ventosa *et. al.*, 1998). This was shown by Cummings and Gilmour (1995) who demonstrated that a *Halomonas* species could grow in salinities up to 2 M NaCl with glycine betaine as its sole carbon source. Growth on glycine betaine was not possible above 2 M, because of the need to use glycine betaine as a compatible solute.

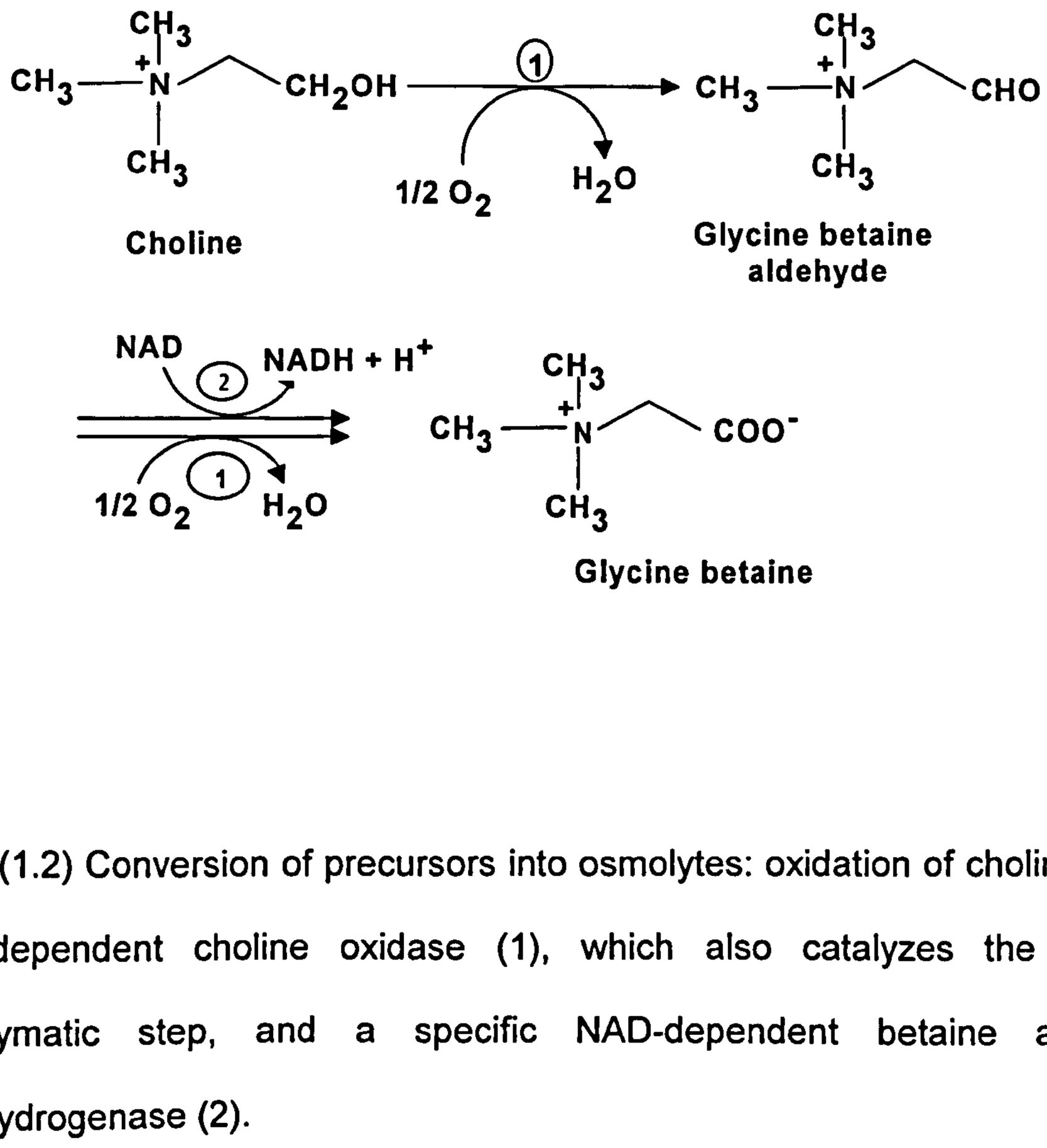


Fig (1.2) Conversion of precursors into osmolytes: oxidation of choline by an O_2-dependent choline oxidase (1), which also catalyzes the second enzymatic step, and a specific NAD-dependent betaine aldehyde dehydrogenase (2).

c) Ectoines:

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) was discovered as a minor component in the phototrophic sulphur bacterium *Ectothiorhodospira halochloris* (Galinski, *et. al.*, 1985). It had escaped detection because of the delocalisation of electrons which meant that it is not detectable by standard amino acid analysis (Figure 1.1e and da Costa *et. al.*, 1998). This solute is without doubt the most abundant osmolyte of aerobic chemoheterotrophic eubacteria and has been found in all halophilic/halotolerant proteobacteria of the γ-sub-division. It seems to be of importance in Gram-positive eubacteria such as *Nocardiopsis* species, some brevibacteria, *Marinococcus* species and others (Severin *et. al.*, 1992; Frings *et. al.*, 1993). Ectoine was found to be the main compatible solute in *Volcaniella eurihalina* and *Deleya salina,* when these organisms were grown in glucose-mineral medium (del Moral *et. al.*, 1994). Ectoine was also found in high concentrations in different *Halomonas* species and is the dominant solute in cells grown in defined medium lacking glycine betaine or its precursor, choline (Canovas *et. al.*, 1997; Wohlfarth *et. al.*, 1990). When glycine betaine was the sole carbon source for a *Halomonas* species grown at 2 M NaCl, ectoine gradually replaced glycine betaine as the main compatible solute as the culture entered stationary phase (Cummings and Gilmour, 1995). Only three enzymatic steps are required to synthesise ectoine from aspartic β-semialdehyde, which is an intermediate in the lysine biosynthesis pathway (Figure 1.3).

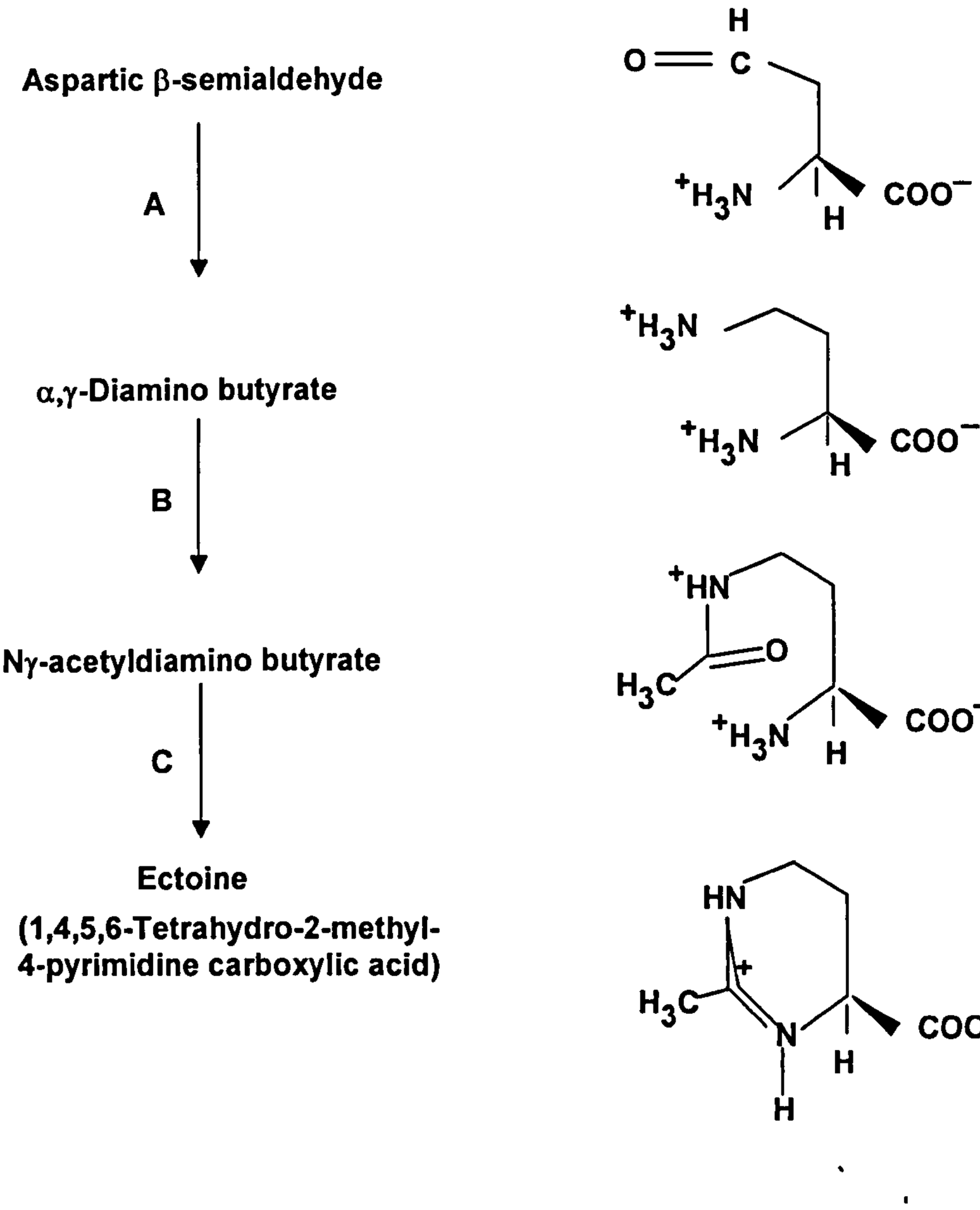


Fig (1.3) Biosynthesis of ectoine. 2,4-diaminobutyrate transaminase (A), 2,4-diaminobutyrate acetylase (B), and Nγ-acetyldiaminobutyrate dehydratase (ectoine synthase) (C).

Ectoines have also been found in non-halophilic antibiotic producing streptomycetes. Da Costa *et al* (1998) have speculated that ectoine may protect the streptomycete strain from its own anti-microbial product (actinomycin D).

d) Proline:

This compatible solute has been found in a number of halophilic algae, mostly Bacillariophyceae (Wegmann, 1986), some halophytic plants (Larher, 1988), and marine invertebrates (Schoffeniels and Gilles, 1970). Proline was originally regarded as the typical osmolyte of halophilic *Bacillus* species, a view primarily based on investigation into *B. subtilis* and closely related species. It was only shown later that the majority of halophilic/halotolerant bacillus-type organisms produce ectoine, either alone or in combination with proline and/or acetylated diamino acids (Deuch, 1994). Further proline producers are found among *Staphylococcus* and *Salinococcus* species. The fact that these organisms are typically moderately halophilic or halotolerant appears to support the view that proline is a solute of lesser compatibility, at least at extreme salt concentrations (da Costa *et. al.*, 1998). The proline homologue, pipecolic acid has been described as an osmolyte in *Corynebacterium ammoniagenes* (renamed *Corynebacterium glutamicum*) (Gouesbet *et. al.*, 1992).

e) N-acetylated diamino acids:

Diamino acids such as ornithine and lysine convert positively charged amino acids into neutral zwitterionic solutes and thus into more compatible osmolytes (da Costa *et. al.*, 1998). The role of Nδ-acetyl-ornithine in osmoadaptation was first documented with strain M96/12b (Wohlfarth *et. al.*, 1993), which is related to typical bacillus-type organisms. Subsequently, this compatible solute was also detected, at least, in minor amounts, in almost all *Bacillus* species under investigation, and also in *Sporosarcina halophila* and *Planococcus citreus* (Severin *et. al.*, 1992). Similarly, the homologous Nε-acetyl-lysine originally isolated and identified from *Sporosarcina halophila*, was also shown to be relatively widespread among bacilli and related organisms (del Moral *et. al.*, 1994). It has also been shown to be a major compatible solute in the methanogen *Methanosarcina thermophila* (Sowers and Gunsalus, 1995).

f) N-derivatized glutamine amides:

This very unusual class of compounds has amidated glutamine as a common structural characteristic (Galinski, 1993). A novel representative of this class of compatible solutes, N-carbamoyl-L-glutamine-1-amide (CGA), has so far only been found in the phototrophic bacterium *Ectothiorhodospira marismortui,* where it amounts to as much as 30% of the solute pool (Galinski and Lippert, 1991). The cytoplasmic concentration reaches more than 0.5 M and it certainly serves an important function as an osmolyte (Oren *et. al.*, 1991). Nα-acetylglutaminylglutamine amide (AGGA) is another

representative of this class of osmolytes and so far the only neutral dipeptide of osmotic function (Galinski, 1993).

g) Sugars and Polyols:

The disaccharide trehalose is very often found in halotolerant or marine organisms (both prokaryotic and eukaryotic) at concentrations of 0.5 M. It is clear that trehalose does alleviate salt stress in these organisms, but the most common view is that trehalose is primarily a stress metabolite associated with unfavourable growth conditions in general and is not specific to salt stress (da Costa *et. al.*, 1998).

Polyols, such as glycerol, arabitol and inositol are typical compatible solutes of algae (Gilmour, 1990) and fungi (Hocking and Norton, 1983). However, their use by bacteria is extremely rare with the best example being the accumulation of sorbitol by *Zymomonas mobilis*, which grows in sugary fruit saps (da Costa *et. al.*, 1998).

1.5.2 The Mode of Action of Compatible Solutes

The general use of only a small number of compounds as compatible solutes, not just by bacteria but also by higher forms of life from amoeba to man (Kinne, 1993), raises the question as to their underlying common principles (Timasheff, 1992). What makes these solutes compatible? From the information available today, three rules seem to emerge: compatible solutes are very soluble, they have no (net) charge and they do not interact with proteins. Compatible solutes are therefore more than just inert molecules because they stabilise protein structure and are excluded from

protein surfaces (Arakawa and Timasheff, 1985; Galinski, 1995). As a consequence, it is primarily free water which responds to osmotic changes of the environment, and that the compatibility of compatible solutes is based on the fact that they specifically adjust the osmotic equilibrium of the free-water fraction. Because of their exclusion from interfaces, they do not interfere with cellular function but at the same time they do restore cell volume (Galinski, 1995).

In contrast to other authors claiming beneficial/protective effects of compatible solutes on cellular macromolecules, Cayley *et al.* (1992) suggested that it is the replacement of other less compatible solutes and the subsequent increase in cytoplasmic volume that is responsible for their beneficial effect. Based on a remarkable correlation between growth rate and cytoplasmic volume (irrespective of its composition), Cayley *et al.* (1992) postulate that the free cytoplasmic volume (unbound water) is the fundamental determinant of growth under hyperosmotic stress and that the secondary effect of volume increase by compatible solute accumulation is the key to their osmoprotective function (at least in *E. coli*).

1.6 The Diversity of Halophilic and Halotolerant Microorganisms

Any organism which can grow in the presence or absence of salt is a halotolerant organism (Gilmour, 1990). The response of microorganisms to salt can also be divided into two additional categories, moderate and extreme halophiles (Gilmour, 1990). However, Kushner (1985) suggested a more complex classification that includes: i) *non halophilic microorganisms* which

grow best in media containing less than 0.2 M (1% w/v) salt; some of them can tolerate high concentrations of salts and are called *halotolerant*; ii) *slight halophiles* which grow best in media with 0.2-0.5 M (1-3% w/v) salt; iii) *moderate halophiles* are those that grow best in media containing 0.5-2.5 M (3-15% w/v) salt; iv) *borderline extreme halophiles* which grow best in media with 1.5-4.0 M (9-23% w/v) salt, and v) *extreme halophiles* which grow best in media with 2.5-5.2 M (15-32% w/v) salt.

1.6.1 Heterotrophic Eubacteria

Halotolerant and moderately halophilic, heterotrophic eubacteria are a diverse group of microbes that share in common their eubacterial classification and their ability to live in high salt (Javor, 1989). The halophilic eubacteria are described as follows:

A) Gram-negative eubacteria

Vibrio. *V.costicola* may be the best known moderately halophilic bacterium because it has served as a model in studies on the effects of salt on nutrition, transport, protein turnover, lipids and osmoregulation (Javor, 1989). *Vibrio* species are common isolates from >10% (w/v) salinities in salterns (Rodriguez-Valera *et. al.*, 1985), but they constituted only 3% of the isolates from hypersaline soil (Rodriguez-Valera, 1986). Recently *Vibrio costicola* has been renamed *Salinivibrio costicola* (Ventosa *et. al.*, 1998).

Pseudomonas, *Alteromonas*, and *Alcaligenes.* These genera include the most common eubacteria isolated from solar salt ponds (Javor, 1989). They may represent nearly 60% of the aerobic heterotrophic eubacteria found in

plate counts in culture media made with brine of 25% (w/v) total dissolved salts (TDS) (Ventosa *et. al.*, 1982). Strains of *Pseudomonas* and *Alcaligenes* are also found in hypersaline soil (Quesada *et. al.*, 1983; Rodriguez-Valera, 1986). These strains all tolerate 20% (w/v) salt, while *P. halosaccharolytica* tolerates 25% (w/v) salt (Hiramatsu *et. al.*, 1980). A moderately halophilic *Pseudomonas* species was found to secrete a soluble protease that demonstrated maximum enzymatic activity in 18% (w/v) salt (VanQua *et. al.*, 1981).

Acinetobacter. Members of this genus have been isolated from hypersaline soils (Quesada *et. al.*, 1983) and solar ponds (Rodriguez-Valera *et. al.*, 1985) where they constitute a minor component in all salinities above 10% (w/v) TDS. A moderate halophilic strain isolated from sea sand produced two soluble amylases with maximum enzyme activity in 1-2 M NaCl (Onishi and Hidaka, 1978).

Deleya. *D. halophila* (now renamed *Halomonas halophila*) is a slightly halophilic rod isolated from hypersaline soils (Quesada *et. al.*, 1984). It grows optimally in 7.5% (w/v) marine salts but tolerates 2-30% (w/v) salinity. The optimum growth for this strain is at 30°-37°C and it is strict aerobe that can grow on a variety of carbon sources.

Chromobacterium. An organism called *Chromobacterium marismortui* was observed as a motile rod in samples from the Dead Sea, but its classification as a species of this genus is uncertain (Ventosa, 1988). It grew optimally in

12% (w/v) salt and produced blue-brown colonies. Strains of *Chromobacterium* were only isolated from brines containing > 25% (w/v) TDS (Rodriguez-Valera *et. al.*, 1985).

<u>*Flavobacterium.*</u> Moderately halophilic strains of *Flavobacterium* have been isolated from hypersaline soils (Quesada *et. al.*, 1983) and from solar salt ponds in > 10% (w/v) TDS brines (Rodriguez-Valera *et. al.*, 1985), where they constituted 8-12% of the total heterotrophic eubacterial isolates.

<u>*Halomonas.*</u> *H.elongata* is a halotolerant rod isolated from a solar saltern (Vreeland and Martin, 1980; Vreeland *et. al.*, 1980). It can grow as a facultative anaerobe by reducing nitrate or fermenting glucose. Growth between 20 and 40°C was recorded, but maximal salt tolerance was noted at 30°C. *H. halodurans*, another halotolerant species, was isolated from seawater (Herbert and Vreeland, 1987). *H. subglaciescola*, an isolate from a hypersaline Antarctic Lake, grows at 0°C and 25°C, while some strains can grow at -5°C (Franzmann *et. al.*, 1987). New species were isolated from Canada and from the Dead Sea, Israel, these two new species have been assigned the names *H. canadensis* and *H. israelensis* (Huval *et. al.*, 1995).

Four obligately anaerobic, heterotrophic eubacteria are known to be moderately halophilic. They are all Gram-negative rods that thrive by fermentation. Their 16S rRNA sequences show that they are all related to each other but are unrelated to any subgroup among the eubacteria (Oren, 1986b). These bacteria are: *Halobacteroides halobius* isolated from Dead

Sea sediment (Oren *et. al.*, 1984b), *Haloanaerobium praevaalens*, isolated from the Great Salt Lake, Utah (Zeikus *et. al.*, 1983), *Sporohalobacter lortetii and Sporohalobacter marismortui* isolated from Dead Sea sediments (Oren *et. al.*, 1987).

B) Gram-positive eubacteria

Micrococcus. Moderately halophilic species of *Micrococcus* include *M. varians*, *M. morrhuae*, and *M. halobius*. *M. varians* var *halophilus*., an isolate from soy sauce mash, produces an extracellular nuclease and an extracellular amylase in medium with 2.5-3.5 M NaCl (Kamekura and Onishi, 1978).

Paracoccus *P. halodenitrificans* was isolated from meat-curing brines (Robinson and Gibbons, 1952). It grew optimally in 4.4-8.8% (w/v) NaCl but tolerated 23.4% (w/v) NaCl. The presence of a low concentration of Ca^{2+} in the medium permitted cells to grow in 1% (w/v) salt (Takahashi and Gibbons, 1959).

Marinococcus and *Planococcus*. *M. halophilus* is a Gram-variable halophilic coccus that produces yellow colonies, motile and strictly aerobic (Novitsky and Kushner, 1976). At lower temperatures it can grow without NaCl, but requires 0.5 M NaCl at 25°C. *M. halophilus* and *Planococcus* sp. are frequently isolated from hypersaline soils and salt ponds (Ventosa *et. al.*, 1983). A halotolerant strain of *Planococcus* was isolated from soil from the Antarctic Dry Valley (Miller and Leschine, 1984).

Pediococcus. *P. halophilus* is a facultative anaerobe that apparently prefers organic-rich hypersaline habitats since it is associated with soy sauce production (Noda *et. al.*, 1980) and salted fish (Villar *et. al.*, 1985).

Staphylococcus. *S. epidermidis*, a halotolerant strain, was isolated as a contaminant in medium containing 25% (w/v) salt. It grows best in medium without NaCl (Komaratat and Kates, 1975). *S. aureus* can grow in salinities up to 3.5 M NaCl and this ability plays an important role in food poisoning outbreaks attributed to this organism (Kaenjak *et. al.*, 1993). The salt tolerance of *S. aureus* allows it to grow in foodstuffs with very low water activities (Townsend and Wilkinson, 1992).

Actinomycetes. *Actinopolyspora halophila* was described as a contaminant in unsterilized medium used to grow extreme halophiles (Gochnauer *et. al.*, 1975). *A. halophila* is essentially an extreme halophile since it requires > 10% NaCl for growth (Johnson and Lanthier, 1986).

Other bacteria. Many other moderately halophilic bacteria have been well studied. Some examples of these bacteria are a *Bacillus*-like, Gram-positive, spore-forming rod which was isolated from a landfill associated with an oil well, brine injection water, and anaerobic sewage sludge (Pfiffner *et. al.*, 1986). It grows anaerobically at up to 50°C in medium with 0% to 10-12% (w/v) salt. *Lactobacillus casei* grows in up to 9-12% (w/v) NaCl (Hegazi,

1984). *Sporosarcina halophila*, a Gram-positive coccus, has been isolated from several hypersaline soils and salt ponds (Ventosa *et. al.*, 1983).

1.6.2 Archaea

All known members of the *Archaea* thrive only in extreme environments: high salt, high temperature, or low redox potential (Javor, 1989). Most extremely halophilic organisms belong to the *Archaea* phylogenetic group. Six genera of non-alkalophilic halobacteria are currently accepted: *Halobacterium*, *Halococcus*, *Haloarcula*, *Haloferax*, *Halorubrum* and *Halobaculum* (Juez, 1988; Kamekura, 1998). Four genera (*Natronobacterium, Natronococcus, Natronomonas* and *Natrialba*) belong to alkaliphilic halophiles (Tindall *et. al.*, 1984; Kamekura, 1998).

In addition, halophilic methanogen isolates have been obtained from hypersaline marine stromatolites and microbial mats (Giani *et. al.*, 1984), solar salt ponds (Mathrani and Boone, 1985), Great Salt Lake, Utah, U.S.A. (Paterek and Smith, 1985), hypersaline Crimean Lagoons (Zhilina, 1986) and hypersaline lakes (Mathrani *et. al.*, 1988). These isolates included *Methanococcus* sp., *Methanosarcina* sp. and *Methanohalophilus* sp.

The discovery of marine, thermophilic, sulphate-reducing bacteria in a novel branch of the *Archaea* Kingdom (Stetter *et. al.*, 1987) could mean that halophilic sulphate reducers may be found. The existence of thermophilic *Archaea* in marine hot springs (Zillig *et. al.*, 1987) also suggests that halophilic extreme thermophiles may also be found.

1.6.3 Cyanobacteria

Cyanobacteria (blue-green algae) constitute a separate group of phototrophic eubacteria (Javor, 1989). Cyanobacteria are common inhabitants of extremely hypersaline habitats, both those of permanently high salinity and those in which evaporation dilution cycles cause broad fluctuations in salinity (Javor, 1989).

A halophilic cyanobacterium from Solar Lake (Sinai Peninsula), *Dactylococcopsis salina*, was described by Walsby *et. al.* (1983). It can grow in salinities of 5-20% (w/v) and at temperatures up to 45°C. It shows optimal growth at salinities between 7.5-15% (w/v) and it survives in 3% (w/v) salinity, but without growth. This species is unique to Solar Lake (Potts, 1980).

The cyanobacterial population of the Great Salt Lake, Utah, is largely confined to the more dilute sections of the southern basin with the exception of *Aphanotheca halophytica* and a species of *Phormidium* or *Oscillatoria* (Post, 1977). Davis (1978) identified coccoid (*Anacystis spp.*, *Aphanothece halophytica* [*Coccochloris elabens*], *Entophysalis*) and filamentous (*Oscillatoria*, *Schizothrix*, *Spirulina*, and *Porphyrosiphon*) forms. Other identified cyanobacteria include coccoid species similar to *Aphanocapsa* or *Coelospherium*, *Dactylococcopsis* or *Synechococcus*, and *Xenococcus* (Golubic, 1980) as well as filamentous species *Microcoleus chthonoplastes* and *Phormidium sp.* (Javor, 1983; Jorgensen and Des Marais, 1986).

1.6.4 Other Phototrophic Eubacteria

Several species of purple sulphur bacteria belonging to the genus *Ectothiorhodospira* are moderately halophilic (Galinski and Truper, 1982). *E. halochloris* is alkaliphilic and was isolated from a soda lake in the Kenyan Rift Valley (Galinski *et. al.*, 1985), whereas *E. marismortui*, which was isolated from a hypersaline sulphur spring on the shore of the Dead Sea, grows best at pH 7-8 (Oren *et. al.*, 1989).

1.6.5 Algae

The algae contain some of the most halotolerant species of eukaryotes known, which are able to survive in very high salinities. The majority of these extremely halotolerant algae belong to the genus *Dunaliella*, which are unicellular biflagellate green algae.

Dunaliella is nearly ubiquitous in hypersaline environments although its presence in the Dead Sea is becoming more sporadic due to increased salinity and limiting levels of phosphate (Oren and Shilo, 1982; Javor, 1983). The genus *Dunaliella* includes species such as, *D. viridis*, *D. parva*, *D. tertiolecta*, *D. minuta*, and *D. salina* (Javor, 1989). When the external NaCl concentration is increased, water flows from the cell causing a decrease in cell volume. This volume change triggers, by an as yet unknown mechanism, the synthesis of glycerol. The volume of the cell subsequently recovers to a value approaching the prestress level and growth recommences with a higher concentration of glycerol inside the cells (Gilmour, 1990). This response seems straight forward, however, the increasing salinity has been shown to severely inhibit photosynthesis, which means that glycerol must be synthesised from the storage product starch (Gilmour *et. al.*, 1982).

1.7 Aims of Project

The major aim of this research project was to compare the mechanism of salt tolerance in Gram positive and Gram negative bacteria. The Gram negative bacterium that was chosen was *Halomonas* strain "Halo", which was the subject of previous work in the laboratory (Cummings, 1991), and was already fairly well characterised. The Gram positive bacterium was a coccus isolated from a sample of soil from Weston Park next to the Department. This was subsequently identified as *Staphylococcus xylosus*.

A comparison of the salt tolerance of *Halomonas* Halo and *S. xylosus* is described in Chapter 3 and the role of compatible solutes in the salt tolerance of both bacteria is described in Chapter 4. Chapter 5 looks at the response of *Halomonas* Halo and *S. xylosus* to different levels of pH, and the interaction between pH and salt stress is examined. The final results chapter tests the hypothesis that higher levels of intracellular compatible solutes protect enzymes against high temperature.

CHAPTER 2

MATERIALS AND METHODS

2.1 Isolation and Selection of Organisms

Three strains of the Gram negative eubacterium *Halomonas* were available in the laboratory, *Halomonas* Halo, *Halomonas* Duncon, and *Halomonas* SPCI. Each strain was inoculated into *Halomonas* defined medium (HDM, see Table 2.1) at a range of salinities from 0.05 to 3.4 M NaCl to determine the most salt tolerant strain (see section 3.2.1). On the basis of this experiment, *Halomonas* Halo was used in all further experiments.

Strain JDP14 was isolated by Jonathan Parkes from Weston Park next to the Department. He isolated this strain from a sample of soil using L-Broth medium (LB, see Table 2.2) containing different amounts of NaCl (0.5, 1, 1.5, 2, and 3 M). He described this strain as a Gram-positive coccus, which grew well up to 3 M NaCl. This strain was subsequently identified as *Staphylococcus xylosus* (see section 3.2.2) and was used to allow a comparison to be made between salt tolerance in Gram-negative and Gram-positive bacteria.

Table 2.1: Composition of *Halomonas* Defined Medium (HDM)

Ingredient	
$Mg\ Cl_2\ 6H_2O$	5.29 g l^{-1}
KCl	0.75 g l^{-1}
Fe EDTA	0.018 g l^{-1}
$(NH_4)_2SO_4$	4.1 g l^{-1}
$K_2HPO_4\ 3H_2O$	1.32 g l^{-1}
Glucose	1.8 g l^{-1}
1 M Tris-HCl buffer pH 7	50 ml l^{-1}
NaCl	Different amounts 5.84 g l^{-1} (0.1 M), 29.2 g l^{-1} (0.5 M), 116.9 g l^{-1} (2.0 M) and 175.3 g l^{-1} (3.0 M)

Table 2.2 Composition of LB Medium

Ingredient	
Tryptone	10 g l^{-1}
Yeast extract	5 g l^{-1}
NaCl	5.84 g l^{-1} (0.1 M), 29.2 g l^{-1} (0.5 M), 116.9 g l^{-1} (2.0 M) and 175.3 g l^{-1} (3.0M)

Table 2.3 : List of ingredients of CDM as devised by Hussain *et al.* (1991)

Chemical	Weight	Chemical	Weight
Solution 1 Dissolved in 1400 ml of distilled water adjusted to pH 7.2			
$Na_2HPO_4.2H_2O$	140 g	L-Lysine	2 g
KH_2PO_4	60 g	L-Leucine	3 g
L-Aspartic Acid	3 g	L-Methionine	2 g
L-Alanine	2 g	L-Phenylalanine	2 g
L-Arginine	2 g	L-Proline	3 g
L-Cystine	1 g	L-Serine	2 g
Glycine	2 g	L-Threonine	3 g
L-Glutamine	3 g	L-Tryptophan	2 g
L-Histidine	2 g	L-Tyrosine	2 g
L-Isoleucine	3 g	L-Valine	3 g
Solution 2 dissolved in 400 ml of distilled water			
Biotin	0.4 mg	Pyridoxal	16 mg
Nicotinic Acid	8 mg	Pyridoxamine di HCl	16 mg
D-Pantothenic Acid	8 mg	Riboflavin	8 mg
Thiamine HCl	8 mg		
Solution 3 dissolved in 2000 ml of 0.1 M HCl			
Adenine Sulphate	800 mg	Guanine HCl	800 mg
Solution 4 Dissolved in 100 ml of 0.1 M HCl			
$CaCl_2.6H_2O$	1 g	$(NH_2)SO_4.FeSO_4.$ $6H_2O$	600 mg
$MnSO_4$	500 mg		
Solution 5 Dissolved in 2000 ml of distilled water			
Glucose	200 g	$MgSO_4.7H_2O$	10 g

Solutions 1, 3 and 4 are mixed and autoclaved together whereas solution 2 is filter sterilised and solution 5 is autoclaved separately and added after cooling.

2.2 Media and Growth Conditions

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

2.2.1 Media

2.2.1.1 *Halomonas* defined medium (HDM)

To produce liquid medium all the ingredients in Table 2.1 were added to 700 ml of distilled water and dissolved except K_2HPO_4 and glucose. The pH was adjusted to 7, the volume was made up to 990 ml with distilled water and the medium autoclaved. The K_2HPO_4 solution (26.4 g in 100 ml) and glucose solution (36 g in 100 ml) were prepared and autoclaved separately. 5 ml from each solution was added to the medium when cool. The medium was stored at room temperature. When required 1 g of yeast extract was added to the medium.

2.2.1.2 L broth (LB) (Miller, 1972)

LB was prepared by adding all the ingredients in Table 2.2 into the desired volume of distilled water. The pH was adjusted to 7 using NaOH and the medium was autoclaved. Oxoid Bacto agar (1.5 % [w/v]) was used for L agar (LA).

2.2.1.3 Chemically defined medium (CDM) (Hussain *et al.*, 1991)

A chemically defined medium (CDM) was used for the growth and ion transport experiments for both organisms. CDM is composed of 5 solutions which are mixed together (Table 2.3).

Solution 1 :

To produce solution 1 the ingredients as indicated in Table 2.3 were added to 1000 ml distilled water and dissolved. The volume was made up to 1400 with distilled water and autoclaved. Once cooled the solution was aliquoted into 70 ml fractions and autoclaved again.

Solution 2:

To produce solution 2 the ingredients as indicated in Table 2.3 were added to 300 ml of distilled water and dissolved. The volume was made up to 400 ml with distilled water. The solution was filter sterilised and stored in the refrigerator.

Solution 3:

Solution 3 was prepared by dissolving the ingredients as indicated in Table 2.3 in 1500 ml of 0.1 M HCl. The volume was made up to 2000 ml with 0.1 M HCl and autoclaved. Once cooled the solution was aliquoted into 100 ml fractions and autoclaved again.

Solution 4:

To produce solution 4 the ingredients as indicated in Table 2.3 were dissolved in 70 ml of 0.1 M HCl and the volume was made up to 100 ml.

Solution 5:

To prepare solution 5 the ingredients as indicated in Table 2.3 were dissolved in 1500 ml distilled water and the volume was made up to 2000 ml distilled water. The solution was aliquoted into 100 ml fractions and autoclaved.

To produce liquid CDM, the following ingredients were dissolved in distilled water to make a final volume of 880 ml and autoclaved.

70 ml of solution 1

50 ml of solution 3

1 ml of solution 4

NaCl - different amounts to give 0.1, 0.5, 2 and 3 M

100 ml of solution 5 and 20 ml of solution 2 were added separately to the medium when cool to give a final volume of 1000 ml. The medium was stored at room temperature.

2.2.2 Batch cultures

HDM + 1 g l^{-1} yeast extract was normally used for growing JDP14 and *Halomonas* in batch cultures, but for some experiments CDM or LB were used. For liquid cultures the cells were grown in cotton wool plugged sterile 250 ml conical flasks containing 100 ml medium. The flasks were placed on rotary shaker (250 rpm) at 30 °C and were normally incubated overnight. The inoculum was normally 1 ml from a culture grown overnight. Growth was monitored by measuring the increase in absorption at 660 nm against a distilled water blank using an LKB Ultraspec spectrophotometer.

2.2.3 Continuous culture

JDP14 was also grown in continuous culture using an LH 500 series fermenter (LH Fermentation Ltd., Stoke Poges, UK). All components of the chemostat were first sterilised in the autoclave and then assembled. HDM + 1 g l^{-1} yeast extract was used to grow the organism in continuous culture. The pH was set at 7.4 and a 100 ml culture of growing cells (HDM + 1 g l^{-1} yeast extract) was used to inoculute 850 ml of medium in the growth vessel.

Temperature was maintained at 30 °C with the combination of a heater and a cold finger, and the cells were constantly mixed at 500 rpm by an agitator. Air was bubbled through the culture at approximately 500 ml min^{-1}.

Fermenter culture was used to allow accurate control of the medium pH. This was achieved by the automatic addition of sterile 1 M NaOH or 1 M HCl, when the pH deviated from pre-set limits. The medium was sterilised in 10 litre batches, contained in glass reservoirs, by autoclaving for 30 min. at 121 °C. Once growth had commenced, medium in the reservoir was pumped into the culture vessel at a constant pre-determined rate to give a flow rate of 52.8 ml / hour (= a dilution rate of 0.056). Cell density was measured spectrophotometrically at 660 nm against distilled water blank. Cells were checked under the microscope every day for any sign of contamination.

2.2.4 Purification and maintenance of the organisms

For both bacteria, the purity was checked by streaking a sample on L broth agar or on nutrient agar (Oxoid, Basingstoke, U.K.) containing 0.5 M or 2 M NaCl. The plates were incubated at 30 °C for 36 hours. After incubation the plates were examined for colony morphology and a single colony was picked off using a sterile loop and streaked onto a second plate. Once this plate was grown individual colonies were aseptically removed and inoculated into 10 ml of HDM + 1 g l^{-1} yeast extract (see Table 2.1), and incubated aerobically at 30 °C for 24 hours. These cultures were then divided into 2.5 ml aliquots and sterile glycerol was added to a final concentration of 50 % v/v and they were frozen at –20 °C. When required the frozen cells were thawed

at room temperature and inoculated into 100 ml of HDM + 1 g l^{-1} yeast extract or CDM in 250 ml conical flask, and incubated overnight at 30 °C.

2.2.5 Effect of sodium concentration on growth

A number of batch cultures were set up in HDM +1 g l^{-1} yeast extract at pH 7 using 50 mM Tris/HCl buffer with different concentrations of NaCl (0.1, 0.5, 2 and 3 M). Flasks were inoculated with 1 ml of well grown cells (HDM +1 g l^{-1} yeast extract at pH 7) which had been washed twice with sodium free medium. They were incubated at 30 °C with shaking at 250 rpm. Growth was monitored by measuring the OD_{660} every 1.5 h against a distilled water blank. The growth curves were plotted against time, and the doubling time was calculated.

2.2.6 Effect of potassium concentration on growth

The same procedure described in section 2.2.5 was employed, but in this case KCl was added instead of NaCl to the medium.

2.2.7 Effect of sodium and potassium concentration on growth

HDM +1 g l^{-1} yeast extract was prepared with equal amounts of NaCl and KCl to make final concentrations of 0.1, 0.5, 2 and 3 M salt. Tris/HCl buffer (pH 7) was used at 50 mM. A number of batch cultures were set up and flasks were inoculated with 1 ml of well grown cells (HDM + 1 g l^{-1} yeast extract at pH 7) which had been washed twice with sodium free medium. They were incubated at 30 °C with shaking at 250 rpm. Growth was monitored by measuring the OD_{660} after 24 and 48 hours against a distilled water blank.

2.2.8 Effect of pH on growth

The effect of pH on the growth of *Halomonas* and JDP14 was examined using batch cultures. A number of flasks were set up with 100 ml of 0.5 M NaCl (HDM + 1 g l^{-1} yeast extract) containing 50 mM phthalic acid buffer (pH 5.5), 50 mM Tris/HCl buffer (pH 7) and 100 mM Tris/HCl (pH 8.5). Flasks were inoculated with 1 ml of well grown cells from a pH 7 culture and incubated at 30 °C with shaking at 250 rpm. Growth was monitored by measuring the OD_{660} every 1.5 h. The growth curves were plotted against time.

2.2.9 Effect of inhibitors on growth

A number of flasks were set up with 50 ml of 0.5 M NaCl (HDM + 1 g l^{-1} yeast extract) containing 250 μl of 20 mM CCCP or 20 mM monensin. Control samples contained the same volume of ethanol where appropriate. Flasks were inoculated with 1 ml of well grown cells from the same medium and incubated at 30 °C with shaking at 250 rpm. The optical density was measured every 1.5 h and growth curves were plotted against time.

2.3 Methods used to identify JDP14

The initial tests performed included:

(a) Gram staining.

(b) Motility.

(c) Catalase test.

(d) Oxidase test.

(e) A series of biochemical tests using an API STAPH kit.

2.3.1 Morphology

Gram stains were conducted on the culture (Kirkpatrick *et al*, 1993); and examined under a light microscope. To determine colony morphology the bacterium was streaked out on nutrient agar containing 0.5 M NaCl. After incubation for 36 hours the colonies were examined using a binocular microscope. The observations made include the colony diameter, pigmentation, elevation and margin, description of form and opacity. Electron microscope pictures were produced from the University of Sheffield Electron Microscope Unit and these images allowed a pictorial view to see the effects of different salinities on the morphology of this bacterium.

2.3.1.1 Electron Microscope Preparation

The samples were fixed in Karnovsky's fixative, (2% paraformaldehyde, 2.5% glutaraldehyde). The samples were then washed in sucrose (10%) in 0.1 M phosphate buffer. The samples were washed three times with 30 minutes intervals at 4 °C. Secondary fixation was carried out in 2% osmium tetroxide aqueous for 1 hour at room temperature.

Dehydration was through a graded series of ethanol:

75% ethanol for 15 mins.

95% ethanol for 15 mins.

100% ethanol for 15 mins.

100% ethanol for 15 mins.

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

All the above steps were carried out at room temperature.

The samples were then placed in an intermediate solvent, propylene oxide, for two changes of 15 minutes duration.

Infiltration was accomplished by placing the samples in a 50/50 mixture of propylene oxide/Araldite resin. The samples were left in this 50/50 mixture overnight at room temperature. The samples were left in full strength Araldite resin for 6-8 hours at room temperature after which they were embedded in fresh Araldite resin for 48 hours at 60 °C.

Ultrathin sections, approximately 70-90 nm thick, were cut on a Reichert Ultracut E ultramicrotome and stained for 15 minutes with 3% uranyl acetate in 50% ethanol followed by staining with Reynold's lead citrate for 2 minutes. The sections were examined using a Philips CM10 Transmission Microscope at an accelerating voltage of 80Kv. Electron micrographs were recorded on Kodak 4489 Electron Microscope film.

2.3.2 Motility

Motility was examined using hanging drop preparations under the light microscope (Allen and Baumann 1971).

2.3.3 Catalase test

Method used was a modified version of the one described by Cappuccino and Sherman (1983). In the presence of oxygen (O_2), most organisms will produce hydrogen peroxide (H_2O_2). This toxic compound is degraded to water and O_2 by the enzyme catalase. This test is often used to determine whether an organism can utilise O_2 because catalase is invariably present in aerobes but never in anaerobic or microaerophilic organisms. The experimental procedure was to add a solution of dilute hydrogen peroxide to

a smear of JDP14 cells on a glass slide or to a plate of JDP14 colonies. If catalase is present bubbles of O_2 will be observed.

2.3.4 Oxidase test

The method used was as described by Collins and Lyne (1985) with some modifications. The oxidase test was used to find out whether the isolate JDP14 possesses cytochrome aa_3 (cytochrome oxidase), which is the last enzyme in the electron transport chain of oxidative phosphorylation, and consists of two molecules of heme and two atoms of copper. In the test for cytochrome oxidase, the indicators are oxidised as they transfer electrons to cytochrome oxidase. The experimental procedure was to soak small pieces of filter-paper in 1 % (w/v) aqueous tetramethyl-p-phenylenediamine dihydrochloride. Then some colonies from a freshly grown plate culture were removed with platinum wire and rubbed on the filter paper. Cytochrome oxidase activity was present if a blue colour appeared within 10 seconds.

2.3.5 API STAPH kit

API STAPH consists of a strip containing dehydrated test substrates in individual microtubes. The tests are reconstituted by adding to each tube an aliquot of API STAPH medium that has been inoculated with JDP14. The strip is then incubated for 18 hours at 37 °C after which the results are read and interpreted with reference to the information contained in the manual. The identification is facilitated by the use of the APILAB software. This work was carried out at Sheffield University Medical School, Department of Medical Microbiology.

2.4 Measurement of Respiration Rate

Respiration rate was measured using a Clarke-type oxygen electrode (Hansatech Scientific Instruments, Kings Lynn, UK) with a Servoscribe 1 S potentiometric chart recorder as described by Delieu and Walker (1972). The electrode consists of platinum wire sealed in plastic as the cathode, and an anode of circular silver wire bathed in a saturated KCl solution. The electrodes were separated from the reaction mixture 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 a 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 was kept at a constant temperature (30 °C) by circulating water from a temperature-controlled water bath (Fig 2. 1).

The calibration of the oxygen electrode was achieved by setting the reading to zero, after the addition of a small amount of sodium dithionite to air saturated water. The dithionite reacts with dissolved O_2 and removes it from the solution as shown below.

$$Na_2S_2O_4 + O_2 + H_2O \longleftrightarrow NaHSO_4 + NaHSO_3.$$

After washing, air saturated water was added and this gives the 100% value. To calculate the respiration rate, you need to know the concentration of O_2 in air saturated water at 30 °C (0.23 μmole ml^{-1}) and the protein content of the bacterial suspension. Then the following equation is used.

$$O_2 \text{ uptake } (\mu \text{ mol } O_2 . mg^{-1} \text{ protein. } h^{-1}) = \frac{O_2 \text{ content of 2 ml medium at 30 °C}}{\text{range in chart units (0 - 100 \%)}} \times \frac{60}{\text{mg protein in sample}} \times \frac{\text{No of chart units change}}{\text{time (minutes)}}$$

To prepare cells for measurements in the oxygen electrode, 100 ml of cells (grown at pH 7 in HDM + 1 g l^{-1} yeast extract) were harvested by centrifugation at 5000 *g* for 10 minutes, and washed twice in the same medium used for measurement. The cells were normally concentrated twofold and the protein content was determined (section 2.5). 2 ml samples of concentrated cells were used each time for measurements in the oxygen electrode. When the effect of sodium or potassium concentrations on the rate of respiration was studied the same procedure was employed except that the washing step was carried out with sodium or potassium free medium. Then the cells were resuspended in different concentrations of sodium or potassium for the measurements in the oxygen electrode. The effect of the inhibitors CCCP (carbonyl cyanide m- chlorophenyl hydrazone) and monensin on the respiration rate was studied. In these experiments, 2 ml of the cells were added to the oxygen electrode and the respiration rate was monitored for a few minutes until stable, then different concentrations of CCCP or Monensin were added to the oxygen electrode using a Hamilton syringe.

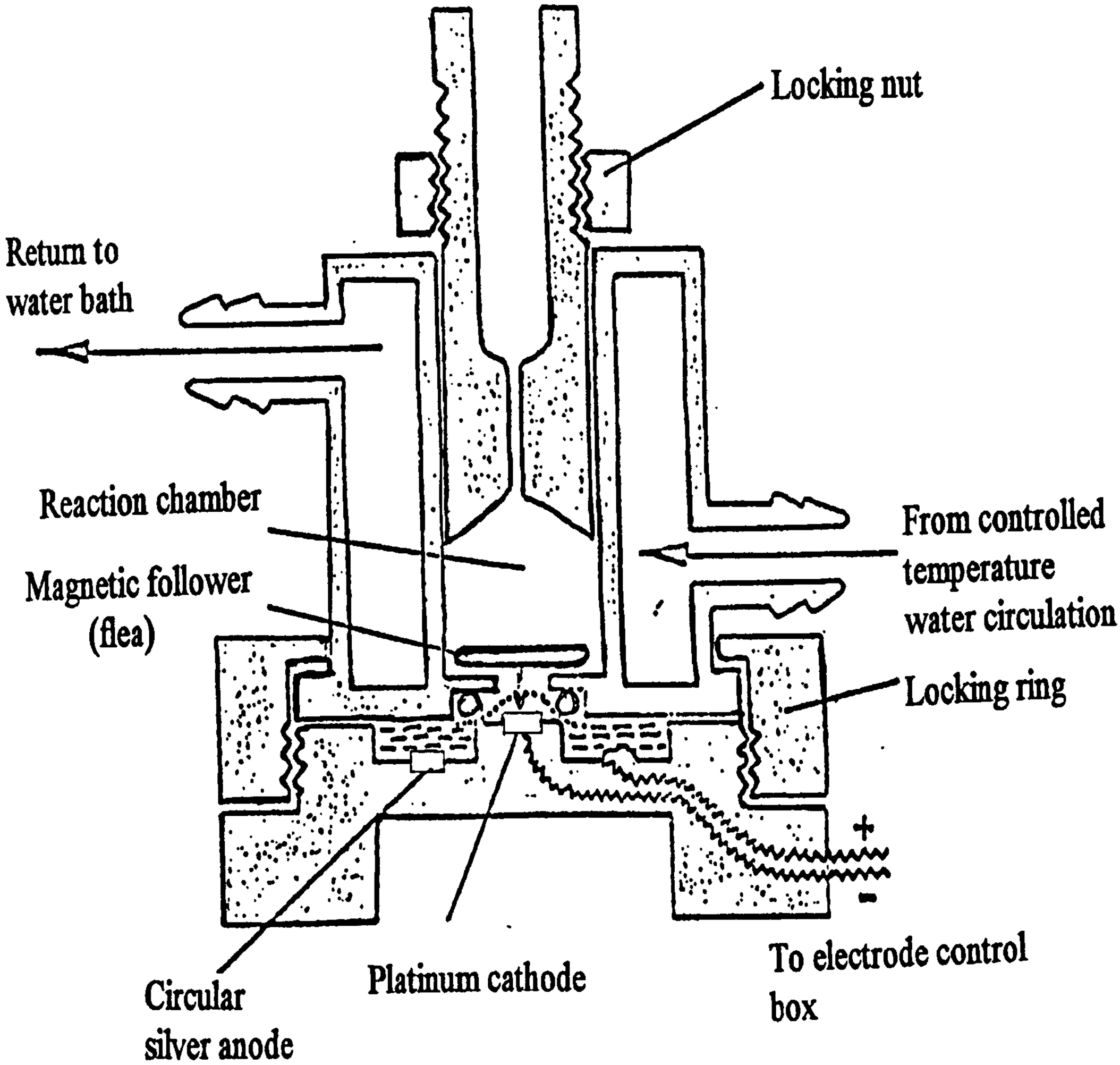


Figure 2.1: A diagram of the oxygen electrode

2.5 Protein determination

Soluble protein was measured using the method described by Bradford (1976). Bovine serum albumin (BSA) was used as the standard. Bradford reagent (from Sigma Chemical Company, UK.) containing Brilliant blue G, phosphoric acid and methanol was used.

To prepare samples for protein determination, 1 ml from both *Halomonas* and JDP14 cells were harvested by centrifugation at 4000 *g* for 15 minutes and resuspended in 50 mM Tris buffer pH 7.4. *Halomonas* cells were disrupted using an ultrasonic disintegrator (MSE Soniprep 150). The sonication vessel was surrounded by an ice-water mixture and the cells disrupted by sonication for 2 minutes, interspersed with 30 second periods of cooling to prevent local warming of the suspension. JDP14 cells were disrupted by adding 25 μl of lysostaphin enzyme (L 0761 from Sigma Chemical Company, UK) and 5 μl DNase (M610A – RQ1 from Promega Company, UK) and were incubated at 37 °C for 30 minutes. Cell debris of both bacteria was removed by centrifugation at 13,000 *g* for 10 minutes at 4 °C. The supernatant fluid was used for protein determination.

To carry out the assay, 0.1 ml of cell extract was added to a test tube. 3 ml of Bradford reagent was added, and the contents were thoroughly mixed. After 5 minutes incubation at room temperature the absorbency was measured at 595 nm against a water blank (3 ml reagent plus 0.1 ml water). The protein content of the sample was determined from a standard curve

obtained by plotting the absorbency of standard solutions containing 100 - 1400 μg protein ml^{-1} (see Appendix A).

2.6 Proline determination

Proline concentrations of 3% w/v TCA extracts of JDP14 cells were determined using the method of Bates *et al* (1973). Acid Ninhydrin Reagent was prepared by warming 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml of 6 M orthophosphoric acid, with agitation until dissolved. This reagent was stored at 4 °C and was used within 24 hours of preparation. 0.1 to 0.3 ml of each extract was reacted with 1 ml of acid ninhydrin and 1 ml of glacial acetic acid for 1 hour at 100 °C. The reaction was then terminated on ice and the reaction mixture was shaken with 2 ml of toluene. The mixtures were then centrifuged for about a minute to break the resultant emulsions. The concentration of the proline-ninhydrin complex was then estimated by measuring the optical density of the upper layer at 410 nm. A standard curve using 0 to 40 μg of proline per 1 ml of 3% TCA was prepared (Appendix B).

2.7 Growth of *Halomonas* Halo and JDP14 on CDM

A number of batch cultures were set up in CDM (Table 2.3) at pH 7 with different concentrations of NaCl (0.1, 0.5, 2 and 3 M). Flasks were inoculated with 1 ml of cells grown overnight. They were incubated at 30 °C with shaking at 250 rpm. Growth was monitored by measuring the OD_{660} every 1.5 h against a distilled water blank. The growth curves were plotted against time.

2.8 Effect of compatible solutes on growth at 3 M NaCl

To determine the effect of compatible solutes on bacterial growth in highly saline media, CDM was prepared containing 3 M NaCl. The compatible solutes to be investigated were added to 100 ml aliquots of this medium to give a final concentration of 20 mM. An inoculum of 1 ml of cells grown in 0.5 M NaCl CDM was then added, and the OD_{660} was monitored over the course of incubation at 30 °C with shaking at 250 rpm.

2.9 ^{14}C-Glycine Betaine Uptake Experiments

2.9.1 Manufacture of ^{14}C-glycine betaine

Synthesis of ^{14}C-glycine betaine from {^{14}C methyl} choline (Amersham, UK) was performed enzymatically using choline oxidase (from *Alcaligenes* sp., Sigma Chemical Co. Poole, Dorset, UK.), according to the method of Lanfald and Strom (1986). The reaction mixture contained 10 μM sodium phosphate pH 8, 6 units choline oxidase, and 530 kBq of ^{14}C-choline in a final volume of 290 μl. This mixture was incubated for 36 hours at 37 °C, and separated using one dimensional TLC (NH_3 : methanol, 1:3 v/v). The radioactive betaine, which migrated identically with an authentic betaine standard, was then scraped of the TLC plate (silica gel 60 F_{254} Merck, Germany), and resuspended in distilled water. The specific activity was assumed to be equal to that of the ^{14}C-choline stock solution. The percentage recovery of ^{14}C-betaine was determined by counting a 1μl aliquot of the sample derived from the TLC plate. This was multiplied by the volume of the betaine solution

to give the total dpm of the solution. Division by 6 x 10^4 gave the number of kBq in the solution (6 x 10^4 dpm = 1 kBq). This could then be used to calculate the percentage recovery of ^{14}C-betaine.

2.9.2 Measurement of betaine uptake

The uptake of ^{14}C-betaine was assayed in cells grown overnight at 0.5 or 3 M NaCl CDM (Table 2.3) in the presence or absence of 20 mM cold betaine. The cultures were then diluted by adding 25 ml from the cultures to 25 ml fresh medium with the same concentration of salt and incubated at 30 °C for 2 h until the OD had increased. The cells were then harvested and washed twice in carbon free CDM by centrifugation (5000 *g*, 15 min). The cells were then concentrated 20 fold in the wash medium and kept on ice. The transport assay used was an extensively modified version of the protocol of Thomson and MacLeod (1973). The assay medium was CDM with no carbon source. 2.5 ml of this medium was placed in the test tube. A 0.5 ml aliquot of the cell suspension was then added and the mixture was incubated for 5 min. The required concentration of cold betaine was added and 0.2 µCi of ^{14}C-betaine. The suspension was whirlimixed and 0.3 ml was removed and vacuum filtered through 0.45 µm nitrocellulose filters (Whatman, UK.). Subsequent samples were removed and filtered at appropriate time intervals and the filters were washed with 3 ml of CDM (no C present). The filters were then placed in 5 ml of scintillation fluid (Safe Fluor S, Lumac, The Netherlands) and counted in a scintillation counter (Beckmann LS1801) using a ^{14}C dpm programme.

2.9.3 Effect of inhibitors on ^{14}C-betaine transport

The effect of the ionophore CCCP (carbonyl cyanide m-chlorophenyl hydrazone), on ^{14}C-uptake was determined. The same procedure described in section 2.9.2 was employed, but in this experiment 15 μl of 20 mM CCCP were added to 3 ml cell suspension before adding cold betaine and ^{14}C-betaine and incubated at room temperature for 30 minutes (the final concentration of CCCP is 50 μM). The procedure was completed as described in section 2.9.2.

2.10 Silicone Oil Technique

Centrifugation through silicone oil was used to completely separate cells from medium (Gimmler and Schirling, 1978). A range of oils with different densities can be produced by mixing individual silicone oils with different densities. Cell suspensions of the density to be used in experiments (but with no radioisotopes 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 during centrifugation (Figure 2.2). In all experiments, Dow Corning 550 oil was used.

2.10.1 Intracellular volume (ICV) determination

Method was based on the one described by Rottenberg (1979). Two 1 ml samples of concentrated cells (0.3 - 0.8 mg protein ml^{-1}) were placed in 1.5 ml Eppendorf tubes. 11μl of $^{3}H_2O$ (1850 kBq ml^{-1}) were added to one sample of concentrated cells to give 20 kBq ml^{-1} and 20 μl of ^{14}C-dextran (MW = 70000, 740 kBq ml^{-1}) were added to the other sample to give 14.5 kBq ml^{-1},

both samples were carefully whirlimixed. After 5 - 15 minutes incubation at room temperature, triplicate samples (0.3 ml) were taken from each Eppendorf and layered onto 0.3 ml of silicone oil in fresh Eppendorf tubes. They were then centrifuged in a microfuge (MSE, Micro-Centaur) at 13,000 *g* for 2 min. From the aqueous supernatant fraction, triplicate samples (50 μl) were taken and added to 5 ml scintillation fluid (Safe Fluor S, Lumac, The Netherlands) in a scintillation vial and counted in a Beckman LS 1801 Liquid Scintillation Counter. The bottoms of the Eppendorf tubes, which contain the pellets of bacteria, were cut off within the oil layer and placed cut end down in Eppendorf tubes containing 300 μl distilled water. They were then centrifuged for 2 min to remove the pellets from the tips and the tips were discarded. The pellets were resuspended in the water and 300 μl samples were placed in scintillation vials containing 5 ml scintillation fluid and dispersed using a vortex agitator. The vials were counted in the liquid scintillation counter.

The $^{3}H_2O$ is evenly distributed throughout the pellet, whereas the ^{14}C-dextran is only found in the spaces between cells in the pellet (Figure 2.3). The pellet volume and the extracellular volume (ECV) were calculated from the ratio of $^{3}H_2O$ and ^{14}C-dextran, respectively, in the cell and supernatant fraction using the following equations.

$$\text{Pellet volume}\,(\mu\text{l}) = \frac{^{3}\text{H}_2\text{O dpm in pellet}}{^{3}\text{H}_2\text{O dpm in supernatant} \times 6} \times 300$$

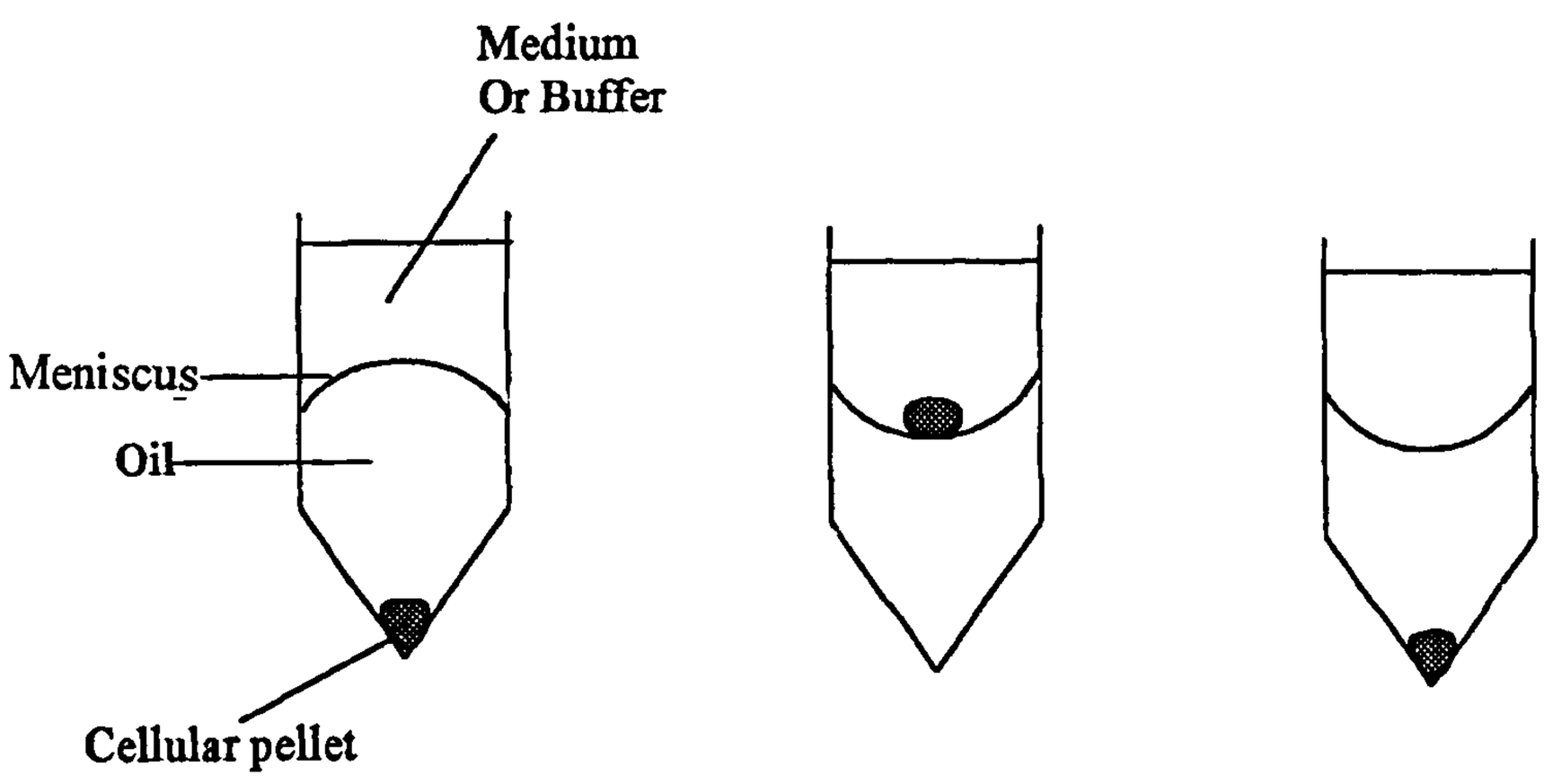


Figure 2.2: Silicone oil density selection for intracellular volume, membrane potential and internal pH determinations.

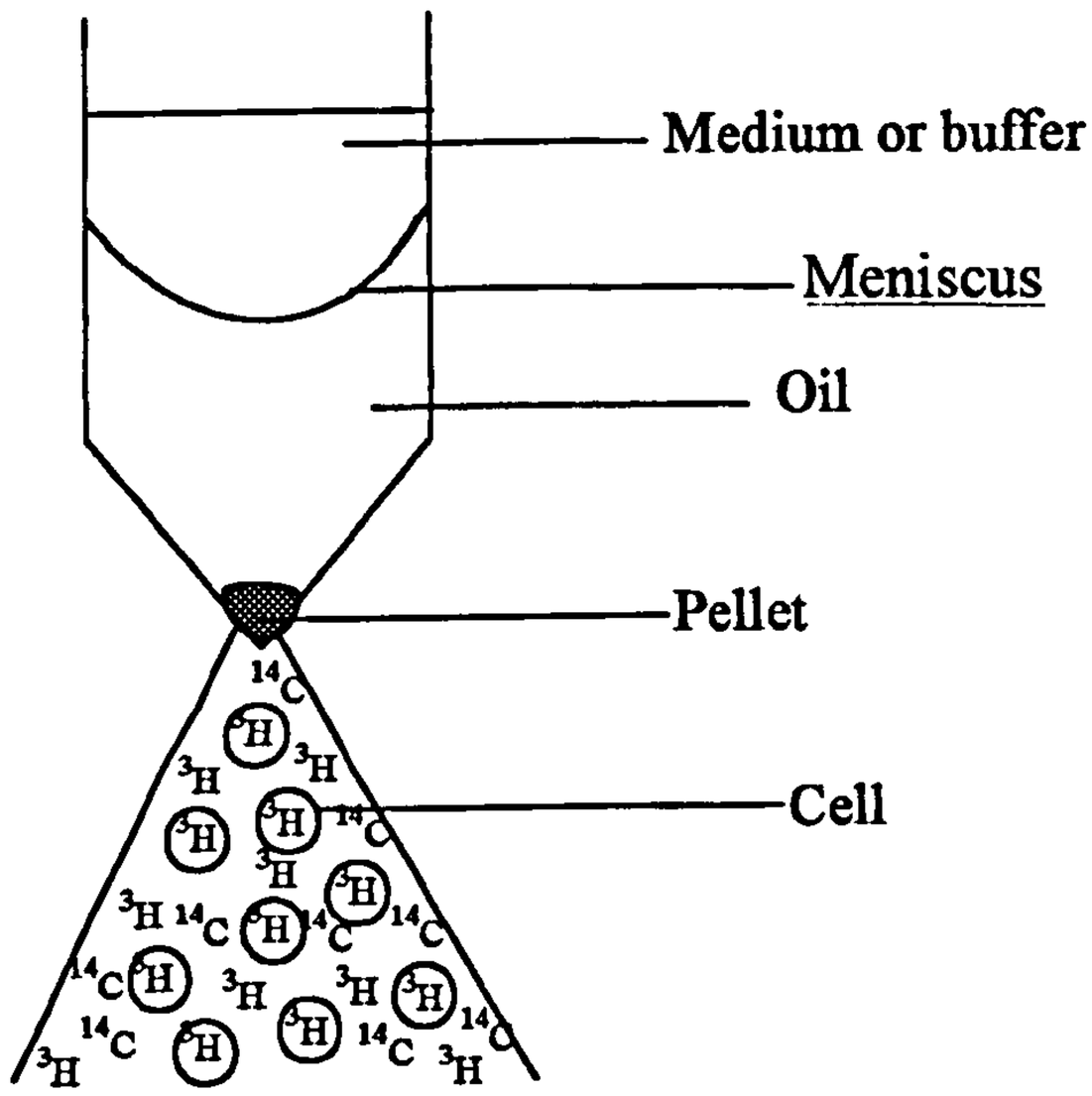


Figure 2.3: ICV determination using $^{3}H_2O$ and ^{14}C-dextran.

The $^{3}H_2O$ distributes throughout the pellet and ^{14}C-dextran is only found in the spaces between cells in the pellet.

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

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

2.10.2 Measurement of Membrane Potential ($\Delta\Psi$)

Method was as described by Rottenberg (1979; 1989).

The membrane potential was determined using ^{3}H-TPP^{+} (tetraphenylphosphonium) in an identical manner to ICV determination method (section 2.10.1).

The experimental procedure was as follows.

1. 5 µl of 800 kBq ^{3}H-TPP^{+} were added to 1 ml of concentrated cells to give a final concentration of 20 kBq ml^{-1} ^{3}H-TPP^{+}.

2. The cell suspension was mixed and incubated at room temperature for 30 min.

3. The cells were centrifuged through silicone oil as in section 2.10.1 and the same aliquots of supernatant and pellet were taken for counting.

The membrane potential was calculated as follows:

a) Dpm ^{3}H-TPP^{+} of supernatant was divided by 50 = dpm ^{3}H-TPP^{+} in 1 µl = **A**

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

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

d) Ratio of **C/A** = concentration of TPP^{+} inside the cells (a_i)/concentration of TPP^{+} outside cells (a_o).

Using the Nernst equation

$$\Delta\Psi(mV) = -\frac{RT}{ZF}\ln\frac{a_i}{a_o}$$

Where,

R = 8.3143 Joules mol^{-1} K^{-1}

T= 303 K (30 °C)

F= 96.487 Joules ml^{-l} mV^{-1}

Z= 1 (charge on ionic species)

At 30 °C and converting from ln to $\log_{10}$ (x 2.303)

$$\Delta\Psi\ (mV) = -58.8 \times 1og\ \frac{a_i}{a_o}$$

2.10.3 Effect of external pH

Cells were grown in batch culture at different pH values (5.5, 7 and 8.5), harvested by centrifugation, concentrated fivefold and resuspended in the same medium at the pH under examination. The ICV and membrane potential were then determined as described in sections 2.10.1 and 2.10.2.

2.10.4 Effect of inhibitors

Inhibitors were added to 1 ml samples of concentrated cell suspensions and incubated for 30 min before the addition of ^{3}H-TPP^{+}, ^{14}C-dextran and $^{3}H_2O$

to parallel samples. The inhibitors used were CCCP (50 μM) and monensin (50μM). Cell suspensions were then treated as described above

.2.10.5 Determination of Internal pH

The internal pH was measured using a weak acid or base (Rottenberg, 1979; 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. On the other hand a weak base (^{14}C-methylamine) was used, when the internal pH was lower than the external pH.

The silicone oil method used was identical to the one used for ICV (section 2.10.1) and membrane potential determination (section 2.10. 2), except that 5μl of 40 kBq 10μl^{-1} ^{14}C-methylamine or ^{14}C-benzoic acid were added to 1 ml cell suspension to give a final concentration of 20 kBq ml^{-1} and left for 30 minutes. The calculations were exactly the same as those used in section 2.10.2 to calculate the ratio a_i/a_o. To determine the internal pH from the a_i/a_o ratio calculated from ^{14}C-methylamine, two equations can be used.

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

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

If the pK is less than 1.5 units above pH outside cells then

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

pK of methylamine = 10.6

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

If pK of the weak acid is more than 1.5 units below pH outside, then the following equation is utilised.

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

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

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

pK of benzoic acid = 4.2

The equation used to calculate the proton motive force is:

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

See section 2.10.2 for definitions of R, T and F.

2.11 Effect of temperature and salt on enzyme activities of *Halomonas* Halo and JDP14

2.11.1 Preparation of cell free extract

Bacteria were grown in 0.5 and 2 M NaCl CDM (see Table 2.3) in batch culture. 20 ml from both *Halomonas* Halo and JDP14 cells were harvested by centrifugation at 4000 *g* for 15 minutes and resuspended in 50 mM Tris buffer pH 7.4 with twenty fold concentration. *Halomonas* Halo cells were disrupted using an ultrasonic disintegrator (MSE Soniprep 150). The sonication vessel was surrounded by an ice-water mixture and the cells disrupted by sonication for 2 minutes, interspersed with 30 second periods of cooling to prevent local warming of the suspension. The probe was used at a power setting of 25 microns (peak to peak). JDP14 cells were disrupted by adding 25 µl of

lysostaphin enzyme and 5 µl DNase and were incubated at 37 °C for 30 minutes. Cells debris for both bacteria were removed by centrifugation at 13,000 *g* for 10 minutes at 4 °C. The supernatant fluid obtained was referred to as the crude cell-free extract.

2.11.2 General assay conditions

Continuous assays of enzyme activity were carried out using a Philips PU 8625 UV/VIS spectrophotometer. All the components were incubated for 30 min at different temperatures (30 –70 °C) in a water bath, except the substrates. The temperature of the cuvettes was maintained at the temperature under examination. The absorbance was monitored and recorded using a linear chart recorder. In all assays the reaction rate was initially linear and proportional to the amount of extract present. Any activity measured before addition of the substrate was subtracted from the reaction rate.

2.11.3 Fumarase (EC 4.2.1.2)

Fumarate + H_2O ⟷ L - malate

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

The reaction mixture contained (values in ml):-

	Halomonas Halo		JDP14	
	0.5 M NaCl	2 M NaCl	0.5 M NaCl	2 M NaCl
50 mM Tris/HCl pH 7	1.5	1.5	1.5	1.5
50 mM L-malate (sodium salt)	0.1	0.1	0.2	0.2
Cell free exract	0.05	0.1	0.05	0.05
Water to final volume	3	3	3	3

The reaction was initiated by the addition of 0.1 or 0.2 ml L-malate, and the increase in absorption at 240 nm (with deuterium lamp and using quartz cuvettes) was recorded against a water blank. Enzyme activity is expressed as µmole fumarate produced min^{-1}.mg. $protein^{-1}$. The extinction coefficient of fumarate at 240 nm is 2.44 L. μmol^{-1}. cm^{-1}.

2.11.4 Malate dehydrogenase (EC 1.1.1.37)

Malate + NAD^+ ◄► Oxaloacetate + $NADH^+$ + H^+

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

The assay mixture contained (values in ml):

	Halomonas Halo		JDP14	
	0.5 M NaCl	2 M NaCl	0.5 M NaCl	2 M NaCl
50 mM Tris/HCl pH 7	1.5	1.5	1.5	1.5
1.5 mM NADH	0.1	0.1	0.1	0.1
7.5 mM Oxaloacetate pH 7.5	0.1	0.1	0.1	0.1
Cell free exract	0.02	0.02	0.05	0.05
Water to final volume	3	3	3	3

The reaction was started by the addition of oxaloacetate and the decrease in absorbance at 340 nm was followed against a water blank. Enzyme activity is expessed as μmole NADH oxidised. min^{-1}. mg $protein^{-1}$. The extinction coefficient of NADH at 340 nm is 6.22×10^3 L. $mole^{-1}$. cm^{-1}.

2.11.5 Acid Phosphatase

Method 1

This assay follows the method described by Gimmler *et al.* (1984) and was used to measure acid phosphatase activity in *Halomonas* Halo.

4-Nitrophenyl phosphate + $H_2O \rightarrow$ 4-nitrophenol + Pi

(pH 4.8 acid phosphatase enzyme) (Moss, 1984)

The reaction mixture contained (values in ml):-

	0.5 M NaCl *Halomonas*	2 M NaCl *Halomonas*
90 mM Citrate/NaCl pH 4.8	1.5	1.5
20 mM 4-nitrophenylphosphate	0.2	0.2
Cell free extract	0.2	0.2
Water to final volume	3.0	3.0

The reaction mixture was incubated at different temperatures for 30 minutes. The spectrophotometer was blanked at 405 nm with water and an increase in absorption was measured against the control to which no 4-nitrophenylphosphate had been added before incubation. The enzyme activity was expressed as nmol 4-nitrophenol produced min^{-1} mg $protein^{-1}$. The extinction coefficient for 4-nitrophenol at 405 nm is 18.51 L. $nmol^{-1}$. cm^{-1}.

Method 2 (Smibert and Krieg, 1994)

This method was used to test for acid phosphatase in JDP14. A very dense suspension of cells in saline (0.85% w/v NaCl) was prepared. 0.3 ml of the suspension was added to 0.3 ml of a substrate solution consisting of 0.01 M citrate buffer (pH 4.8) containing 0.01 M disodium 4-nitrophenyl phosphate. The mixture was incubated for up to 6 h at different temperatures. Then 0.3 ml of 0.04 M glycine NaOH buffer (pH 10.5) was added to stop the reaction. The spectrophotometer was blanked at 405 nm with water and an increase in absorption was measured against the control to which no cells had been

added. The enzyme activity was expressed as nmole 4-nitrophenol produced min^{-1} mg $protein^{-1}$. The extiction coefficient for 4-nitrophenol at 405 nm is 18.51 L. $nmol^{-1}$. cm^{-1}.

2.11.6 β-Galactosidase (EC 3.2.1.23)

Lactose + H_2O ↔ D-Galactose + D-Glucose

The procedure used was described by Miller (1972).

The reaction mixture contained (values in ml):-

	0.5 M NaCl JDP14	2 M NaCl JDP14
Z buffer	0.8	0.8
Cell free extract	0.2	0.2
4 mg/ml ONPG	0.2	0.2
1 M Na_2CO_3	0.5	0.5

0.8 ml of Z buffer (Z buffer contains 0.1 M sodium phosphate [pH 7.0], 10 mM KCl, 1 mM $MgSO_4$, and 50 mM mercaptoethanol) was added to a test tube. 0.2 ml cell free extract was added to the tube and the solution was incubated for 10 min at 30 °C in a water bath to equilibrate. 0.2 ml of o-nitrophenyl-β-D-galactoside (ONPG) was added to the mixture and incubated for 30 min at different temperatures. 0.5 ml of 1 M Na_2CO_3 was added to stop the reaction and intensify the colour. The mixture was shaken and the absorbance at 420 nm was read against a blank which contained no cell free extract. Enzyme activity is expessed as nmole o-nitrophenol produced. h^{-1} .mg $protein^{-1}$. The product concentrations were calculated from a standard curve of o-nitrophenol (0 – 100. $\mu g.ml^{-1}$) (Appendix C).

2.12 Statistics

All experiments were repeated at least three times and error bars represent standard error of the means. If no error bars are shown, they were smaller than the symbol used to represent the mean.

CHAPTER 3

SALT TOLERANCE OF *HALOMONAS* HALO AND *STAPHYLOCOCCUS XYLOSUS*

3.1 INTRODUCTION

Microorganisms show a wide range of salt tolerance and some demonstrate a requirement for salt (see section 1.6). Bacteria belonging to the genus *Halomonas* are known to be either halotolerant or moderately halophilic, the distinction depends to a certain extent on whether you look at the range of salt concentrations tolerated or at the salt concentration that is optimal for growth (Vreeland, 1987; Cummings and Gilmour, 1995; Canovas *et. al.* 1998). Nevertheless, it is important to know whether a bacterium requires NaCl to be present.

The halotolerant (euryhaline) bacterium *Halomonas elongata* (Vreeland *et al.* 1980; Vreeland and Martin 1980; Martin *et al.* 1983; Vreeland *et. al.*, 1984) has been shown to grow in the presence of as little as 0.05 M NaCl and to grow optimally in media containing any NaCl concentration between 0.375 and 1.4 M. The latter concentration represents a saturated NaCl solution and it suggests that *H. elongata* is one of the most salt tolerant organisms known (Vreeland, 1987).

Another *Halomonas* species, *H. canadensis* was used by Huval *et al.* (1995) to demonstrate that temperature has an important effect on salt tolerance. At temperatures between 15 to 30 °C it grows well from 3 to 25 % w/v NaCl (0.5 - 4.3 M), but at 45 °C, it grows in media containing 8 to 32 % NaCl (1.4 - 5.5 M). *H. canadensis* cannot grow below 5 °C, it also grows poorly below 1.0 % w/v NaCl (0.2 M) and it cannot grow at all unless some NaCl is added to the medium. KCl cannot substitute for NaCl and does not support growth in the absence of NaCl (Huval *et al.*, 1995).

The other organism studied in this chapter is a Gram positive bacterium subsequently identified as *Staphylococcus xylosus.* The genus *Staphylococcus* is known to contain species that are capable of growth at salinities in excess of 3 M NaCl (Townsend and Wilkinson, 1992). *S. aureus* is the most studied member of the genus, growing at water activities as low as 0.86 (equivalent to 3.5 M NaCl) (Graham and Wilkinson, 1992). Integral with the above is the ability of *S. aureus* to tolerate and grow in media or food products containing a high concentration of NaCl (Jay, 1992). *S. aureus* naturally contains high levels of cytoplasmic K^+ and glutamate (Kunin and Rudy, 1991).

Staphylococcus arlettae was found to be extremely halotolerant, growing well at salt concentrations from 0.06 M NaCl, to 4.5 M NaCl. However, this strain grew over a wide range of temperature from 8 to 45 °C, and the optimal growth conditions were found to be at 0.4-0.6 M NaCl and 30-32 °C (Vilhelmsson *et al.*, 1997).

In this Chapter, the salt tolerance of *Halomonas* strain Halo is compared with the new isolate JDP14 and JDP14 was identified as *Staphylococcus xylosus*.

3.2 RESULTS

3.2.1 Isolation and Selection of Organisms

Three strains of *Halomonas* were available in the laboratory: *Halomonas* Halo, *Halomonas* Duncon, and *Halomonas* SPCI. 1 ml of each strain was inoculated into 100 ml of HDM (see Table 2.1) at a range of salinities from 0.05 to 3.4 M NaCl. Flasks were incubated for 72 hours on an orbital shaker at 250 rpm in a 30°C constant temperature room. Optical density readings are shown in Table 3.1. It is clear from these data that *Halomonas* Halo was the most salt tolerant strain and it was used in all further studies.

A Gram positive strain (JDP14) was available in the laboratory. It had been isolated from a sample of Weston Park soil using LB medium (see Table 2.2) containing up to 3 M NaCl. It appeared that JDP14 had a similar range of salt tolerance as *Halomonas* Halo and it was decided to further characterise JDP14. The first step was to identify JDP14.

Table 3.1. The Optical Density (660 nm) of *Halomonas* Species in Different Concentrations of NaCl

Concentration of NaCl	*Halomonas* Halo			*Halomonas* SPCl			*Halomonas* Duncon		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
0.05 M	0.065	0.452	0.606	0.008	0.071	0.3	0.003	0.03	0.078
0.1 M	0.166	0.725	0.65	0.025	0.316	0.409	0.011	0.111	0.396
0.4 M	0.705	0.637	0.678	0.031	0.619	0.587	0.003	0.216	0.518
1.37 M	0.717	0.663	0.744	0.011	0.193	0.833	0.002	0.019	0.045
2.5 M	0.087	0.791	0.892	0.02	0.073	0.175	0.01	0.047	0.108
3.4 M	0.018	0.078	0.2	0.001	0.025	0.043	0.009	0.02	0.02

3.2.2 Identification of JDP14

Examination by light microscopy showed the bacterium to be non-motile, non-spore forming cocci occurring singly and in pairs with dimensions less than 1 µm. The bacterium was examined after being grown for 24 hours on L broth agar, colonies were irregular, yellow to orange, flat, shiny and opaque. The ability of JDP14 to grow under anaerobic conditions was examined and the bacterium was found to be a strictly aerobic, catalase positive, oxidase negative organism.

The Api STAPH kit was used to identify this strain (see section 2.3.5) and the results are shown in Table 3.2. JDP14 can produce acids due to the utilization of carbohydrates such as glucose, fructose, mannose, maltose, lactose, trehalose, mannitol, xylose and saccharose. Also, it can reduce nitrate to nitrite. The identification was facilitated by the use of the APILAB software in which the pattern of the reactions obtained must be coded into a numerical profile corresponding to positive and negative reactions. From these results this strain is *Staphylococcus xylosus.*

3.2.3 Growth of *Halomonas* Halo and *S. xylosus* at Different Salinities

Figure 3.1 shows the effect of different concentrations of NaCl on the growth of *Halomonas* Halo. From this figure the optimal concentration of salt appears to be 0.5 M NaCl with a generation time (g) of 1.8 h, but growth was also good at 0.1 (g = 2 h) and 2 M NaCl (g = 1.9 h). On the other hand

growth is significantly slower at 3 M NaCl (g = 3.5 h), with a prolonged lag phase of about 8 hours.

Figure 3.2 shows that *S. xylosus* grows well at salinities of 0.1 (g = 1.2 h) and 0.5 M NaCl (g = 1.1 h). Growth at 2 M NaCl was also good after a 4.5 hour period of slow growth with an exponential phase of about 3 hours (g = 1.6 h) and after that it became slower again. At 3 M NaCl (g = 3.8 h) there was a lag phase of 6 hours before growth commenced. It is interesting to note that once growth had started at 3 M NaCl, *S. xylosus* showed a faster growth rate than *Halomonas* (Figures 3.1 and 3.2).

3.2.4 Electron Micrographs of *S. xylosus* Cells Grown at Different Salinities

Figure 3.3A shows an electron micrograph of *S. xylosus* cells grown in 0.1 M NaCl HDM + 1 g l^{-1} yeast extract. The cells are cocci, single, non-spore forming and approximately 1 µm in diameter. The thick cell wall can be easily observed in Figure 3.3B, it is due to the thick peptidoglycan layer characteristic of Gram-positive cells. In Figure 3.3C cell division is taking place and it shows DNA (in left hand daughter cell) and ribosomes clearly. Figures 3.4A, B and C show electron micrographs of *S. xylosus* cells grown in 0.5 M NaCl HDM + 1 g l^{-1} yeast extract. From these figures the cell size was 0.93 µm in diameter, but there were no other obvious morphological changes. Figures 3.5A, B and C show electron micrographs of *S. xylosus* cells grown in 2 M NaCl HDM + 1 g l^{-1} yeast extract, the cell size had decreased to 0.85 µm in diameter.

Table (3.2) Identification of JDP14 using API STAPH kit

Test	Substrate	Reaction/Enzyme	Result
0	No substrate	(Negative control)	Red (-)
GLU	D-Glucose	(Positive control) Acidification due to carbohydrate utilisation	(+)
FRU	D-Fructose		(+)
MNE	D-Mannose		(+)
MAL	Maltose		(+)
LAC	Lactose		(+)
TRE	D-Trehalose		(+)
MAN	D-Mannitol		(+)
XLT	Xylitol		(-)
MEL	D-Melibiose		(-)
NIT	Potassium Nitrate	Reduction of nitrate to nitrite	(+)
PAL	β-Naphthyl acid phosphate	Alkaline phosphatase	(+)
VP	Sodium Pyruvate	Production of acetyl methylcarbinol	(-)
RAF	Raffinose	Acidification due to carbohydrate utilisation	(-)
XYL	Xylose		(+)
SAC	Saccharose		(+)
MDG	α-Methyl-D-glucoside		(-)
MAG	N-Acetyl-glucosamine		(+)
ADH	Arginine	Arginine dihydrolase	(-)
URE	Urea	Urease	(+)
LSTR		Lysostaphin resistant	(-)

This strain is *Staphylococcus xylosus*

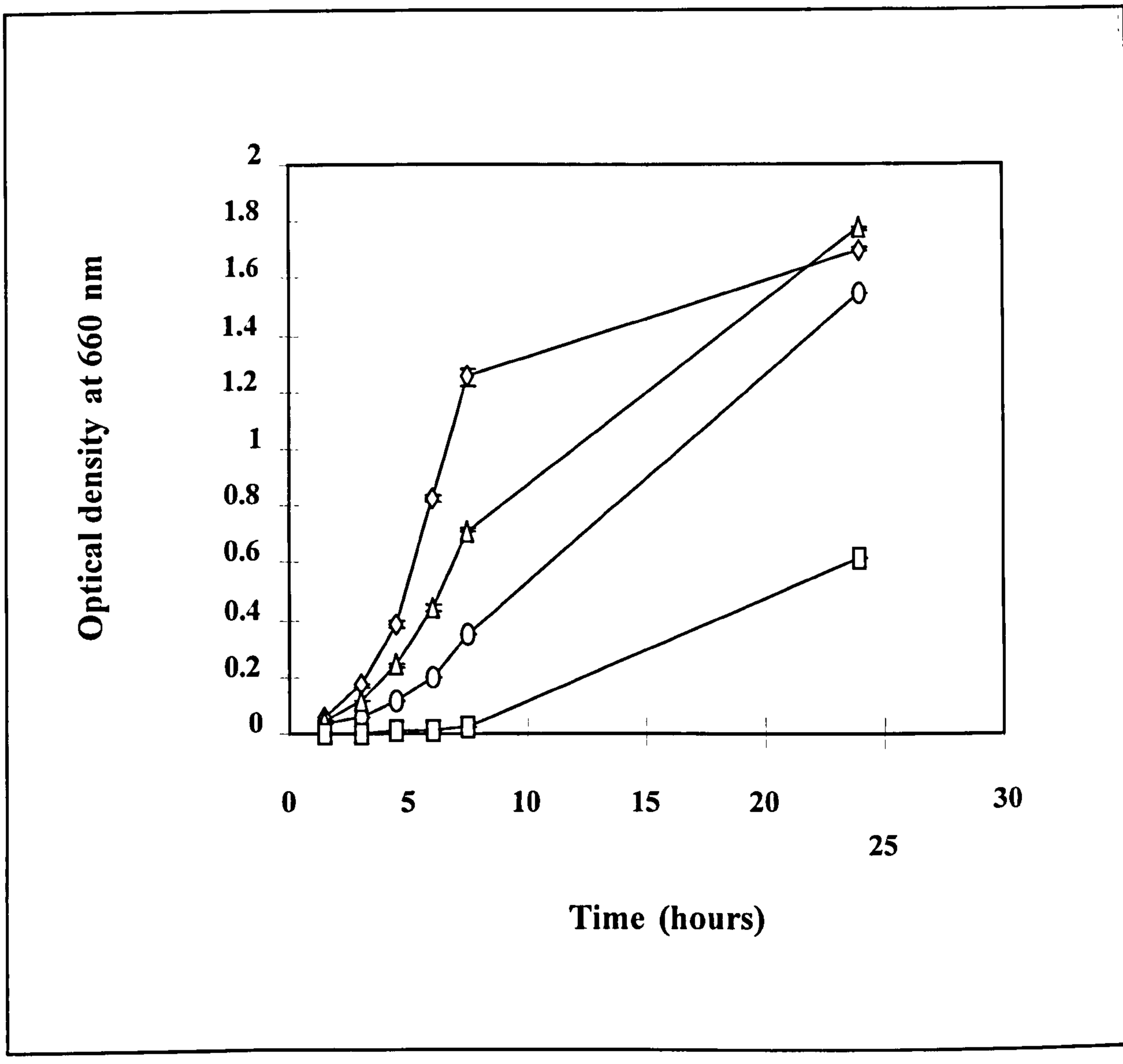


Fig: (3.1) The growth of *Halomonas* Halo at different salinities on *Halomonas* defined medium +1 g l^{-1} yeast extract. Cells grown at 0.5 M NaCl were washed twice with sodium free medium before inoculation into the medium.

—O— 0.1 M NaCl, —◊— 0.5 M NaCl, —Δ—2 M NaCl, —□—3M NaCl

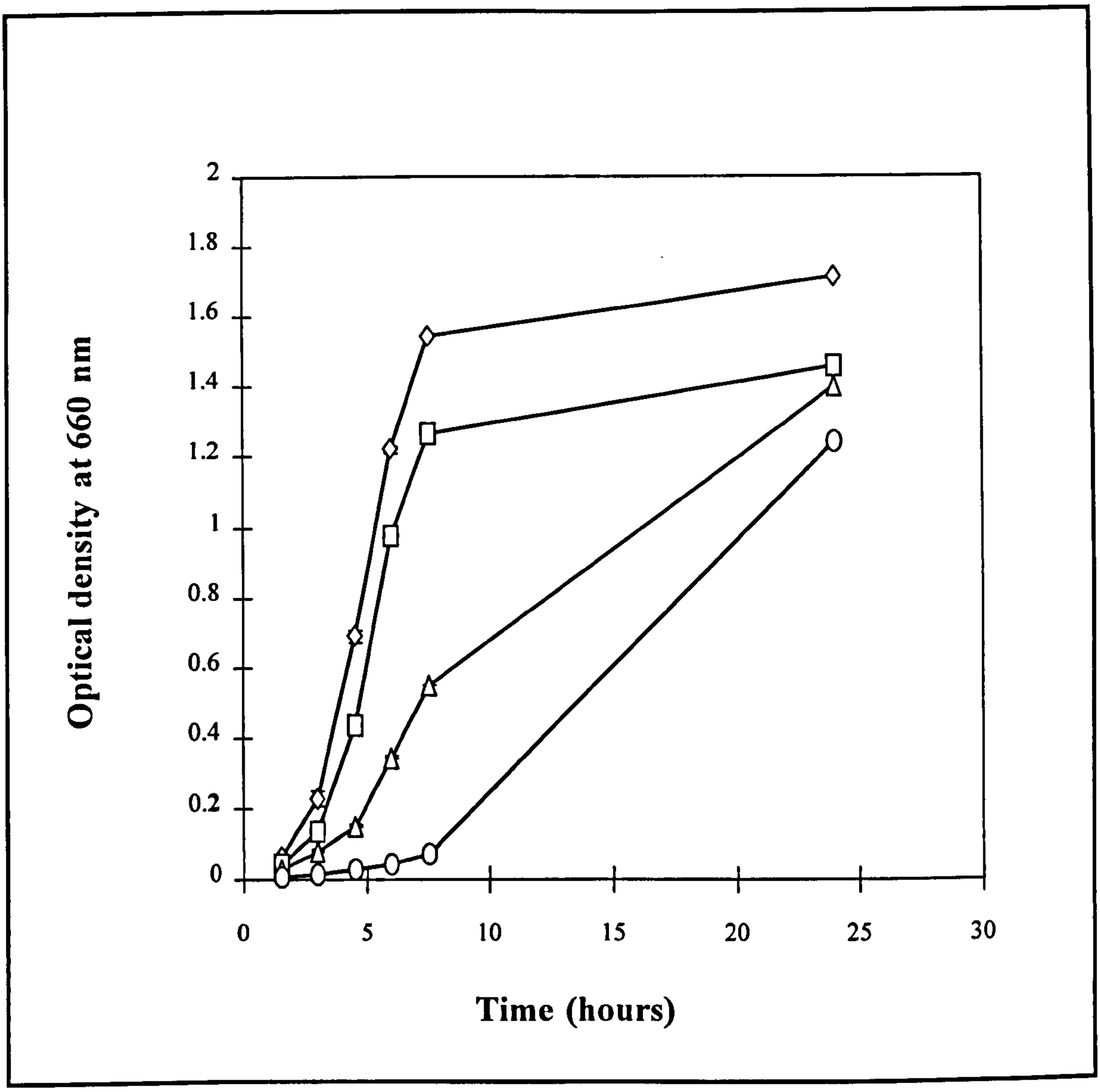


Fig: (3.2)The growth of *Staphylococcus xylosus* at different salinities on *Halomonas* defined medium + 1 g l^{-1} yeast extract. Cells grown at 0.5 m NaCl were washed twice with sodium free medium before inoculation into the medium.

—◊—0.1 M NaCl, —□—0.5M NaCl, —Δ—2M NaCl, —O—3 M NaCl

Finally the cells which were grown in 3 M NaCl HDM + 1 g l^{-1} yeast extract. Figures 3.6A, B and C show small cells with sizes about 0.8 µm in diameter. These results suggest that the increasing salinity has an influence on the size of *S. xylosus* cells, but that no other major morphological changes were taking place.

On the other hand Figures 3.7A, B, C and D show electron micrographs of *S. xylosus* cells grown in 0.1, 0.5, 2 and 3 M NaCl chemically defined medium. The cells grown at 0.1 M and 0.5 M NaCl were approximately 0.8 µm in diameter (estimate from 5 cells). For cells grown at 2 M NaCl the cell size was 0.95 µm and cells grown at 3 M NaCl were about 1 µm in diameter. These results, showing an increase in cell size with increasing salinity, were the opposite to those found with cells grown at different salinities in HDM + 1 g l^{-1} yeast extract (Figures 3.3 to 3.6). It should be noted that the cells grown on the chemically defined medium were incubated for 48 hours to reach a high cell density, whereas the HDM + 1 g l^{-1} yeast extract grown cells were incubated overnight. In general the size of *S. xylosus* cells was between 0.8-1 µm in diameter.

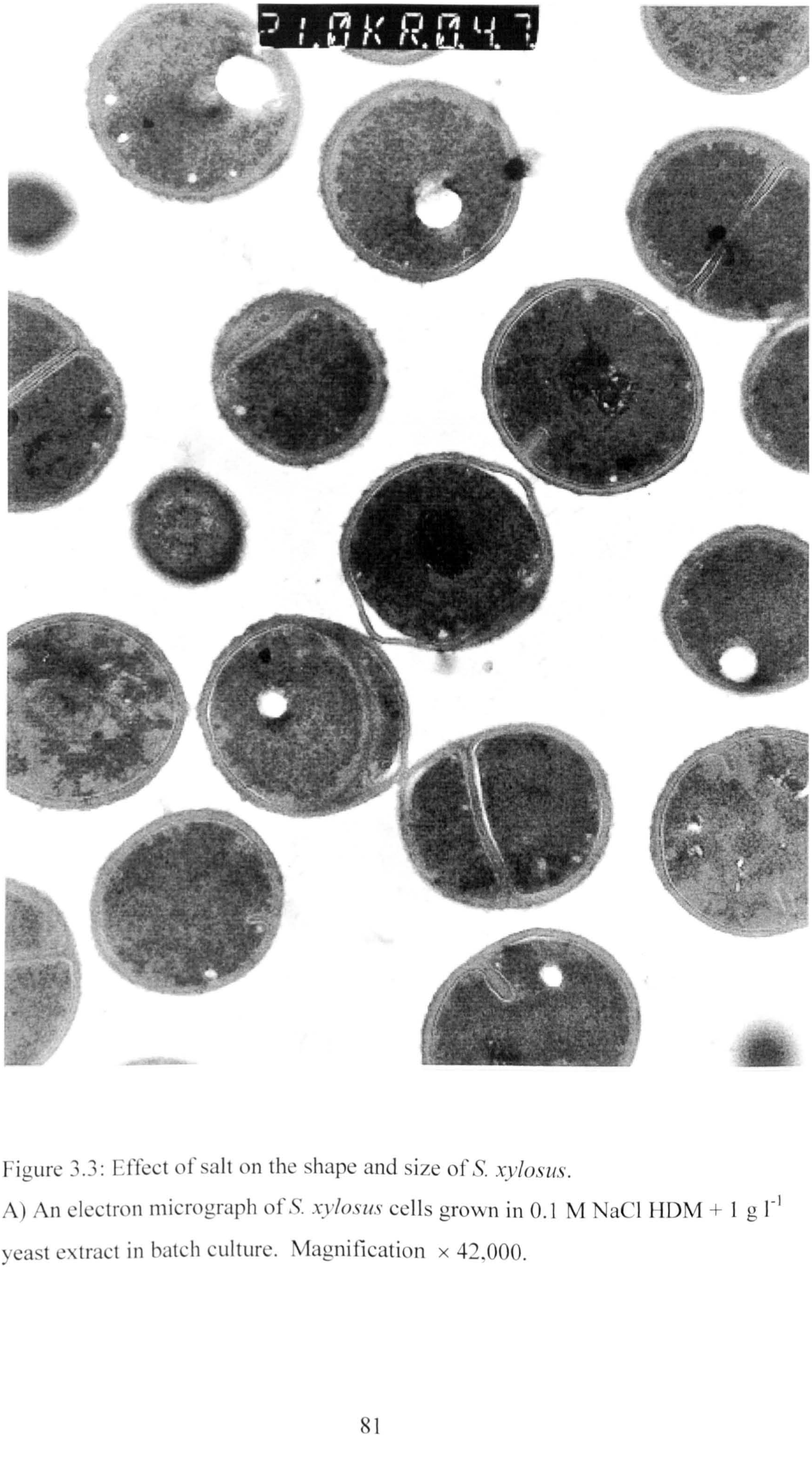


Figure 3.3: Effect of salt on the shape and size of *S. xylosus*.

A) An electron micrograph of *S. xylosus* cells grown in 0.1 M NaCl HDM + 1 g l^{-1} yeast extract in batch culture. Magnification × 42,000.

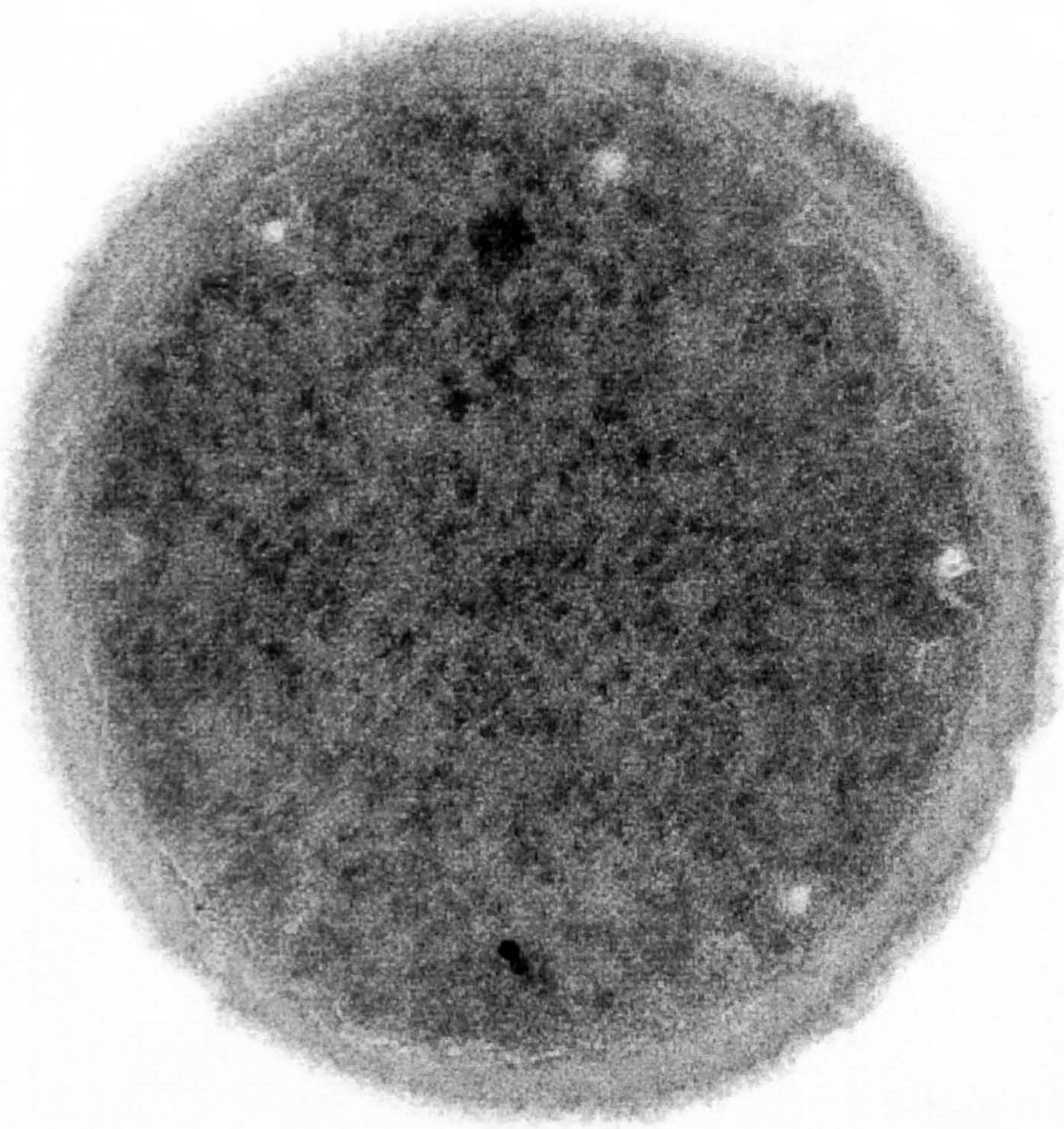


Figure 3.3: (continued)

B) An electron micrograph of *S. xylosus* showing the morphology characteristics. Cells were grown in 0.1 M NaCl HDM + 1 g l^{-1} yeast extract in batch culture. Magnification × 146,000.

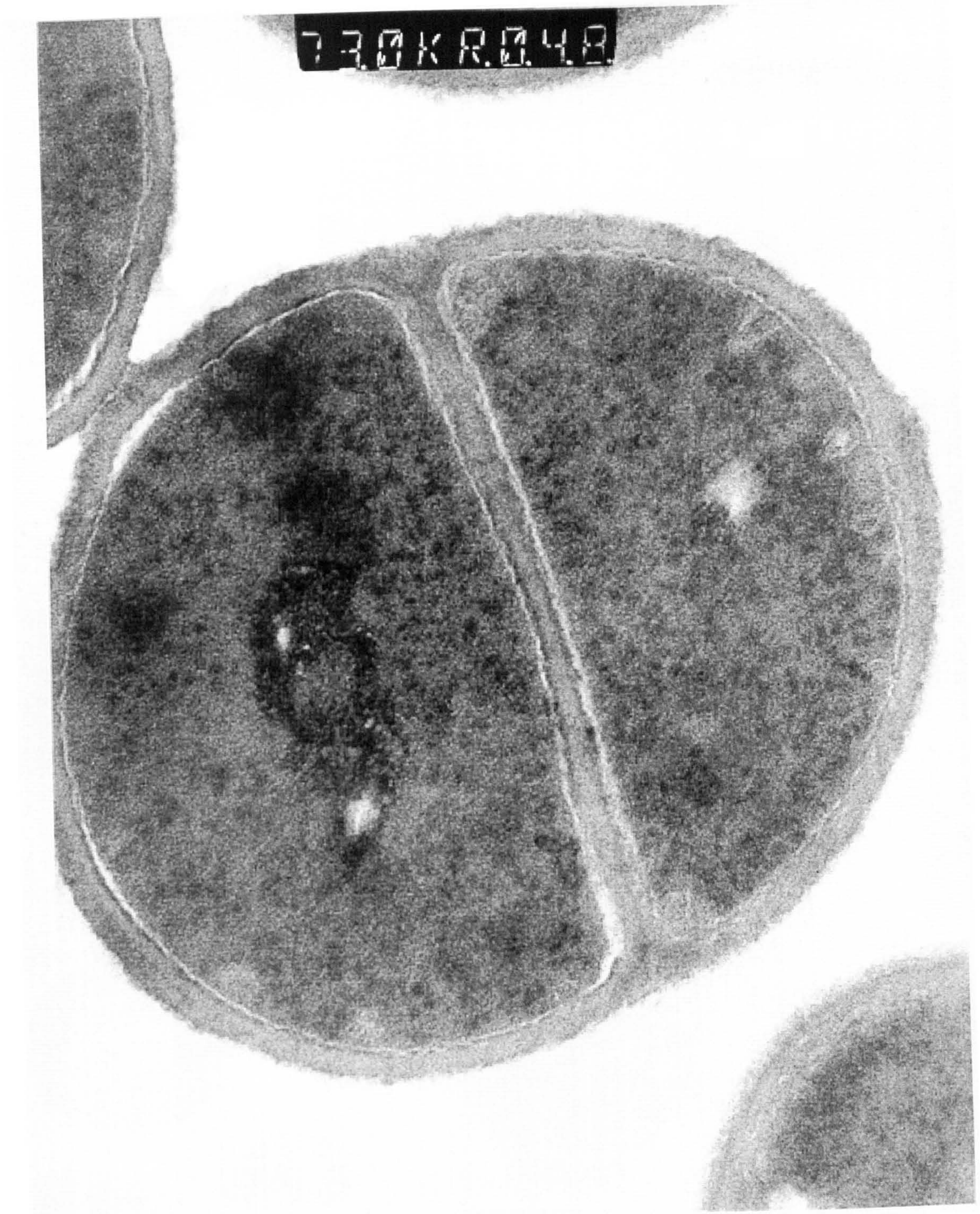


Figure 3.3: (continued)

C) An electron micrograph of *S. xylosus* showing cell division. Cells were grown in 0.1 M NaCl HDM + 1 g l^{-1} yeast extract in batch culture. Magnification × 146,000.

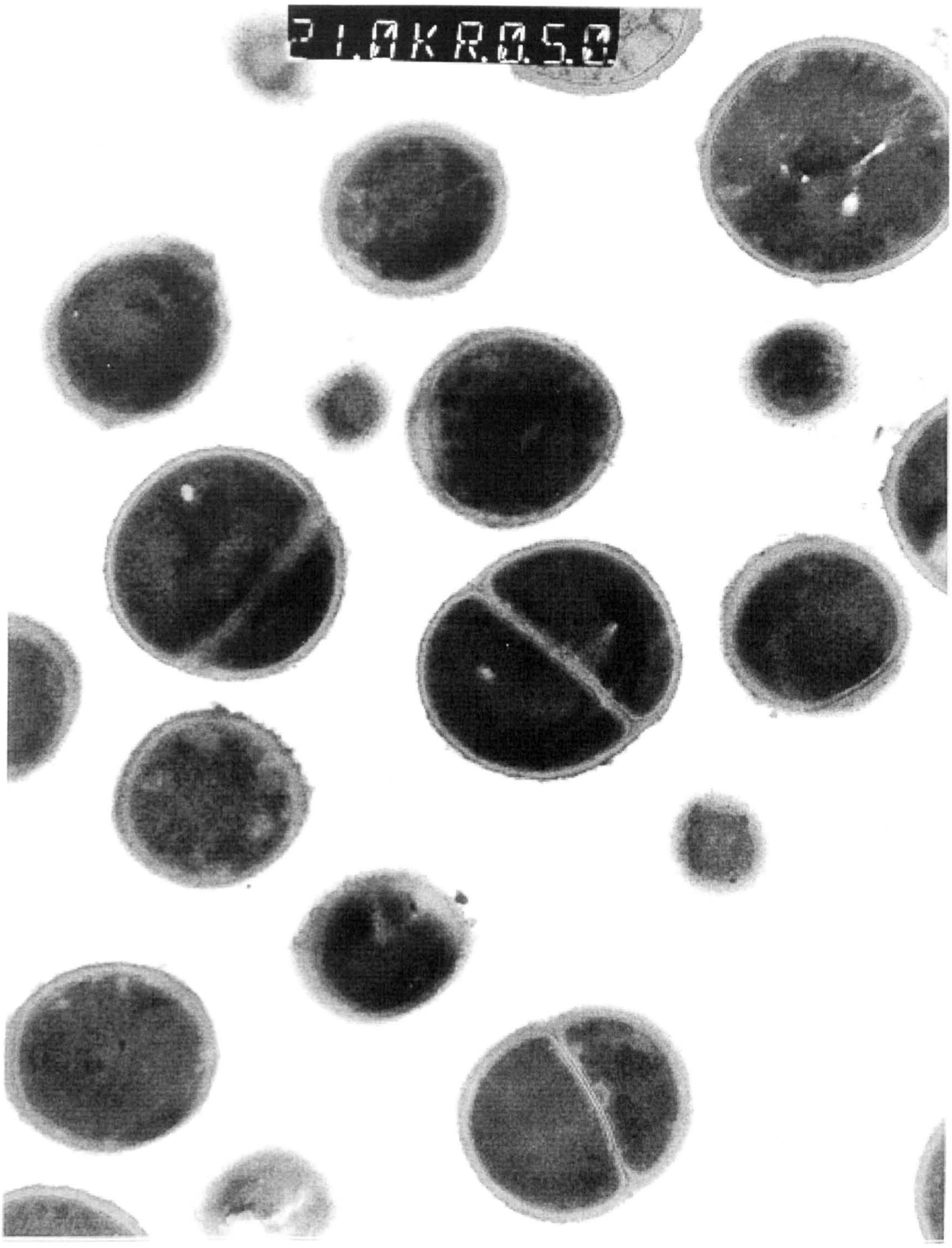


Figure 3.4: Effect of salt on the shape and size of *S. xylosus*.

A) An electron micrograph of *S. xylosus* cells grown in 0.5 M NaCl HDM + 1 g l^{-1} yeast extract in batch culture. Magnification × 42,000.

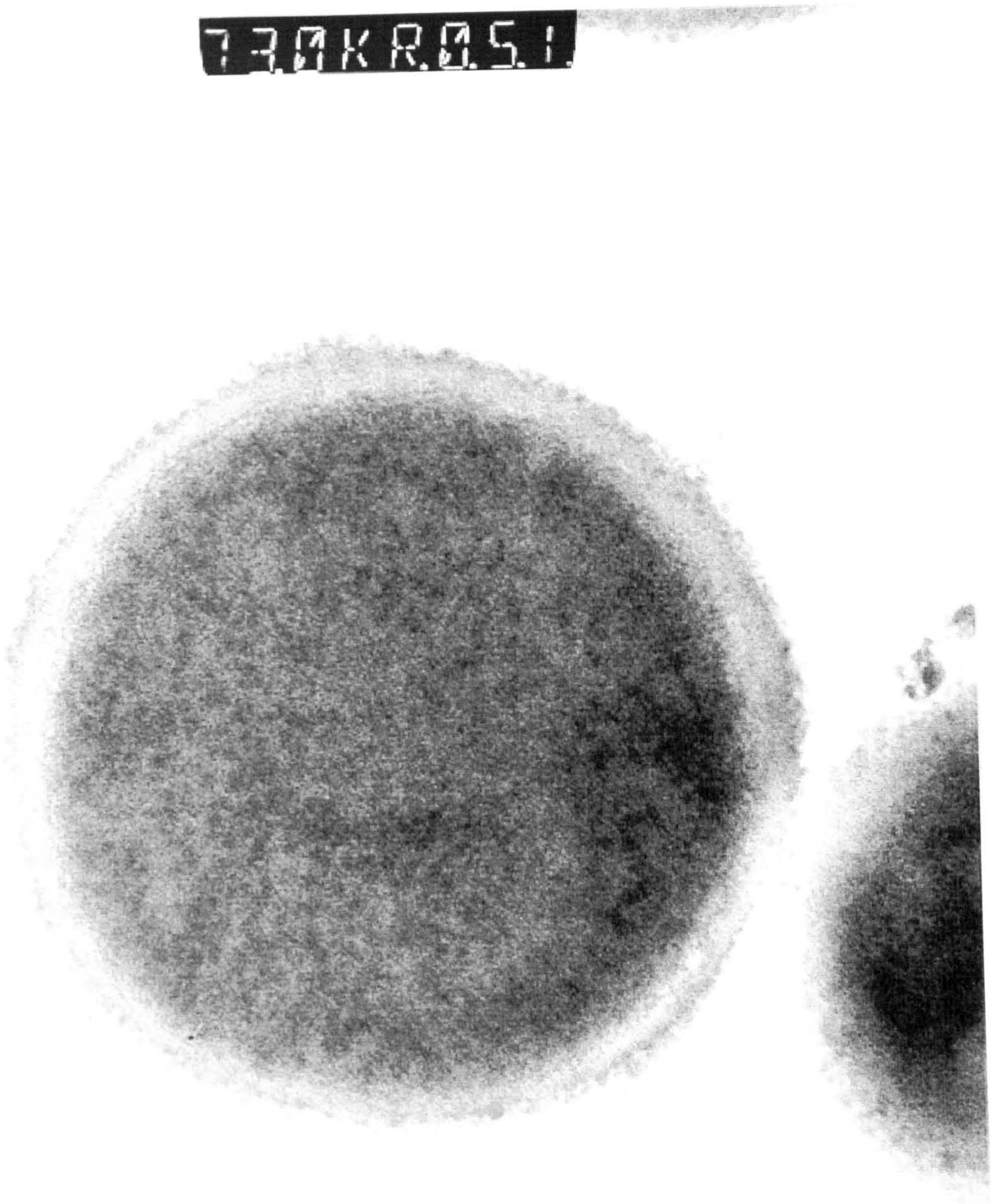


Figure 3.4: (continued)

B) An electron micrograph of *S. xylosus* showing the morphology characteristics. Cells were grown in 0.5 M NaCl HDM + 1 g l^{-1} yeast extract in batch culture. Magnification × 146,000.

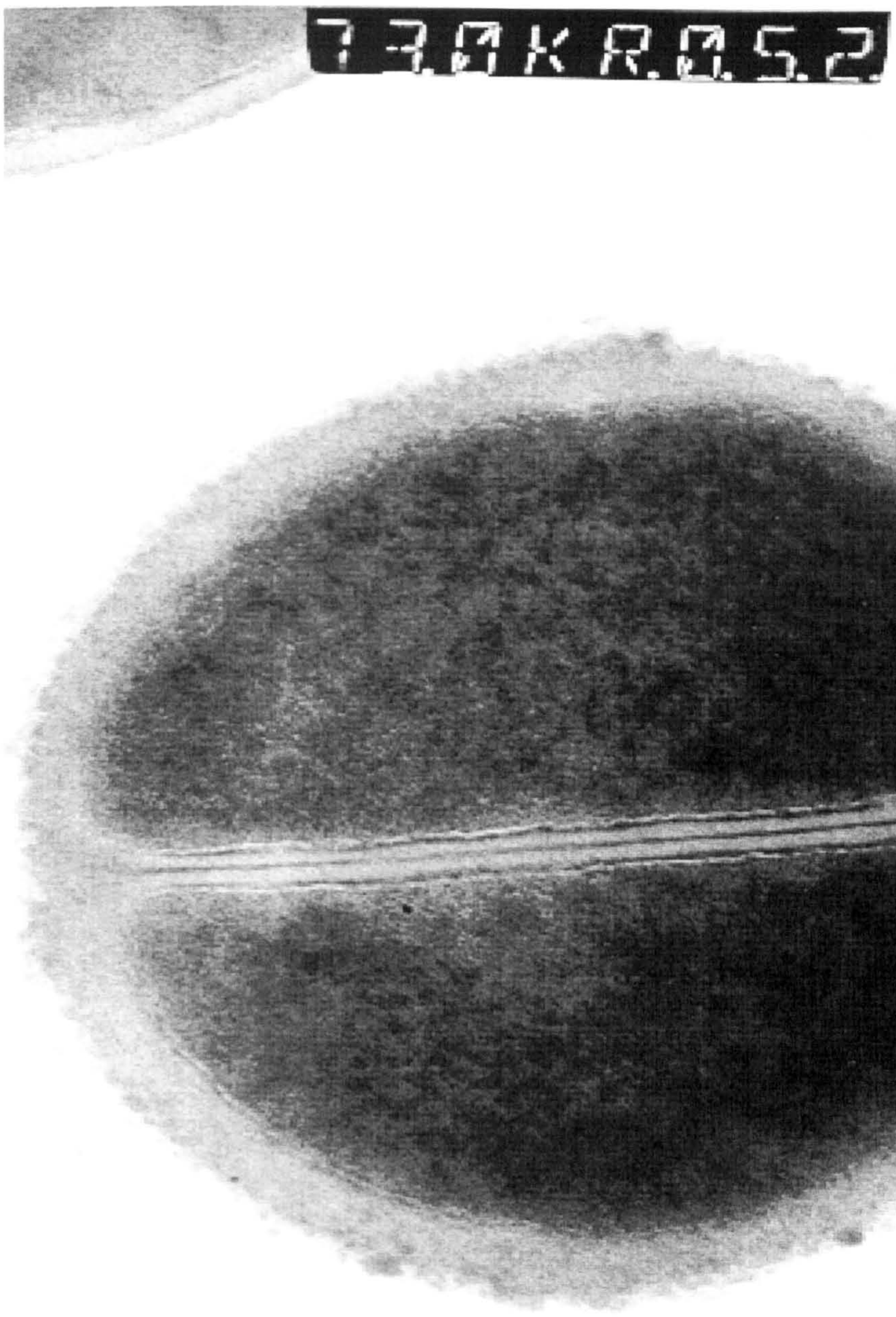


Figure 3.4: (continued)

C) An electron micrograph of *S. xylosus* showing cell division. Cells were grown in 0.5 M NaCl HDM + 1 g l^{-1} yeast extract in batch culture. Magnification × 146,000.

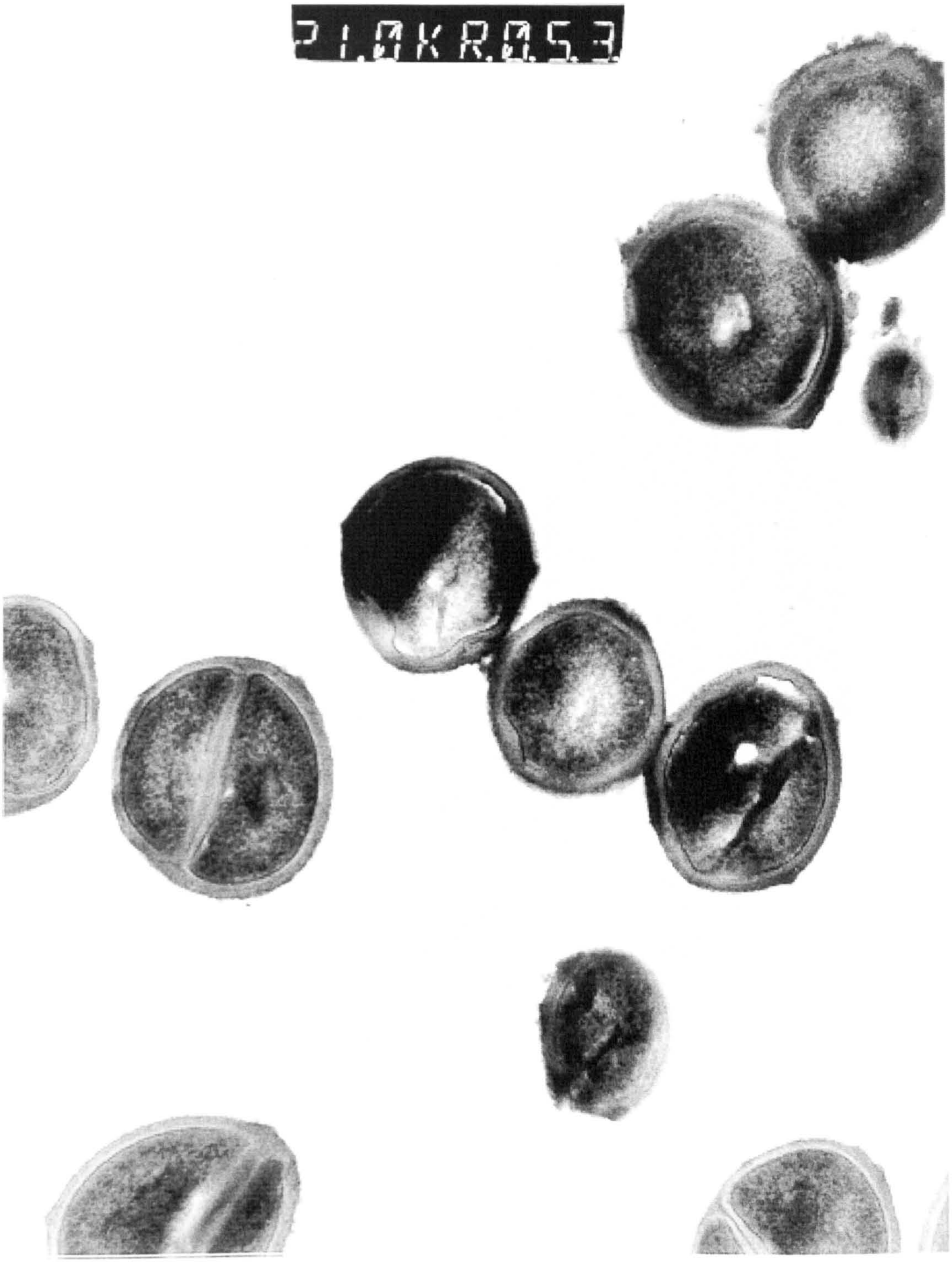


Figure 3.5: Effect of salt on the shape and size of *S. xylosus*.

A) An electron micrograph of *S. xylosus* cells grown in 2 M NaCl HDM + 1 g l^{-1} yeast extract in batch culture. Magnification × 42,000.

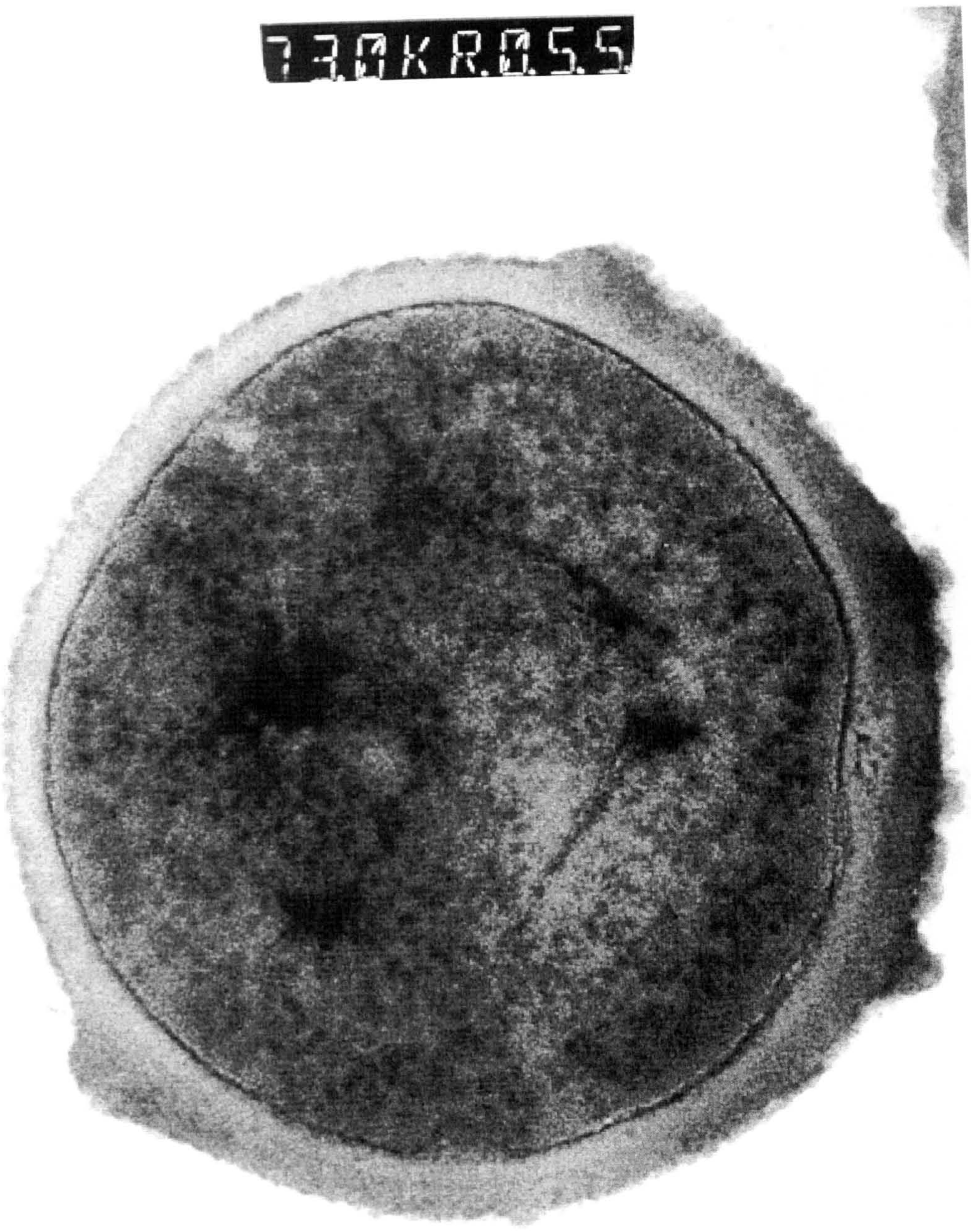


Figure 3.5: (continued)

B) An electron micrograph of *S. xylosus* showing the morphology characteristics. Cells were grown in 2 M NaCl HDM + 1 gl^{-1} yeast extract in batch culture. Magnification × 146,000.

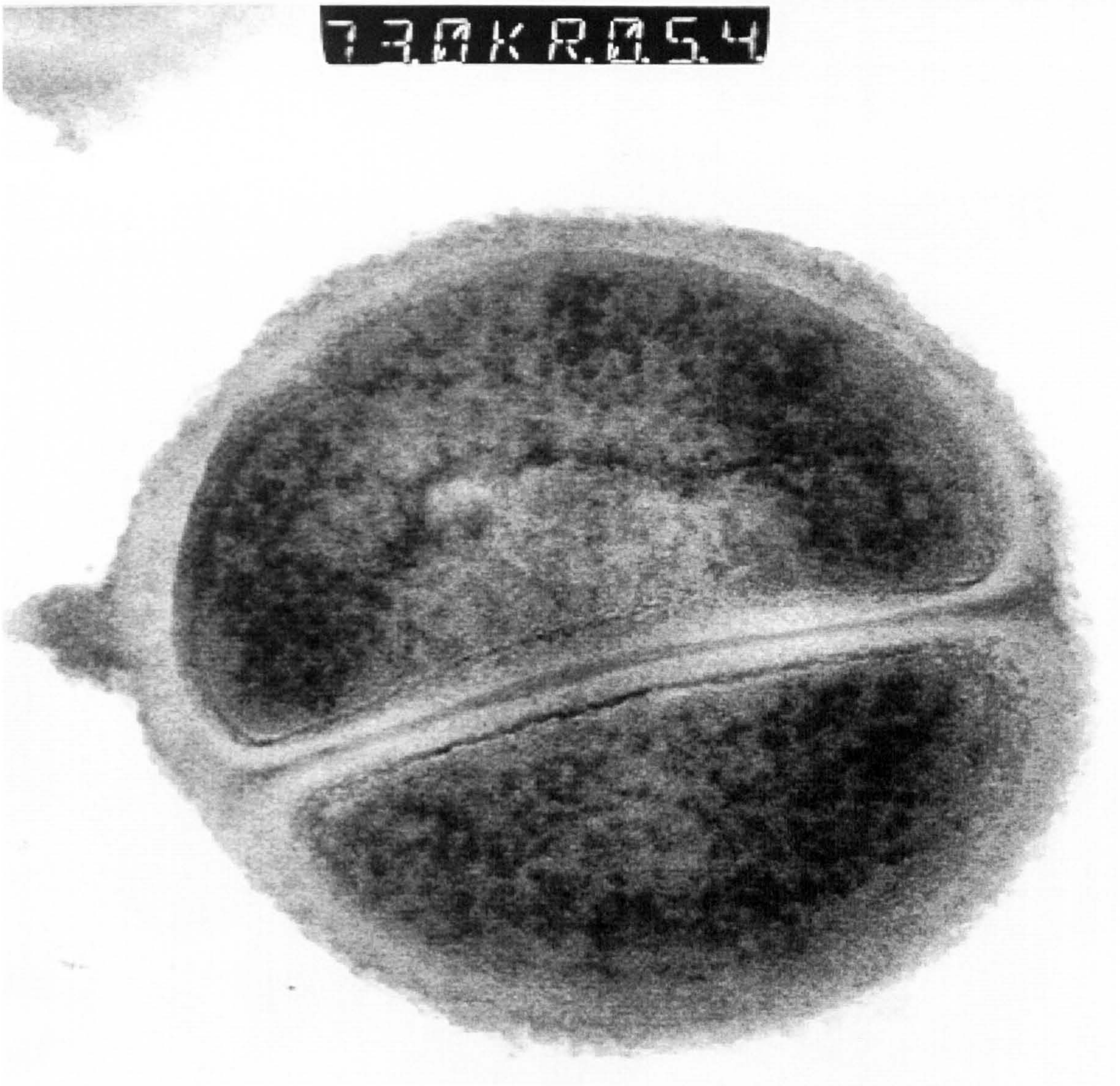


Figure 3.5: (continued)

C) An electron micrograph of *S. xylosus* showing cell division. Cells were grown in 2 M NaCl HDM + 1 g l^{-1} yeast extract in batch culture. Magnification × 146,000.

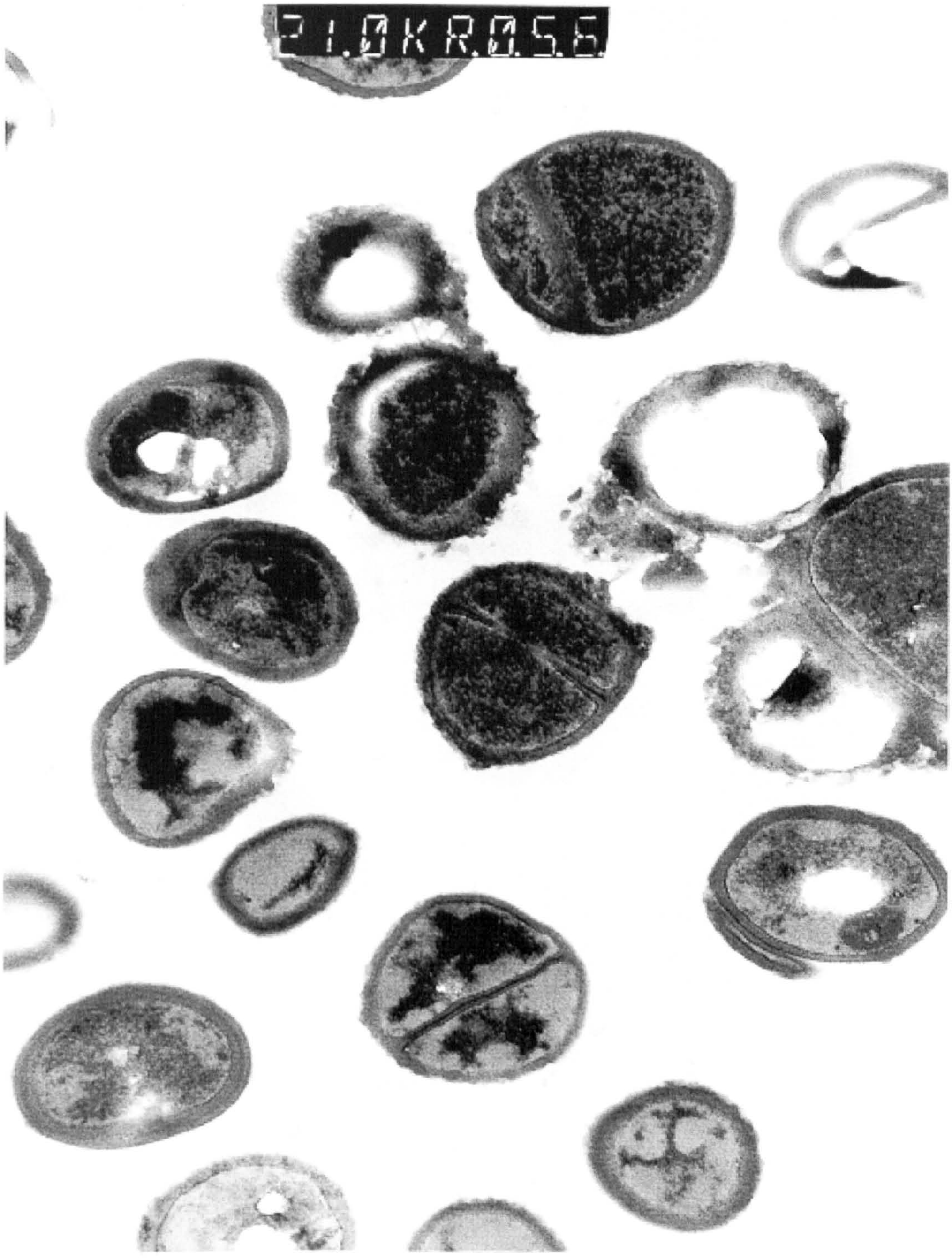


Figure 3.6: Effect of salt on the shape and size of *S. xylosus*.

A) An electron micrograph of *S. xylosus* cells grown in 3 M NaCl HDM + 1 g l^{-1} yeast extract in batch culture. Magnification × 42,000.

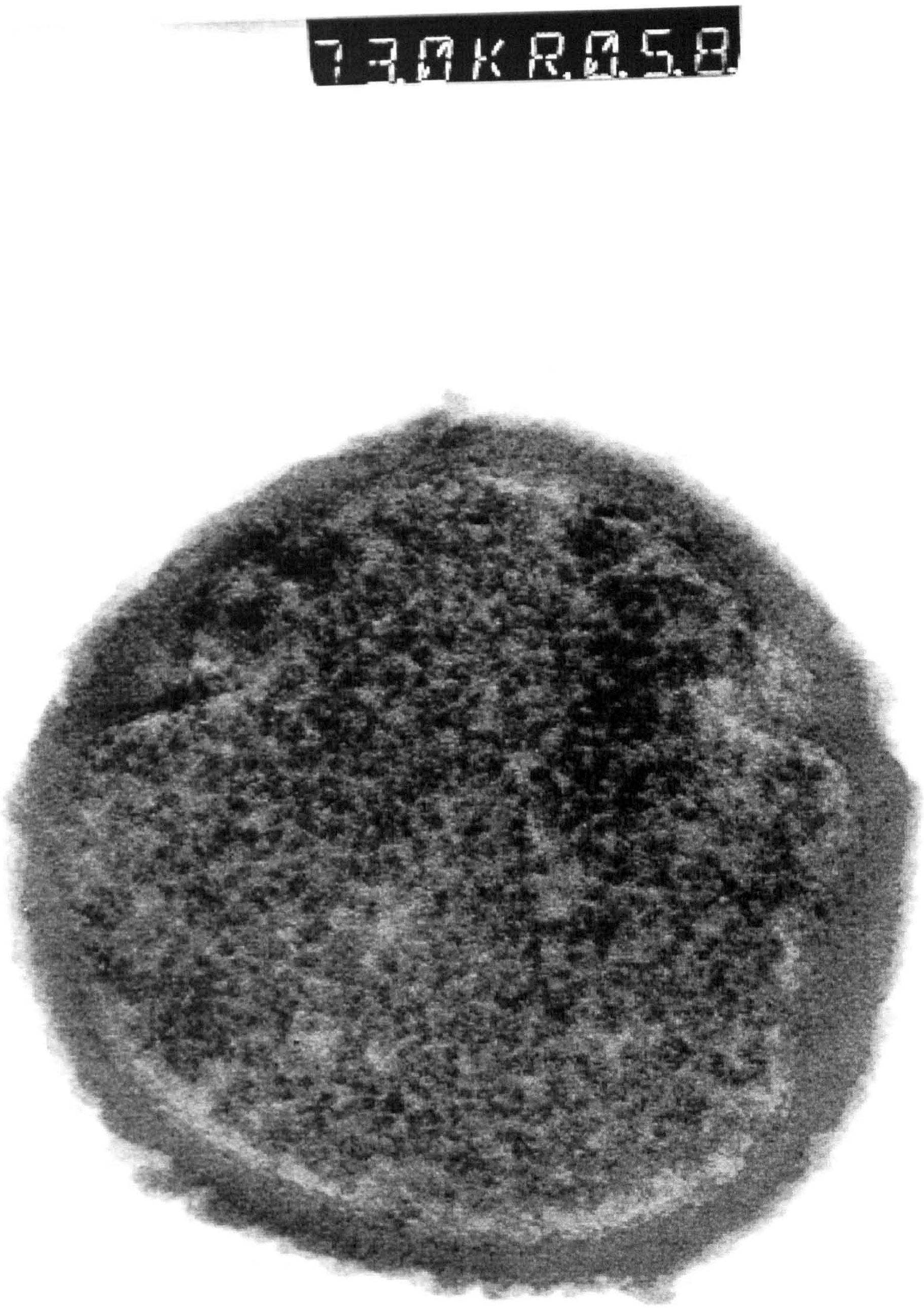


Figure 3.6: (continued)

B) An electron micrograph of *S. xylosus* showing the morphology characteristics. Cells were grown in 3 M NaCl HDM + 1 g l^{-1} yeast extract in batch culture. Magnification × 146,000.

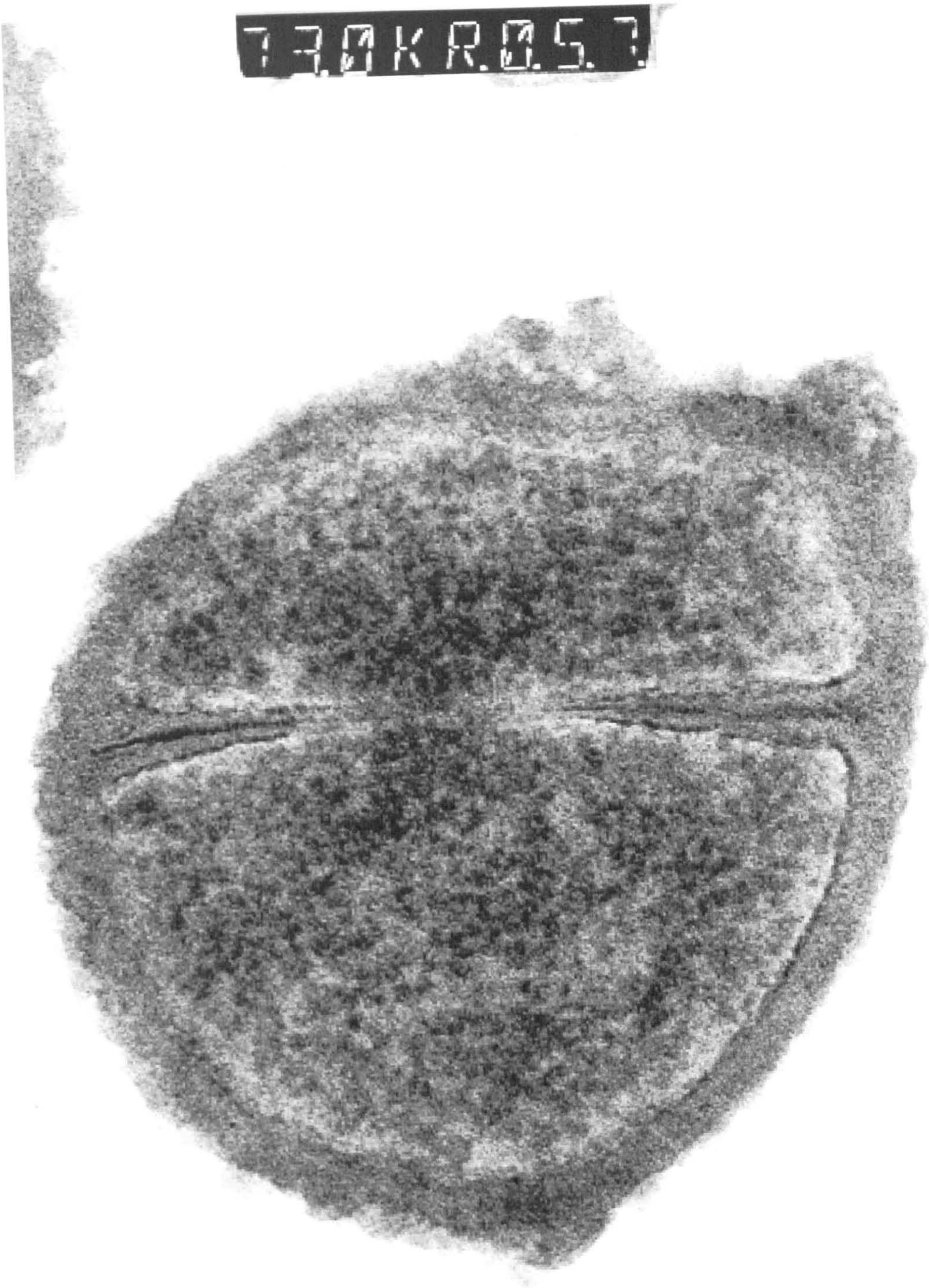


Figure 3.6: (continued)

C) An electron micrograph of *S. xylosus* showing cell division. Cells were grown in 3 M NaCl HDM + 1 g l^{-1} yeast extract in batch culture. Magnification × 146,000.

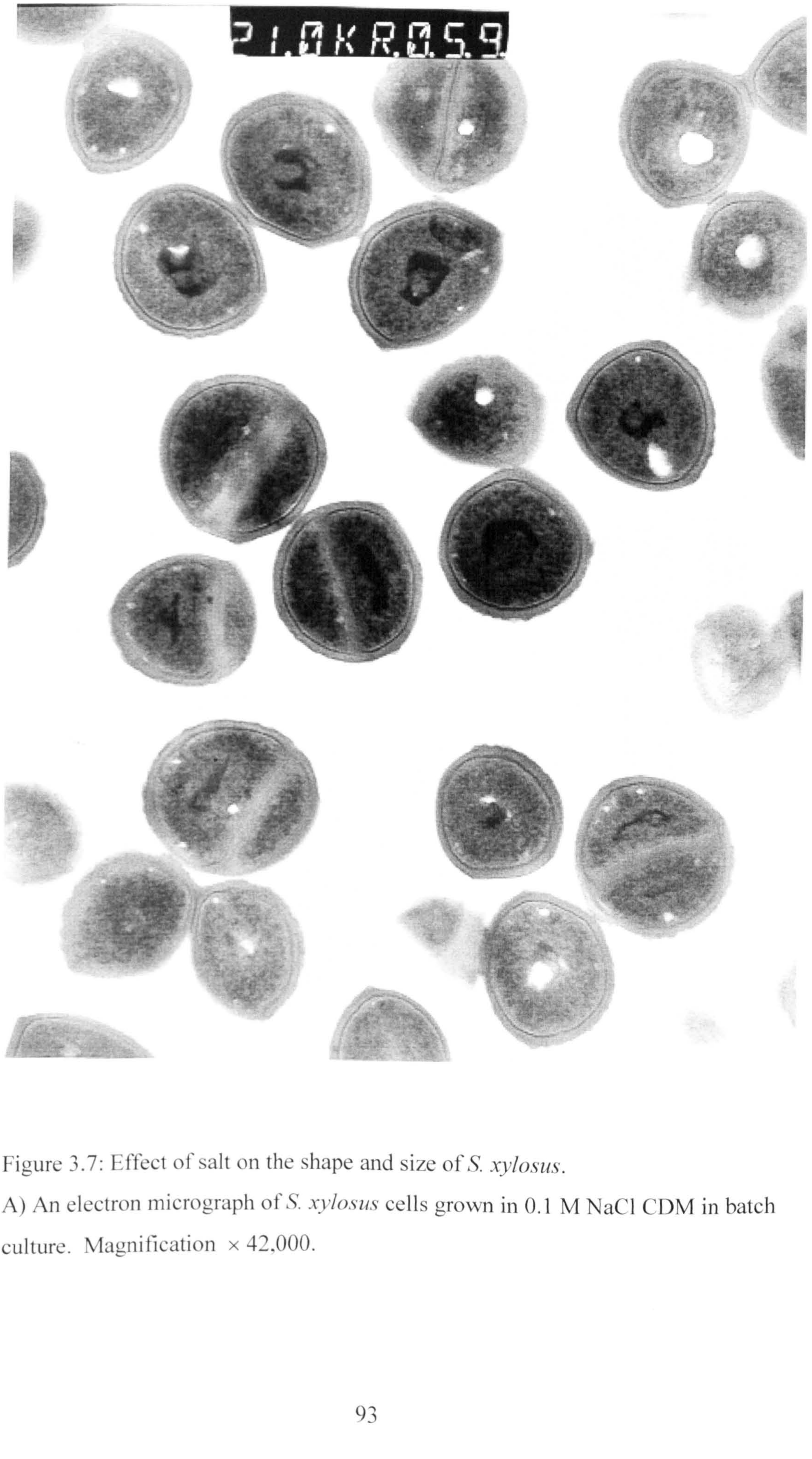


Figure 3.7: Effect of salt on the shape and size of *S. xylosus*.

A) An electron micrograph of *S. xylosus* cells grown in 0.1 M NaCl CDM in batch culture. Magnification × 42,000.

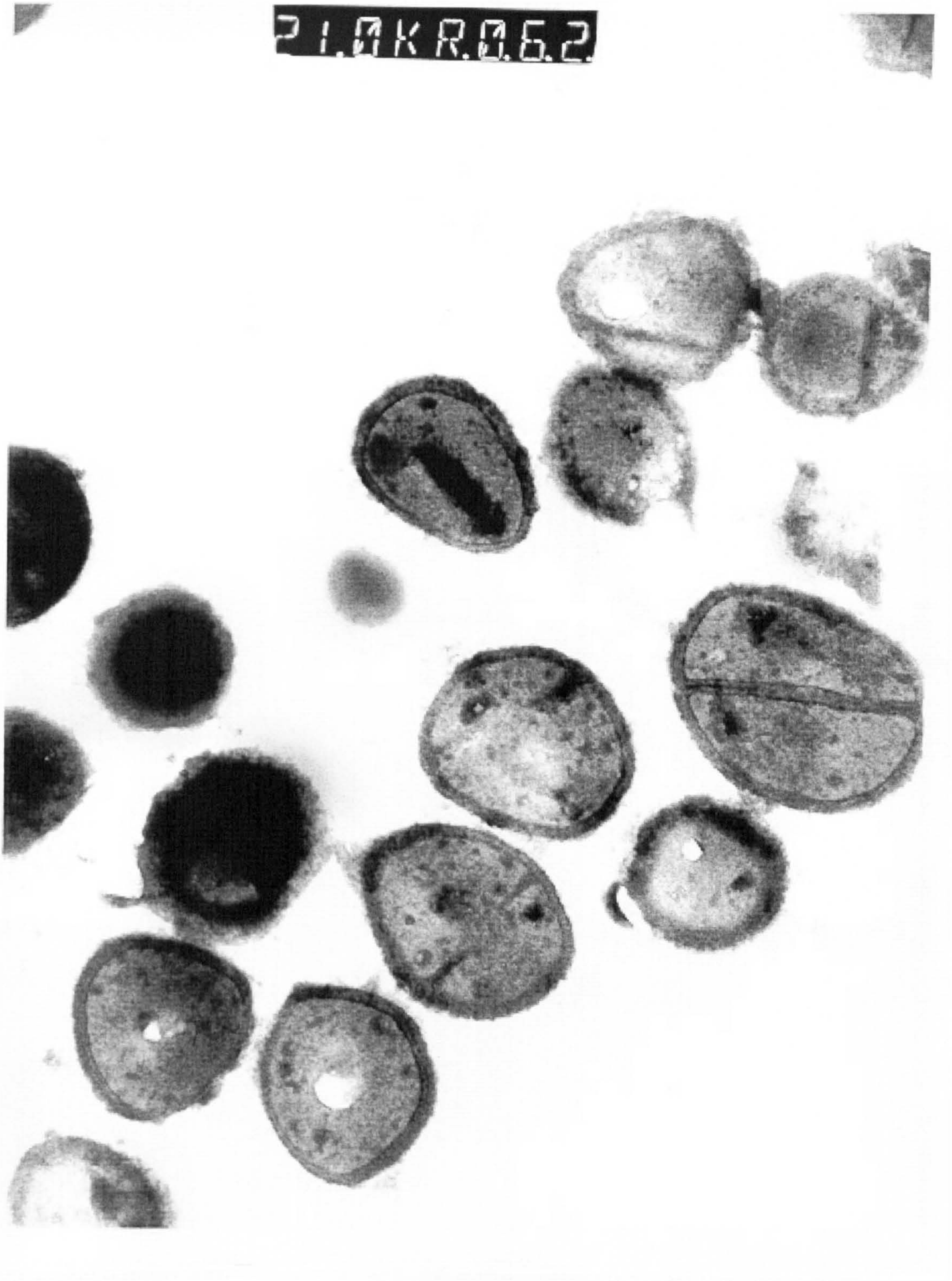


Figure 3.7: (continued).

B) An electron micrograph of *S. xylosus* cells grown in 0.5 M NaCl CDM in batch culture. Magnification × 42,000.

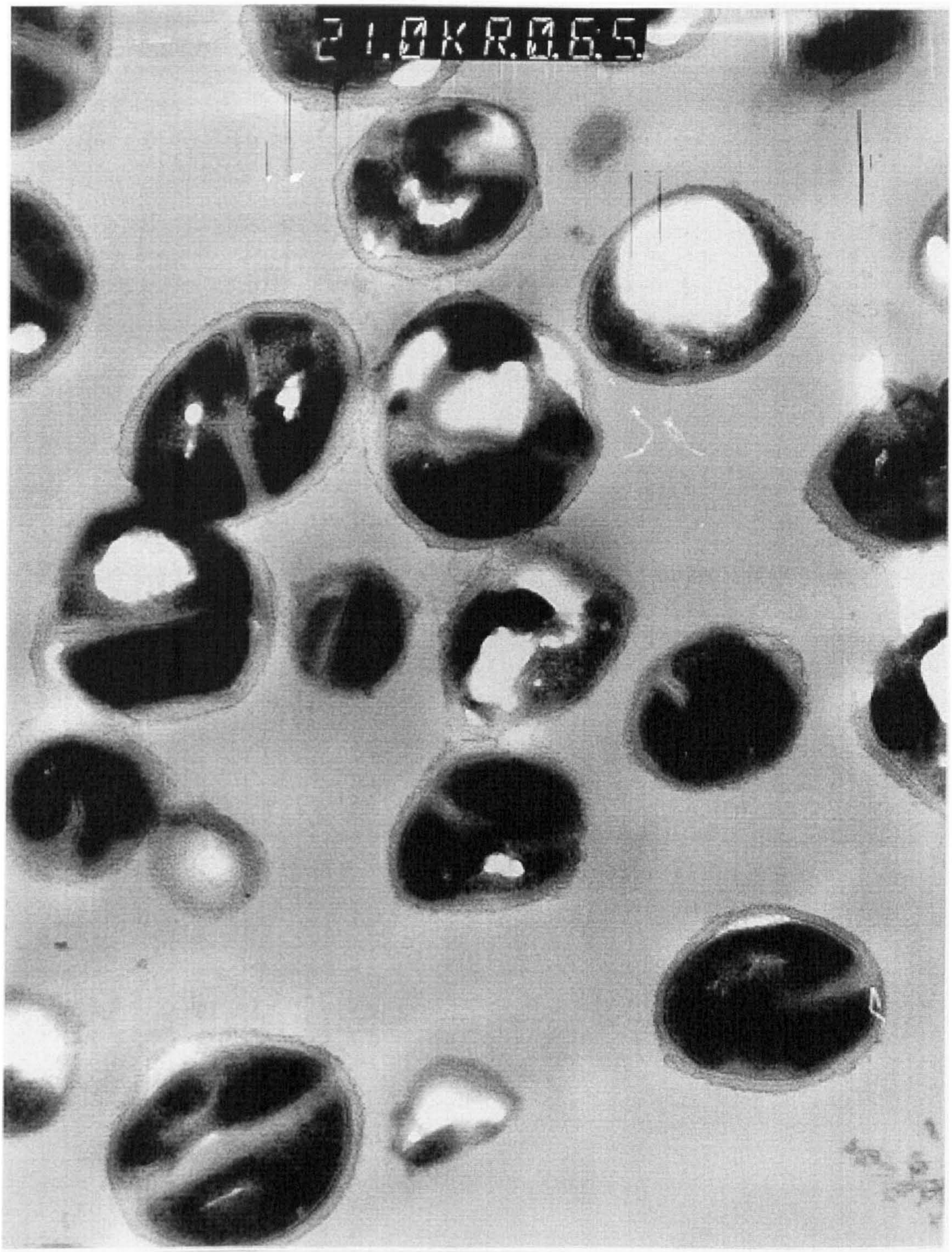


Figure 3.7: (continued).

C) An electron micrograph of *S. xylosus* cells grown in 2 M NaCl CDM in batch culture. Magnification × 42,000.

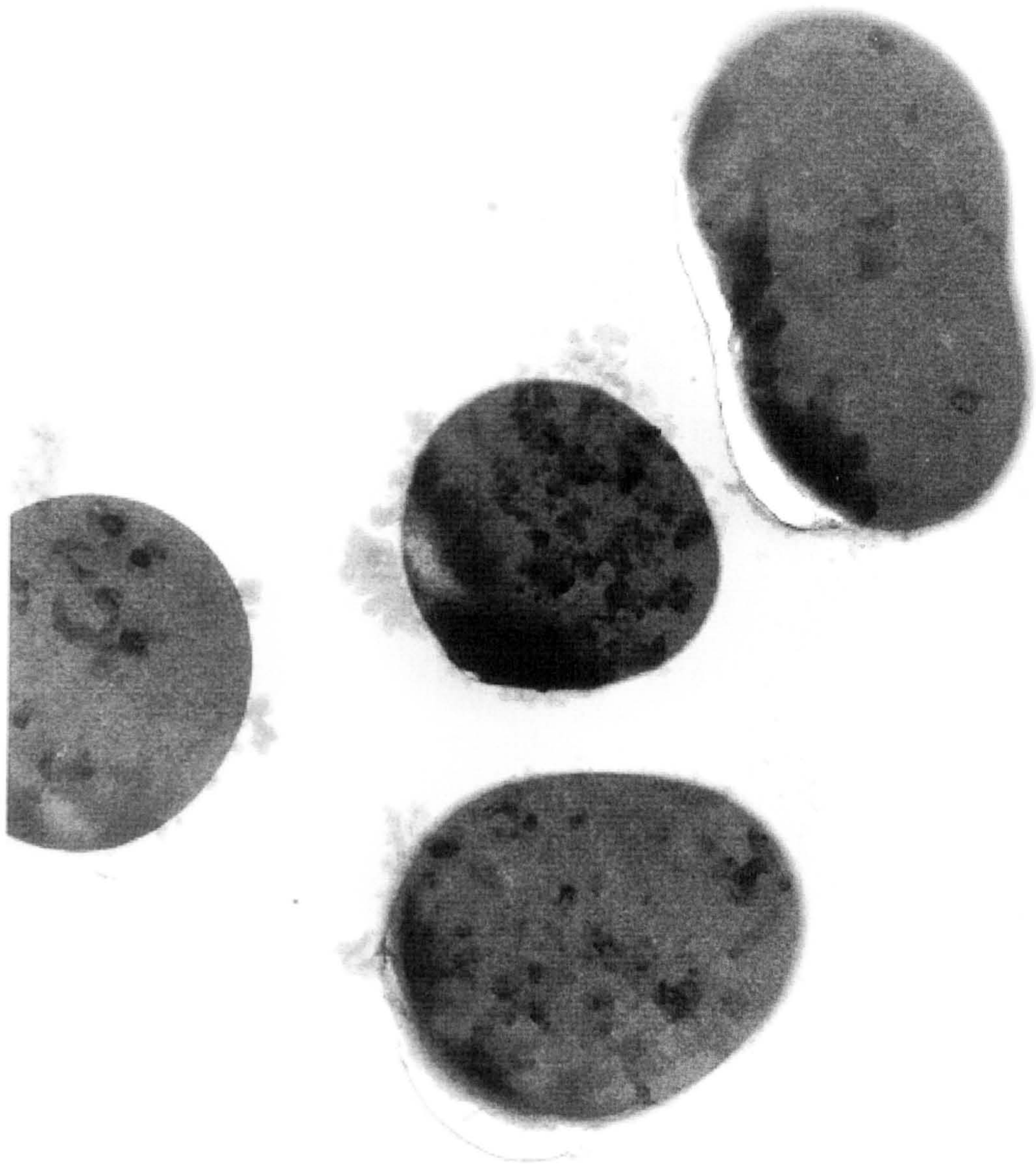


Figure 3.7: (continued).

D) An electron micrograph of *S. xylosus* cells grown in 3 M NaCl CDM in batch culture. Magnification × 42,000.

3.2.5 Effect of KCl on Growth of *Halomonas* and *S. xylosus*

Figure 3.8 shows that *S. xylosus* grows well in the absence of NaCl with up to 0.5 M KCl (g = 1.4 h) in the medium. The presence of 2 M KCl (g = 1.8 h) in the medium significantly reduced the growth rate, but after a lag phase of about 3 hours growth started and reached the same final OD as growth at 0.1 or 0.5 M KCl (Figure 3.8). Growth is very limited at 3 M KCl (g = 4.8 h), with a lag phase of 9 hours.

Figure 3.9 shows that *Halomonas* Halo behaves in a similar to *S. xylosus* when subjected to increasing levels of KCl in the absence of NaCl. It is worth noting that growth of *Halomonas* Halo is slower than *S. xylosus* at 0.1 M (g = 1.7 h), 0.5 M (g = 3.1 h), 2 M (g = 3.6 h) and 3 M KCl (g = 9 h) (Figures 3.8 and 3.9). This may indicate a stronger requirement for NaCl for growth of *Halomonas* Halo.

3.2.6 Effect of KCl and NaCl on Growth of *Halomonas* Halo and *S. xylosus*

Figures 3.10 and 3.11 show the effect of KCl and NaCl on the growth of *S. xylosus* and *Halomonas* Halo respectively. In these experiments, OD readings were only taken after 24 and 48 hours which makes it difficult to directly compare the results with Figures 3.8 and 3.9. However, it is clear that after 24 hours in 1.5 M NaCl and 1.5 M KCl, both *S. xylosus and*

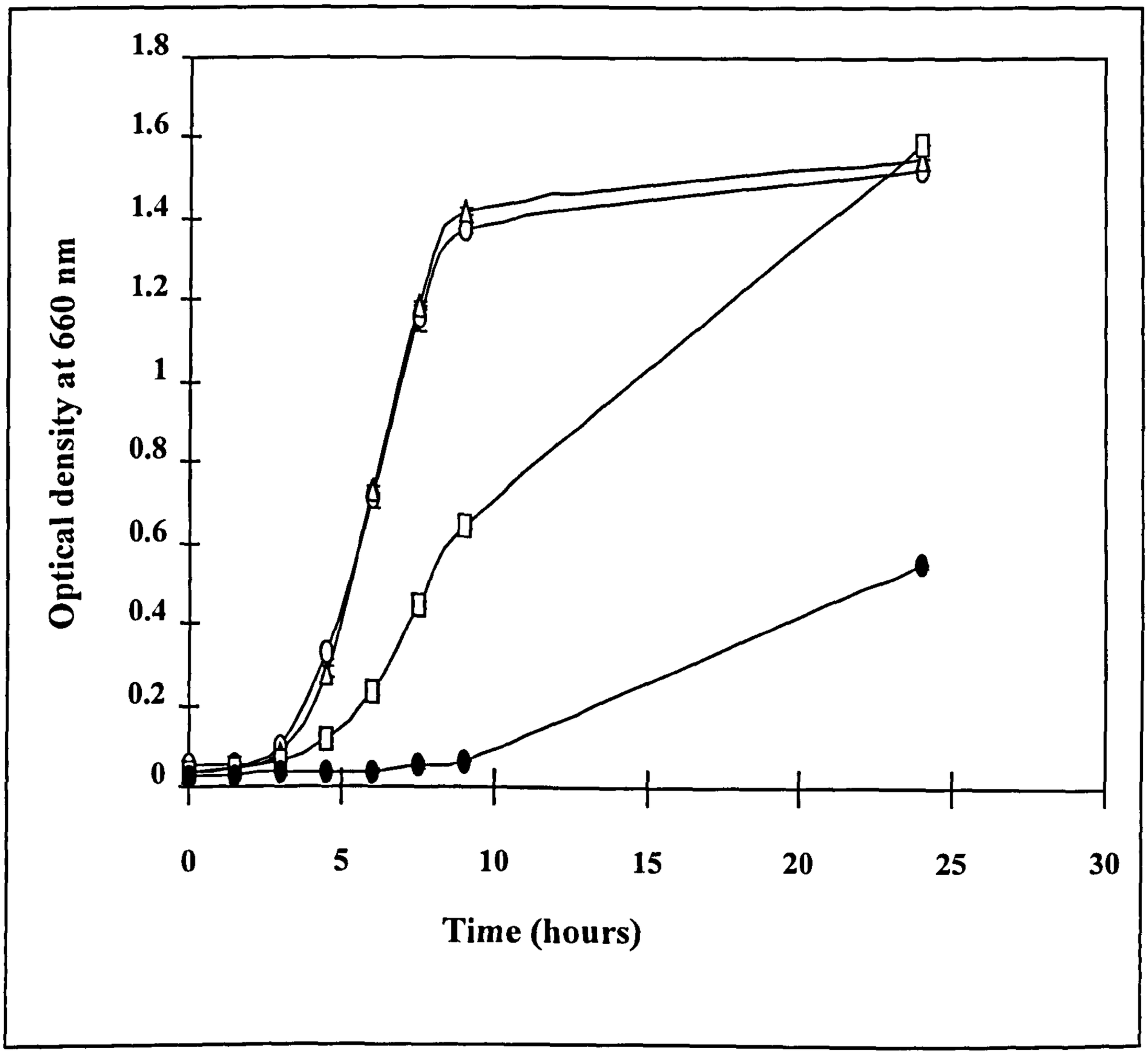


Fig: (3.8) Effect of potassium chloride (KCl) on the growth of *Staphylococcus xylosus*. The cells were grown on *Halomonas* defined medium + 1g l^{-1} yeast extract at different salinities of KCl (NaCl free).

—o— 0.1 M KCl —Δ— 0.5 M KCl —□— 2 M KCl —•— 3 M KCl

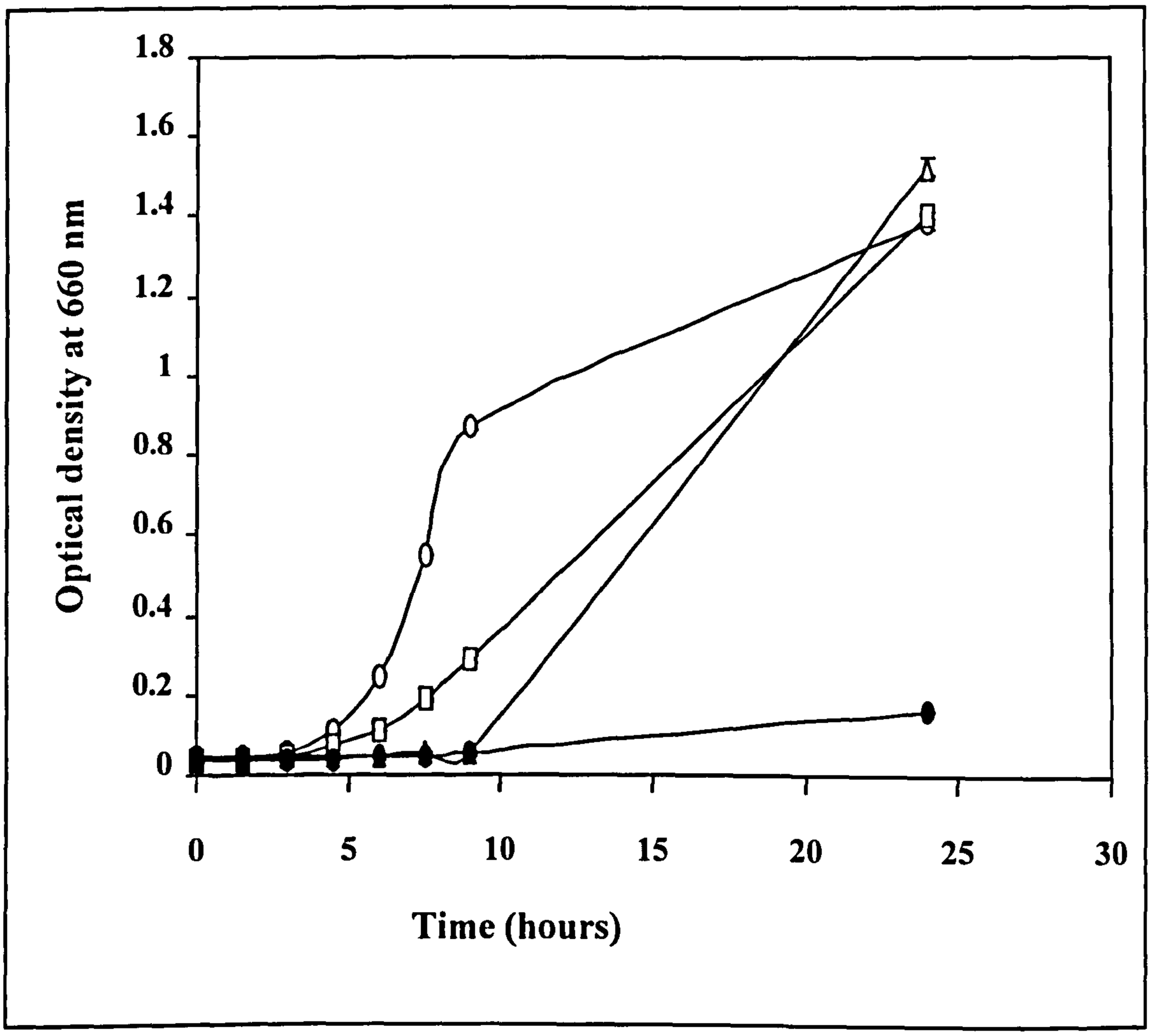


Fig: (3.9) Effect of potassium chloride (KCl) on the growth of *Halomonas* Halo. The cells were grown on *Halomonas* defined medium + 1g l^{-1} yeast extract at different salinities of KCl (NaCl free).

—o— 0.1 M KCl —Δ— 0.5 M KCl —□— 2 M KCl —●— 3 M KCl

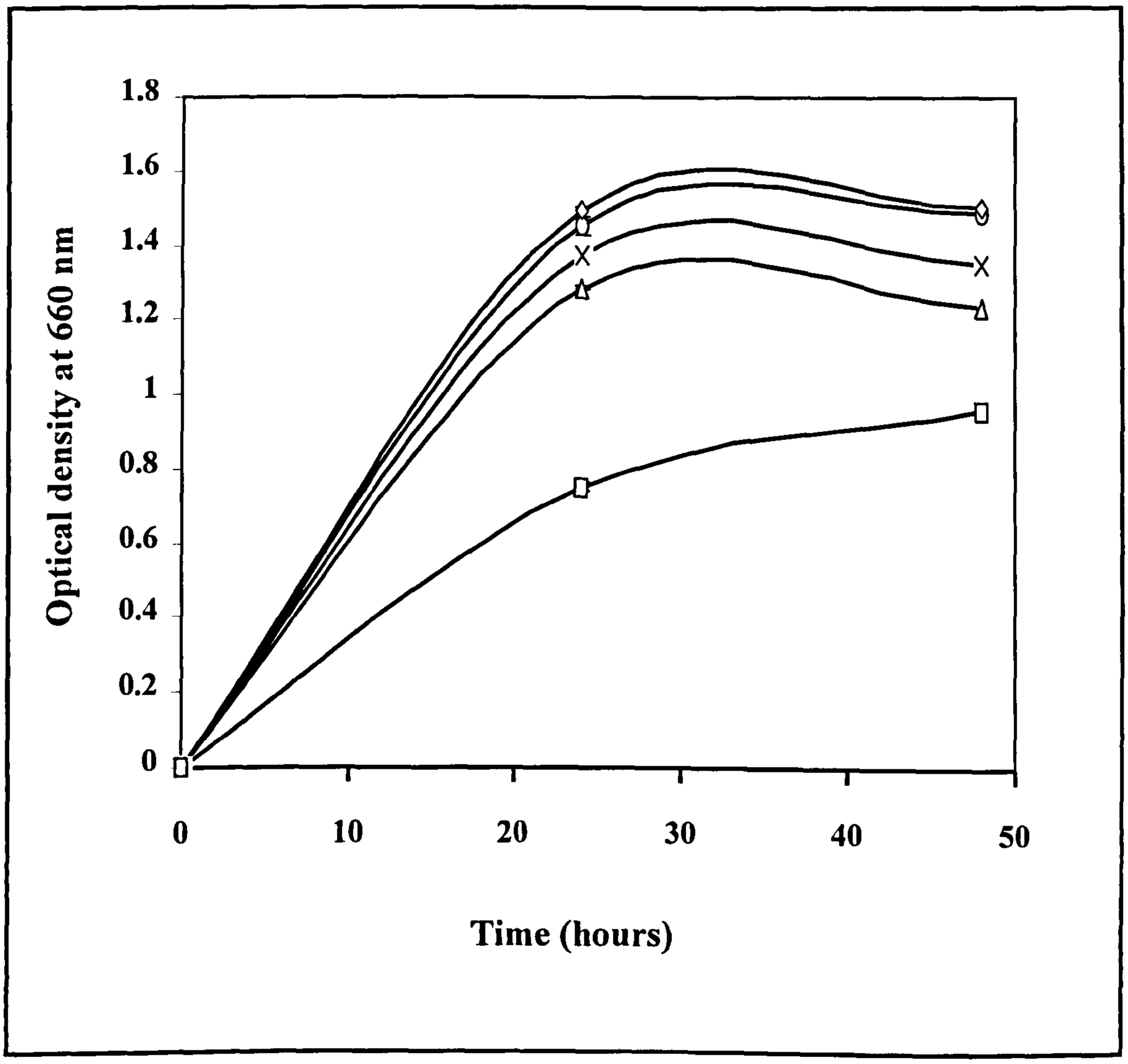


Fig: (3.10) Effect of sodium and potassium on the growth of *Staphylococcus xylosus* at different salinities on *Halomonas* defined medium + 1 g l^{-1} yeast extract. —o— 0.05 M NaCl + 0.05 M KCl, —◊— 0.25 M NaCl + 0.25 M KCl,—×— 0.5 M NaCl + 0.5 M KCl, —Δ— 1 M NaCl + 1 M KCl, —□—1.5 M NaCl + 1.5 M KCl

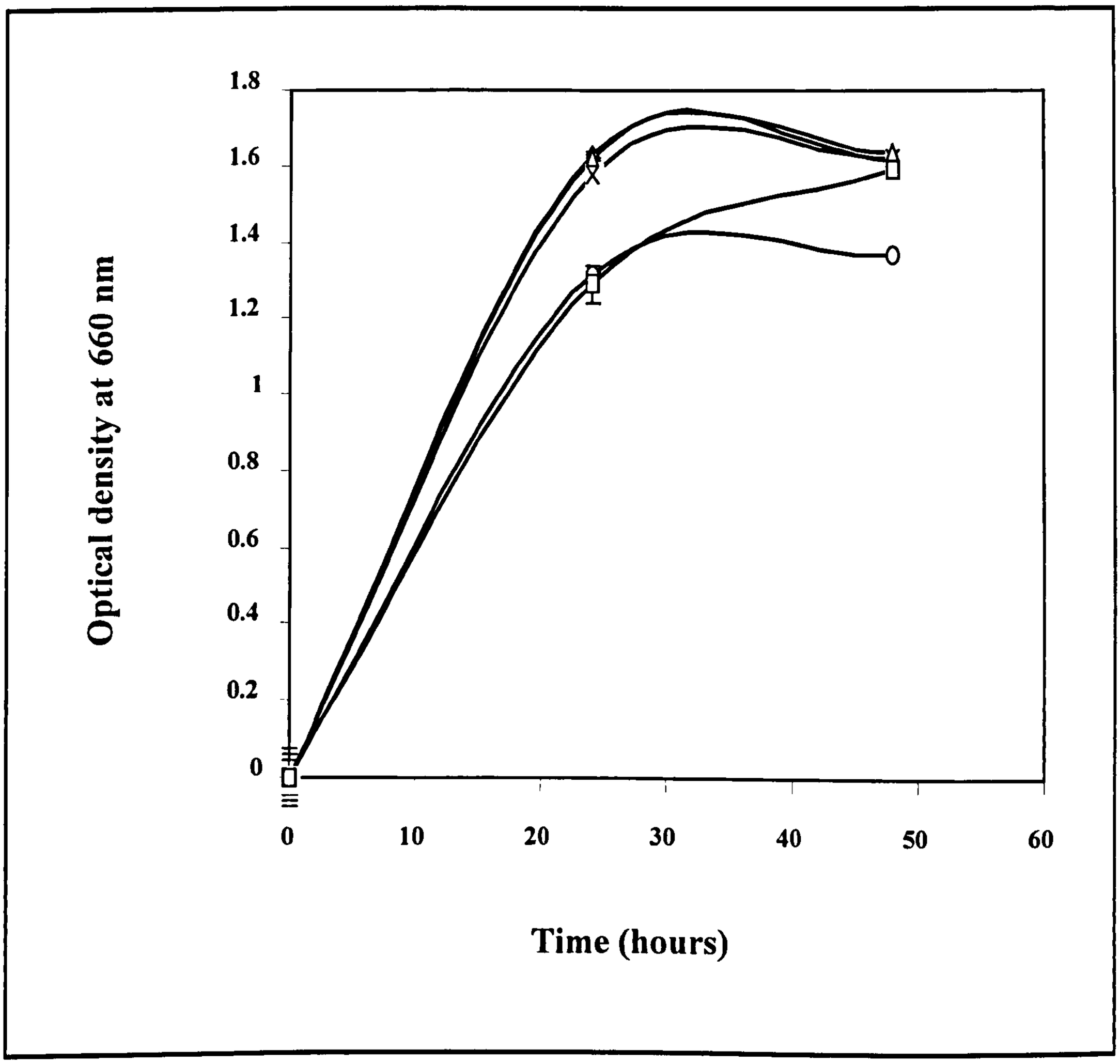


Fig: (3.11) Effect of sodium and potassium on the growth of *Halomonas* Halo at different salinities on *Halomonas* defined medium + 1 g l^{-1} yeast extract. —o—0.05 M NaCl + 0.05 M KCl, —◊— 0.25 M NaCl + 0.25 M KCl —×— 0.5 M NaCl + 0.5 M KCl, —Δ— 1 M NaCl, + 1 M KCl —□— 1.5 M NaCl + 1.5 M KCl

Halomonas Halo showed higher cell densities than when they were exposed to 3 M KCl (Figures 3.8 - 3.11). This effect is particularly evident for *Halomonas* Halo again indicating its greater requirement for NaCl.

3.2.7 Na^+ Requirement for Respiration in *Halomonas* Halo and *S. xylosus*

The difference in response to the presence of NaCl shown by *S. xylosus* and *Halomonas* may be due to a specific requirement for Na^+ ions. To investigate this possibility, the respiration rate of both organisms was measured in the presence of mM amounts of NaCl to find out the minimum requirement that these organisms have for NaCl (Figure 3.12). For *Halomonas* when the NaCl concentration was increased from 0 - 100 mM the rate of respiration also increased suggesting that this strain does require salt for proper functioning of respiration and therefore for good growth. On the other hand, for *S. xylosus* when the NaCl concentration was increased the respiration rate decreased, this means that this strain probably does not require salt for growth (Figure 3.12).

3.2.8 Effect of KCl on the Respiration Rate of *Halomonas* Halo and *S. xylosus*

S. xylosus cells were resuspended in different amounts of KCl and the respiration rate was measured immediately and after 1.5 and 3 hours (Figure 3.13). The results show that the respiration rates of *S. xylosus* cells resuspended in 0.1 M KCl or 0.5 M KCl were high and increased slightly over the three hour period of the experiment. However, the rate of respiration was

much lower for cells resuspended in 2 M or 3 M KCl, and it showed no sign of increasing during the 3 hour measurement period.

Figure 3.14 shows that essentially the same results were found for *Halomonas* when cells were exposed to KCl for 3 hours. The only difference was that the respiration rate of *Halomonas* Halo cells resuspended in 0.1 or 0.5 M KCl increased significantly over the course of the experiment (Figure 3.14).

3.2.9 Effect of NaCl and KCl on the Respiration Rates of *Halomonas* Halo and *S. xylosus*

Figures 3.15 and 3.16 show that initially there is a large effect on the respiration rates of both organisms at high concentrations of salts (3 M NaCl and 1.5 M NaCl + 1.5 M KCl) compared to cells resuspended in 0.5 M NaCl. However for both organisms the rate of respiration declined in 0.5 M NaCl over a 24 hour period, and in the case of *S. xylosus* cells the respiration rate under all three conditions was similar after 24 hours incubation (Figures 3.15 and 3.16).

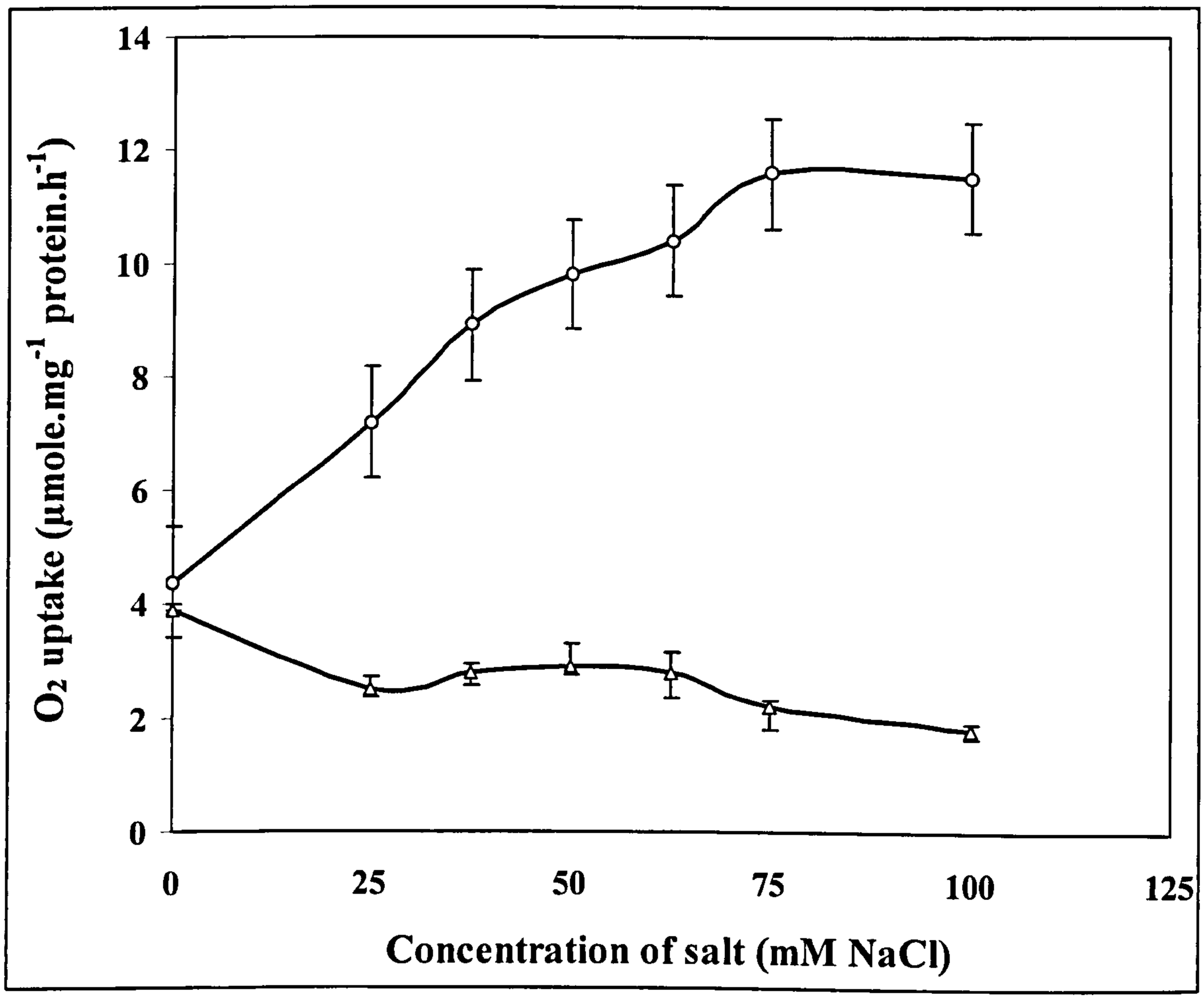


Fig: (3.12) Na^+ requirement for respiration *in Halomonas* halo and *Staphylococcus xylosus*. —o— *Halomonas* —Δ— *S. xlosus*.

The cells were resuspended in HDM + 1 g l^{-1} yeast extract with different concentrations of NaCl and rate of respiration was measured immediately.

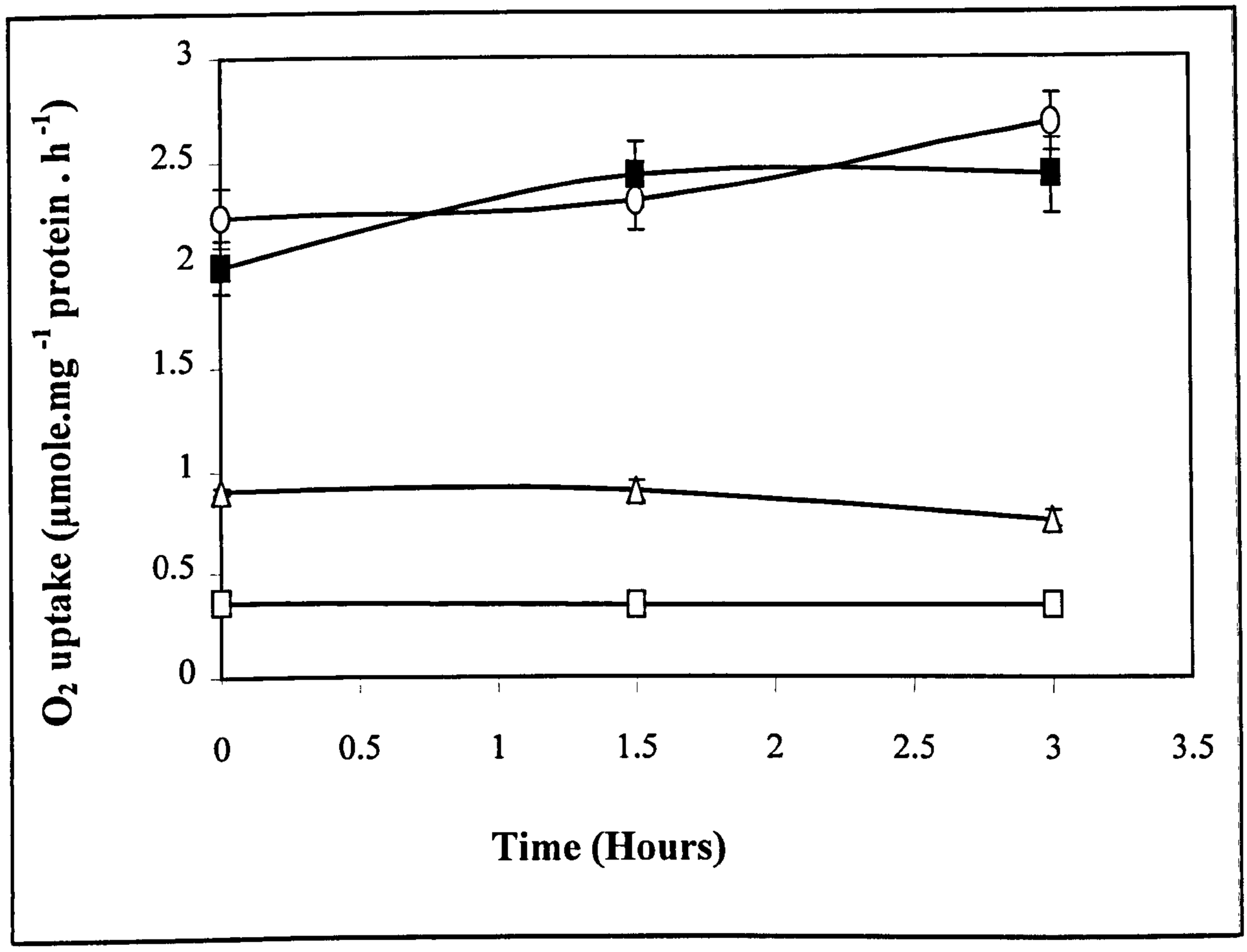


Fig: (3.13) Effect of potassium chloride (KCl) on the respiration rate of *Staphylococcus xylosus*. The cells were grown overnight on HDM + 1 g l^{-1} yeast extract at different salinities of KCl (NaCl free) and resuspended in the same salinity and rate of respiration was measured.

—○— 0.1 M KCl —■— 0.5 M KCl —Δ— 2 M KC —□— 3 M KCl

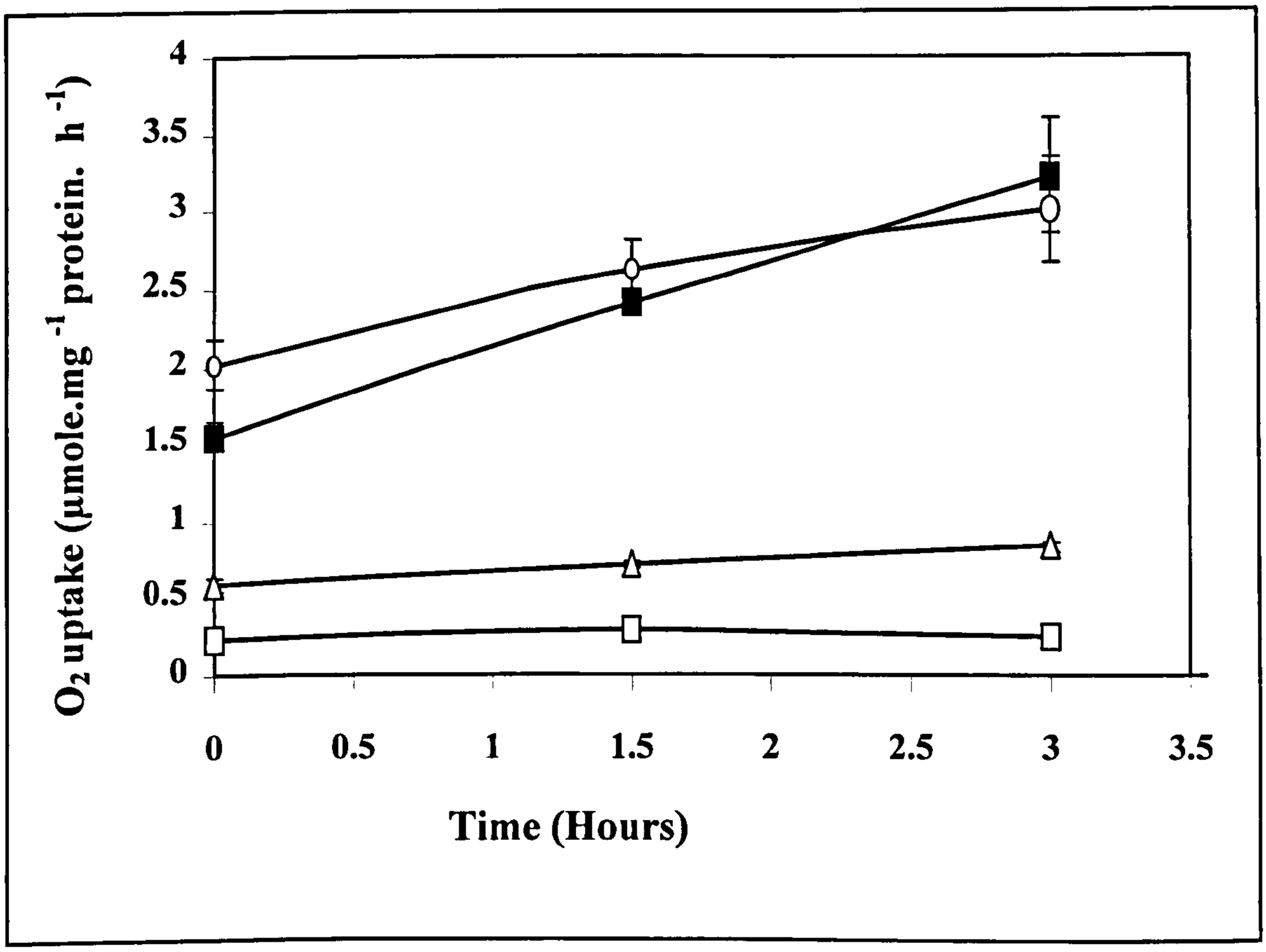


Fig: (3.14) Effect of potassium chloride (KCl) on the respiration rate of *Halomonas* Halo. The cells were grown overnight on HDM + 1 g l^{-1} yeast extract at different salinities of KCl (NaCl free) and resuspended in the same salinity and rate of respiration was measured.

—○— 0.1 M KCl —■— 0.5 M KCl —Δ— 2 M KCl —□— 3 M KCl

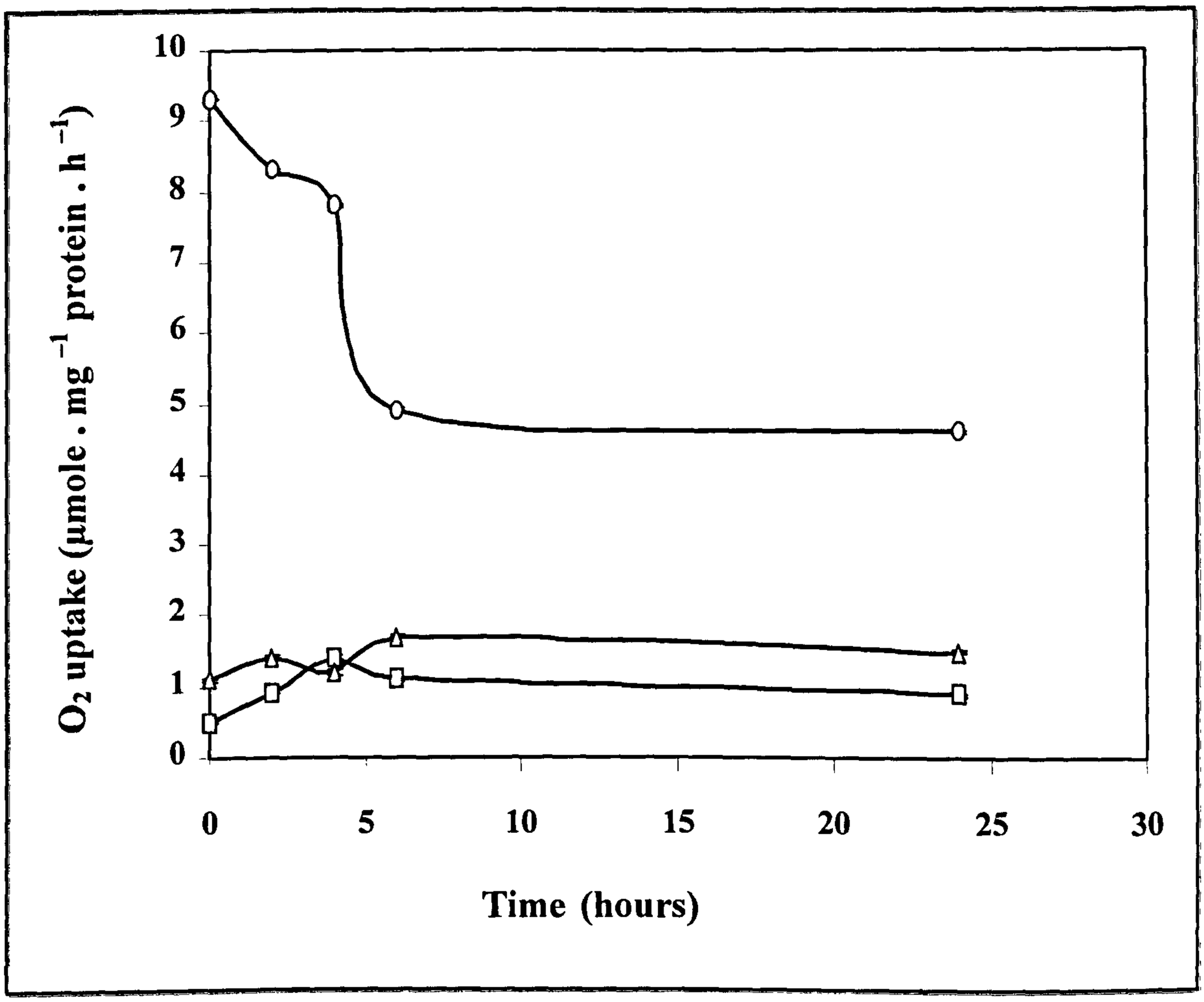


Fig: (3.15) Effect of NaCl and KCl on the respiration rate of *Halomonas* Halo.

—o— 0.5 M NaCl —□— 3 M NaCl —Δ— 1.5 M NaCl + 1.5 M KCl

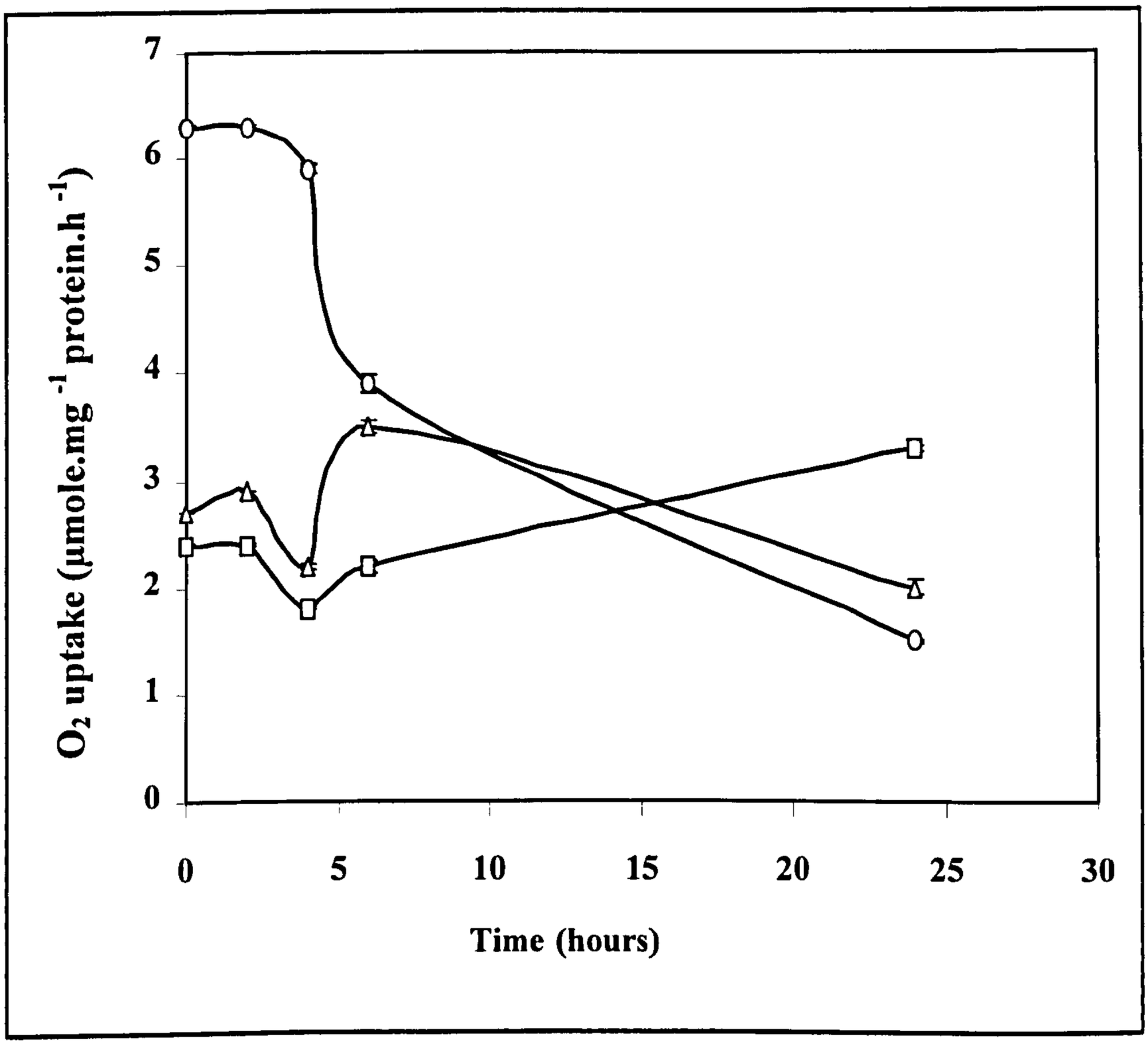


Fig: (3.16) Effect of NaCl and KCl on the respiration rate of *Staphylococcus xylosus*. —o— 0.5 M NaCl —□— 3 M NaCl —Δ— 1.5 M NaCl + 1.5 M KCl

3.3 DISCUSSION

Halomonas Halo had been studied by a previous PhD student (Cummings, 1991). It was originally isolated as a contaminant of a culture of the halotolerant alga *Dunaliella* and the strain was designated "Duncon" due to its provenance. By the time the present work started in 1995, there were three separate strains of *Halomonas* kept in the laboratory, but it was unclear whether or not these strains were significantly different. It was decided to test their salt tolerance and the results in Table 3.1 showed clearly that *Halomonas* strain "Halo" was the most salt tolerant and therefore this strain was used for all future work.

Strain JDP14 was isolated from a soil sample using LB medium containing up to 3 M NaCl. This strain was identified as *Staphylococcus xylosus* using API STAPH kit. To confirm this identification, it would be advisable to check the 16s rRNA sequence. The original description of *S. xylosus* (Schleifer and Kloos, 1975) stated that it is a Gram-positive, non-motile, non-spore forming, coccus-shaped bacterium, occurring singly and in pairs. Cells were about 1.2 μm in diameter and colonies were raised to slightly convex and yellow/orange in colour. This description agrees very well with the description of JDP14 given in section 3.2.2 with only minor differences in colony morphology. Schleifer and Kloos (1975) further state that *S. xylosus* is facultatively anaerobic and JDP14 was found to be strictly aerobic. However, Schleifer and Kloos acknowledge that anaerobic growth of *S. xylosus* is poor.

Two species of staphylococci, which are closely related to *S. xylosus*, were also described by Schleifer and Kloos (1975) i.e. *S. cohnii* and *S. haemolyticus*. *S. xylosus* can be differentiated from both of these species by its ability to metabolise xylose and this is also a feature of JDP14 (Table 3.2). Therefore, the evidence is very strong that JDP14 is indeed a strain of *S. xylosus.*

S. xylosus has been isolated from dry cured Iberian ham (Rodriguez *et al.*, 1996) and from fully cured and dried cod (Vilhelmsson *et al.*, 1997). This was of particular interest because of their enterotoxigenic potential in these foodstuffs. It also demonstrated the ability of *S. xylosus* to grow at very low water activities.

The next step in the present work was to compare the salt tolerance of *Halomonas* Halo with *S. xylosus*. Figures 3.1 and 3.2 show that *S. xylosus* grew better at 3 M NaCl than *Halomonas* Halo in the rich medium used. Both organisms showed near optimal growth at 0.1-0.5 M NaCl, but there was a significant reduction in growth rate at 2 M NaCl.

The strain of *S. xylosus* isolated from Iberian ham by Rodriguez *et al.* (1996) showed optimal growth at 0.5-0.9 M NaCl, with good growth at 0.17, 1.7 and 2.6 M NaCl. Growth was very poor at 3.4 M NaCl and there was no growth above this salinity. It appears that the Iberian ham isolate is slightly more salt tolerant than our soil isolate of *S. xylosus* which did not grow above 3 M NaCl. In contrast the strain of *S. xylosus* isolated from dried cod by

Vilhelmsson *et al.* (1997) is significantly more salt tolerant showing near optimum growth between 0.05 and 2 M NaCl and growing, albeit slowly, at 4.5 M NaCl. Therefore, it appears that the salt tolerance of strains of *S. xylosus* depends on the environment from which they were isloated.

The salt tolerance of *Halomonas* strains has been well studied by Vreeland and co-workers, in particular *H. elongata* has been shown to tolerate essentially the complete range of salinities from 0.05-5.5 M NaCl (Vreeland, 1987). However, it is interesting to note that in most publications from Vreeland's group, the maximum salinity that *H. elongata* is exposed too is 3.4 M NaCl, implying that growth is very slow above this salinity (Vreeland *et al.*, 1983; Hart and Vreeland, 1988). This agrees well with the previous Sheffield work on *Halomonas* Duncon, which had a maximum salinity for growth of 3.25 M NaCl (Cummings and Gilmour, 1995). In the present work, *Halomonas* Halo had a similar growth range to the published values for *Halomonas* Duncon (Figure 3.1).

Another important factor which determines the salinity range of *Halomonas* species is the composition of the medium. In the experiments reviewed by Vreeland (1987), H. elongata was grown in rich medium and grew in salinities from 0.05 - 5.5 M NaCl. However, Canovas *et al.* (1996) showed that in minimal medium H. elongata required at least 0.5 M NaCl for growth and no growth was found above 3 M NaCl. In the present work *Halomonas* Halo grew poorly above 3 M NaCl even though yeast extract was present in the medium.

The Na^+ requirement for growth of *Halomonas* Halo was investigated by replacing NaCl in the medium with KCl (Figure 3.9). Due to the presence of yeast extract in the medium, it was not completely Na^+ free, but the results did demonstrate that mM amounts of Na^+ could support good growth of *Halomonas* Halo in the presence of up to 2 M KCl (Figure 3.9). This was fairly surprising since such high levels of K^+ are usually toxic to cells especially in the virtual absence of Na^+ (Ventosa *et al.*, 1998). In the presence of 3 M KCl, growth of *Halomonas* Halo was very poor (Figure 3.9), but when half of the KCl was replaced with NaCl, growth rate was completely restored (Figure 3.11). Clearly, the balance between the external concentrations of Na^+ and K^+ is important for good growth of *Halomonas* Halo.

To further investigate the requirement of Na^+ for growth of *Halomonas* Halo, the effect of Na^+ on respiration rate was measured (Figure 3.12). It is clear that as the Na^+ concentration increased from 0 - 100 mM, the rate of respiration significantly increased. Therefore, *Halomonas* Halo appears to have a requirement for Na^+ for optimal growth, but that growth is possible in the presence of low concentrations of Na^+.

As part of the identification procedure for *S. xylosus,* a series of electron micrographs were prepared of cells growing in different salinities. Figures 3.3-3.6 showed that *S. xylosus* cells grown at 0.1, 0.5, 2, and 3 M NaCl in HDM + 1 g l^{-1} yeast extract medium had no obvious morphological

differences apart from the cells becoming smaller as the salinity increased. On the other hand Figure 3.7 provides evidence that *S. xylosus* cells grown at 0.1, 0.5, 2 and 3 M NaCl in the chemically defined medium increased in size with increasing salinity. This contradiction may be explained by the different rates of growth in the rich HDM medium compared to the defined medium, because cells were grown over a 48 hour period in the defined medium against only 18-24 hours in the rich medium. More experiments are required to resolve this problem.

In a parallel set of experiments to those already discussed for *Halomonas* Halo, the requirement of Na^+ for growth of *S. xylosus* was investigated. Figure 3.8 shows that *S. xylosus* grows very well in the absence of NaCl with up to 0.5 M KCl in the medium. The presence of 2 M KCl in the medium significantly reduced the growth rate and growth was very limited at 3 M KCl, nevertheless *S. xylosus* grew significantly better in all concentrations of KCl than *Halomonas* Halo (Figures 3.8 and 3.9). This indicates that *S. xylosus* has a lesser requirement for Na^+ than *Halomonas* Halo.

The data shown in Figures 3.10 and 3.11 slightly contradicts this conclusion, because in the presence of equimolar amounts of NaCl and KCl up to 3 M total salinity, growth of *Halomonas* Halo was better than that of *S. xylosus*. On the other hand, the conclusion is strongly supported by the lack of any positive effect on the respiration rate of *S. xylosus* by increasing the Na^+ concentration from 0 - 100 mM (Figure 3.12). On balance, it appears that *S. xylosus* does not require Na^+ to be present in the medium above trace levels.

In a final set of experiments the effects of KCl and equimolar KCl/NaCl solutions on the respiration rate of both *Halomonas* Halo and *S. xylosus* were examined (Figures 3.13 - 3.16). The results followed the same pattern as the growth experiments with both organisms showing high respiration rates at 0.1 and 0.5 M KCl and much lower respiration rates at 2 and 3 M KCl (Figures 3.13 and 3.14). The final two figures (3.15 and 3.16) followed the respiration rates of both organisms over a 24 hour period. At the beginning respiration rates at 0.5 M NaCl were much higher than those at 3 M NaCl or at 1.5 M NaCl + 1.5 M KCl. This remained true for *Halomonas* Halo after 24 hours (Figure 3.15), but for *S. xylosus* the respiration rates in all three salinities were similar after 24 hours (Figure 3.16). More experiments will be required to fully interpret these results.

It can be concluded from the work described in this chapter that both *Halomonas* Halo and *S. xylosus* are halotolerant organisms growing in salinities up to 3 M NaCl. They appear to differ in their requirement for Na^+, with only *Halomonas* Halo definitely requiring mM levels for optimum growth. In the next chapter, the physiological mechanisms used by *Halomonas* Halo and *S. xylosus* to grow at high salinities will be compared.

CHAPTER 4

ROLE OF COMPATIBLE SOLUTES IN SALT TOLERANCE OF *HALOMONAS* HALO AND *STAPHYLOCOCCUS XYLOSUS*

4.1 Introduction

Microorganisms must be able to adapt to changes in the osmolarity of their environment. To adapt to these changes, bacteria accumulate some compounds, named compatible solutes, that confer protection against the deleterious effect of the low water activity (Galinski, 1995). Compatible solutes are best described as organic osmolytes responsible for osmotic balance and at the same time compatible with cell metabolism (Galinski, 1993).

The cytoplasmic membrane of bacteria is permeable to water, but forms an effective barrier for most solutes present in the medium and metabolites present in the cytoplasm. A lowering of the external water activity (hyperosmotic condition) causes a rapid efflux of water and loss of turgor; ultimately, the cells may plasmolyse, i.e. the cytoplasmic membrane may retract from the cell wall (Poolman and Glaasker, 1998). Bacterial cells that can adapt to increased salinities (lowered water potential) avoid the loss of water and turgor pressure by synthesising or accumulating a compatible

solute. The most important compatible solutes were described in section 1.5.1.

To cope with changes in the salt concentration of the environment, *H. elongata* is able to synthesise ectoine and hydroxyectoine and to take up a variety of organic compounds which can serve as osmoprotectants when present externally (Galinski, 1995). Accordingly, exogenous glycine betaine (betaine), choline and choline-O-sulfate have been shown to play an osmoprotective role in *H. elongata* (Canovas *et. al*, 1996). Cummings and Gilmour (1995) found that a *Halomonas* species could grow in salinities up to 2 M NaCl with betaine as its sole carbon source, this implies a dual role for betaine as compatible solute and carbon source. In other species of *Halomonas,* e.g. *H. halophila*, intracellular betaine concentration increased with salinity (del Moral *et. al.*, 1991).

Accumulation of compatible solutes by *Staphylococcus aureus* in response to hyperosmotic stress has been examined in some detail over the past 10 years (Graham and Wilkinson, 1992). Transport systems for betaine (Pourkomailian and Booth, 1994) and proline (Bae and Miller, 1992) are activated, choline transport is induced (Kaenjak *et. al*, 1993), and betaine and proline accumulate to high concentrations (Townsend and Wilkinson, 1992). Further proline producers are found among *Staphylococcus* species (da Costa *et al.*, 1998).

The osmoregulatory role of choline and its derivative betaine has been investigated exclusively at the physiological level in staphylococci. Choline enters the staphylococcal cell by a specific uptake system (Kaenjak *et. al*, 1993) and is subsequently converted to betaine, a potent osmoprotectant in *S. aureus* (Graham and Wilkinson, 1992) and in *S. xylosus* (Rosenstein *et. al*, 1999). High salinity induces an increase in cell size, changes to the structure of peptidoglycan and the formation of pseudomulticellular cells in *S. aureus*. All of these abnormal morphological changes can be reversed by adding betaine to the medium (Vijaranakul *et al.*, 1995; 1997).

In the present chapter, the effect of compatible solutes, in particular betaine, on the growth of *Halomonas* Halo and *S. xylosus* will be investigated.

4.2 Results

4.2.1 Growth of *S. xylosus* and *Halomonas Halo* on Chemically Defined Medium at Different Salinities.

Figure 4.1 shows that *S. xylosus* grows optimally at salinities up to 0.5 M NaCl in the chemically defined medium. Growth was also good at 2 M NaCl after a 6 hour lag phase. However, growth is very limited at 3 M NaCl (Figure 4.1). *Halomonas* Halo showed slower growth and longer lag phases than *S. xylosus* on chemically defined medium containing up to 0.5 M NaCl (Figure 4.2). Due to this slower growth at low salinities, there was little difference in growth rate for *Halomonas* Halo up to 2 M NaCl. Interestingly, growth of *Halomonas* Halo on defined medium at 3 M NaCl was, although slow, better than *S. xylosus* at 3 M NaCl (Figures 4.1 and 4.2)

To understand the significance of these results, they must be compared with Figures 3.1 and 3.2 respectively, because in these earlier figures both organisms were exposed to the same salinities in the presence of yeast extract. It is clear from Figures 3.2 and 4.1 that *S. xylosus* grows very poorly at 3 M NaCl in the absence of yeast extract. However, a comparison of Figures 3.1 and 4.2 shows a much less significant effect of yeast extract on the growth of *Halomonas* Halo at 3 M NaCl.

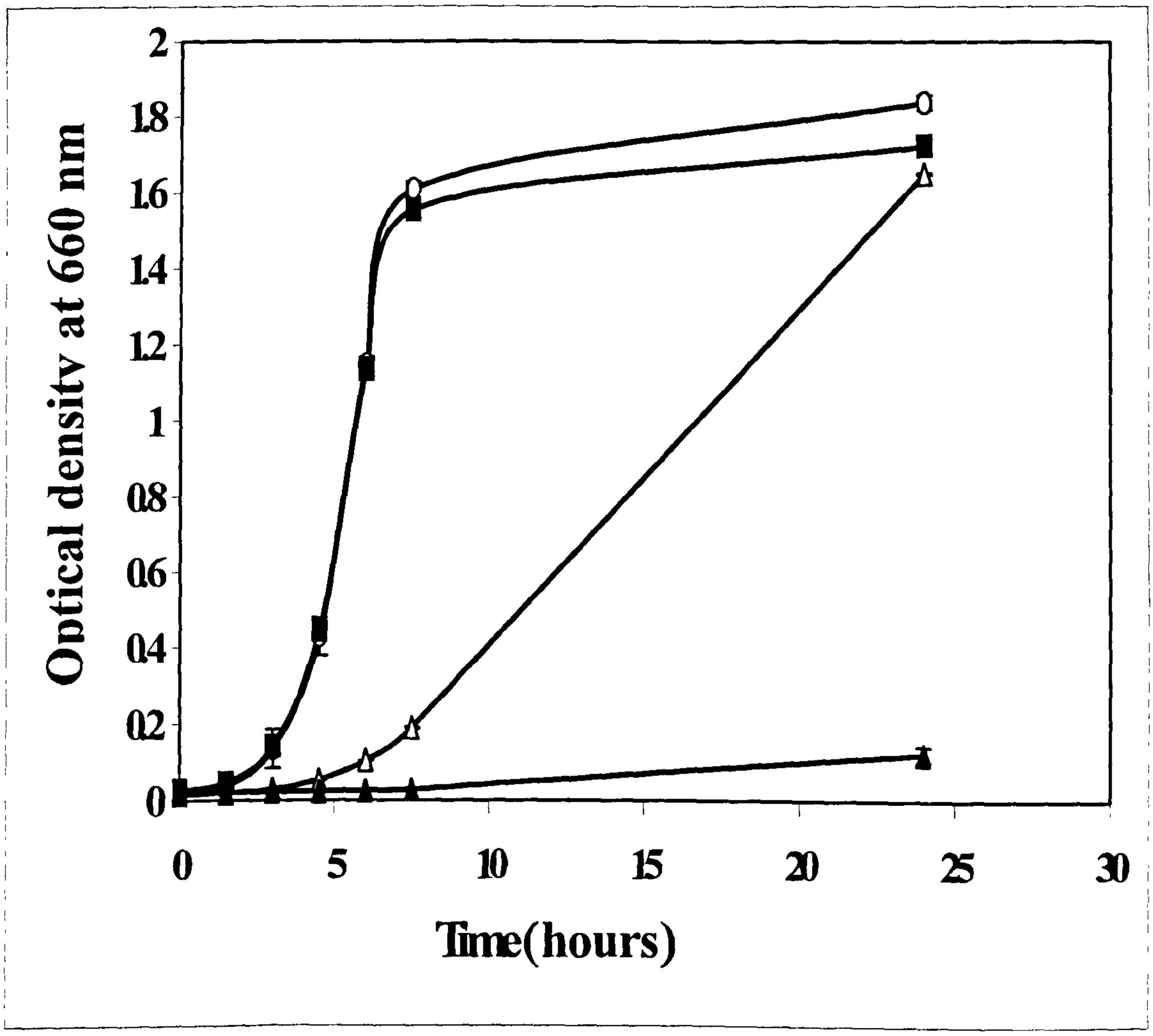


Fig: (4.1) The growth of *Staphylococcus xylosus* on chemically defined medium at different salinities. —o— 0.1 M NaCl —■— 0.5 M NaCl —Δ— 2 M NaCl —▲— 3 M NaCl

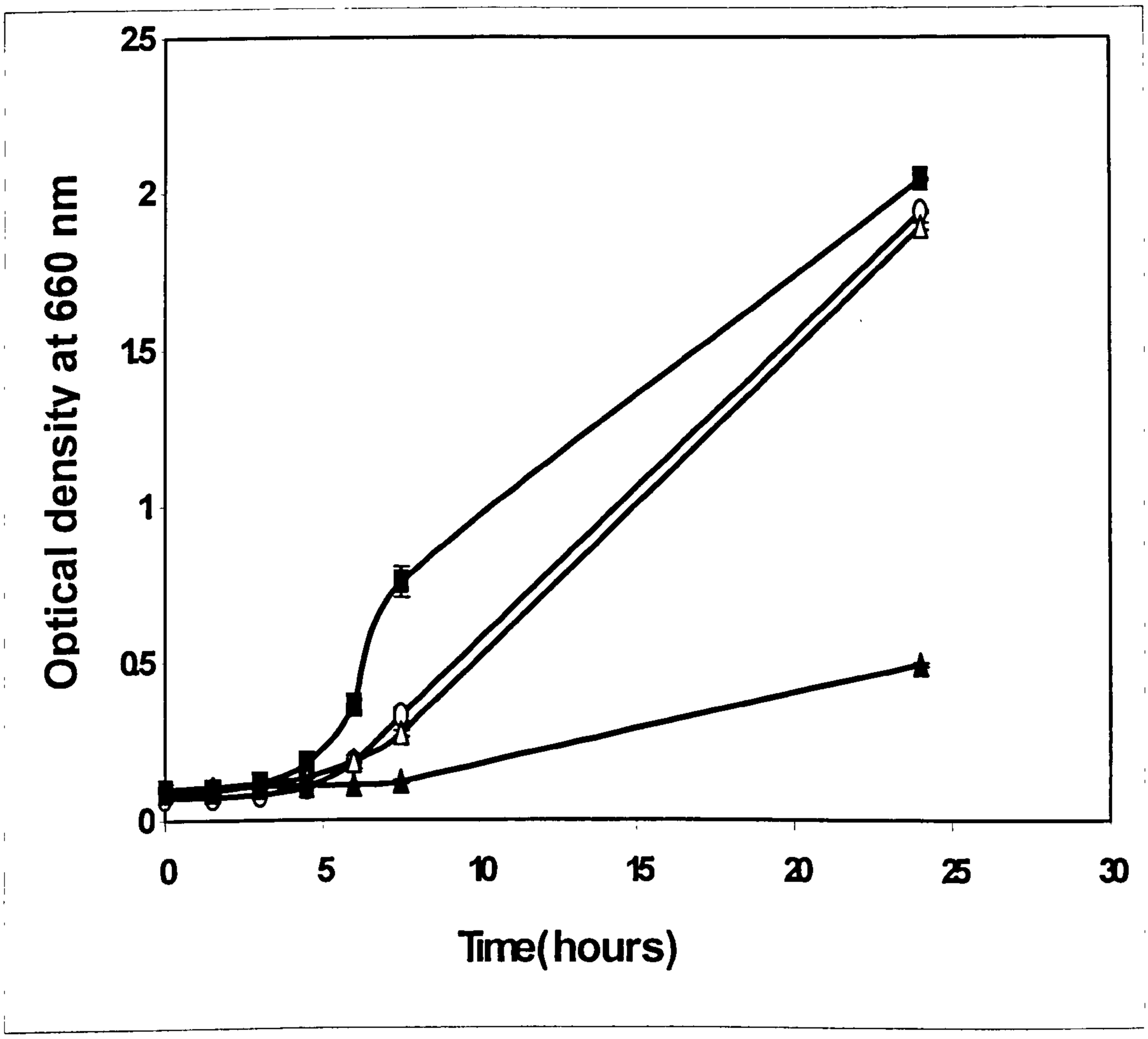


Fig: (4.2) The growth of *Halomonas* Halo on chemically defined medium at different salinities —O— 0.1 M NaCl —■— 0.5 M NaCl —Δ— 2 M NaCl —▲— 3 M NaCl

4.2.2 Effect of Compatible Solutes on Growth of *S. xylosus* and *Halomonas* Halo.

By using the chemically defined medium without the addition of yeast extract, the effect of a range of organic compounds was tested on the growth of *S. xylosus* at 3 M NaCl (Table 4.1). After 24 hours growth, it was clear that the well known compatible solute glycine betaine was the most effective compound for promoting growth of *S. xylosus* at 3 M NaCl. The precursor of betaine, choline, was also effective at stimulating growth but to a much leser extent (16 % simulation as against 113 % for betaine). The other three compounds tested (proline, alanine glutamine) actually decreased growth rate after 24 hours by about 50 %. After 48 hours, a similar trend was found, but with increasing time of incubation, the stimulation of betaine and choline began to decrease as the control culture "caught up" and after 120 hours incubation it had disappeared completely. This demonstrates that the addition of betaine and choline speeds up the growth at 3 M NaCl, but in their absence *S. xylosus* can growth and eventually reach the same maximum optical density (Table 4.1). The inhibition by the other compounds tested also decreased with time, but only in the case of glutamine has it virtually disappeared after 120 hours growth (Table 4.1).

Table 4.2 shows very similar results for *Halomonas* Halo except that the stimulation by betaine and choline after 24 hours growth was even more marked (over 200 % stimulation). Once again the other compounds tended to be inhibitory. At the end of 120 hours growth, all cultures had attained essentially the same optical density.

Table (4.1) Effect of compatible solutes on the growth of *Staphylococcus xylosus*. The cells were grown on 3 M NaCl chemically defined medium with 20 mM compatible solutes. The values are OD_{660} reading ± standard error (n = 3).

Time (h)	Control	Betaine	Choline	Proline	Alanine	Glutamine
24	0.286 ±0.02	0.608 ±0.04 112.6%	0.332 ±0.05 16.1%	0.144 ±0.01 -49.6%	0.146 ±0.01 -49.9%	0.126 ±0.004 -55.9%
48	0.752 ±0.09	1.237 ±0.06 64.5%	1.093 ±0.05 45.3%	0.356 ±0.02 -52.6%	0.338 ±0.02 -55%	0.351 ±0.03 -53.3
72	1.05 ±0.09	1.193 ±0.02 13.6%	1.157 ±0.02 10.2%	0.595 ±0.04 -43.3%	0.543 ±0.04 -48.3%	0.679 ±0.1 -35.3%
96	1.257 ±0.08	1.237 ±0.03 -1.6%	1.222 ±0.03 -2.8%	0.913 ±0.07 -27.3%	0.81 ±0.06 -35.5%	1.104 ±0.1 -12.2%
120	1.319 ±0.07	1.249 ±0.04 -5.3%	1.16 ±0.02 -12%	1.039 ±0.07 -21.2%	0.948 ±0.06 -28.1%	1.302 ±0.1 -1.3%

Table (4.2) Effect of compatible solutes on the growth of *Halomonas* Halo. The cells were grown on 3 M NaCl chemically defined medium with 20 mM compatible solutes. The values are OD_{660} reading ± standard error (n = 3).

Time (h)	Control	Betaine	Choline	Proline	Alanine	Glutamine
24	0.261 ±0.01	0.823 ±0.02 215.3%	0.81 ±0.04 210.3%	0.171 ±0.001 -34.5%	0.16 ±0.004 -38.7%	0.177 ±0.01 -32.2%
48	0.875 ±0.03	1.468 ±0.02 67.8%	1.493 ±0.03 70.6%	0.79 ±0.03 -9.7%	0.714 ±0.04 -18.4%	0.81 ±0.07 -7.4%
72	1.523 ±0.01	1.787 ±0.01 17.3%	1.716 ±0.02 12.7%	1.402 ±0.02 -7.9%	1.464 ±0.05 -3.9%	1.597 ±0.07 4.8%
96	1.802 ±0.01	1.875 ±0.01 4%	1.826 ±0.02 1.3%	1.705 ±0.02 -5.4%	1.775 ±0.04 -1.5%	1.887 ±0.05 4.7%
120	1.931 ±0.01	1.952 ±0.01 1.1%	1.883 ±0.02 -2.5%	1.903 ±0.02 -1.4%	1.923 ±0.02 -0.4%	1.957 ±0.05 1.3%

The results in Tables 4.1 and 4.2 suggest that betaine and choline are more effective compatible solutes for *Halomonas* Halo than for *S. xylosus.* This may be related to their ability to transport betaine into the cell and this hypothesis will be investigated in the next section.

4.2.3 Betaine Uptake by *Halomonas* Halo in The Presence or Absence of CCCP in 0.5 M NaCl Chemically Defined Medium.

The results in the previous section demonstrated that adding betaine into the medium resulted in better growth of *Halomonas* Halo at 3 M NaCl. This implies that betaine must be transported into the cells. Figure 4.3 shows that *Halomonas* Halo takes up betaine when grown at the relatively low salinity of 0.5 M NaCl. This figure also demonstrates that the uptake is dependent on energy, because transport is largely inhibited by the proton motive force inhibitor CCCP. In these experiments the cells were grown in the absence of betaine before harvesting and concentrating for the experiment. It was decided to repeat the experiment with cells grown in the presence of 20 mM cold betaine and Figure 4.4 shows that similar results were achieved. However, the presence of cold betaine in the culture medium and in the experimental medium led to a slower rate of betaine transport into the *Halomonas* Halo cells (Figures 4.3 and 4.4). It is also interesting that in the presence of cold betaine, the inhibition by CCCP was not complete (Figure 4.4).

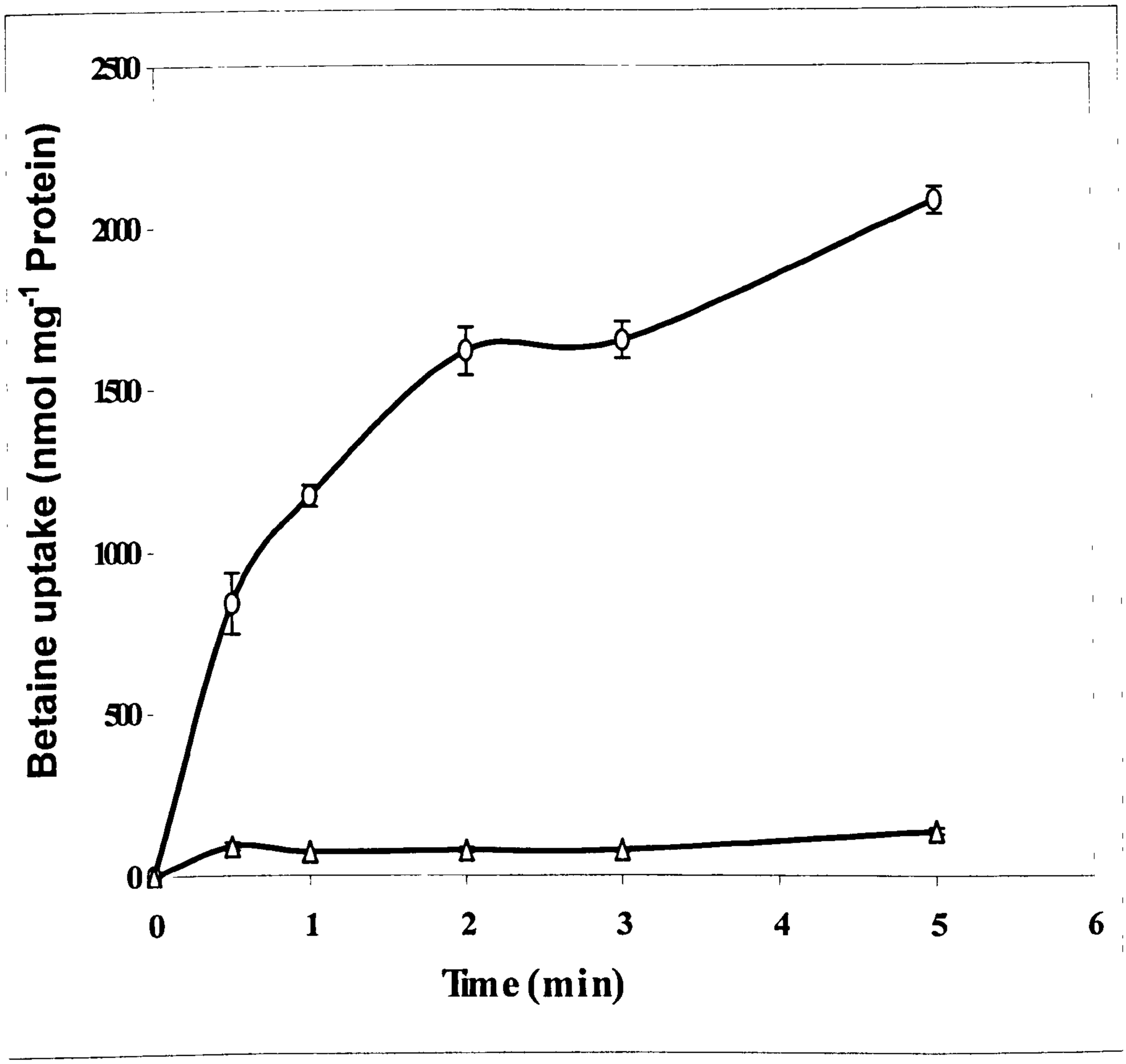


Fig: (4.3) Betaine uptake by *Halomonas* Halo in the presence or absence of 50 µM CCCP. The cells were grown in 0.5 M NaCl chemically defined medium in the absence of betaine. —o— Control. —Δ— 50 µM CCCP

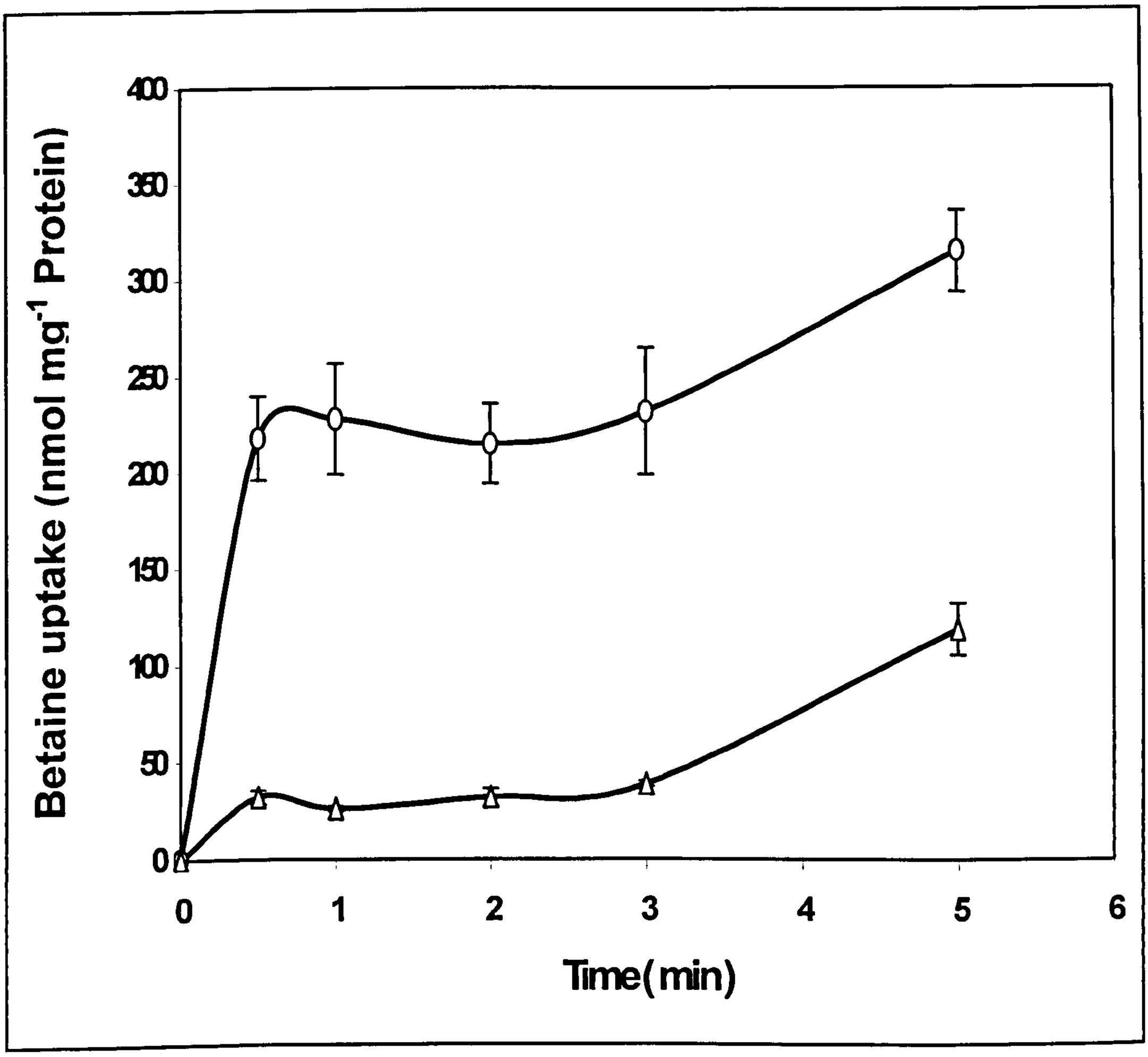


Fig: (4.4) Betaine uptake by *Halomonas* Halo in the presence or absence of CCCP. The cells were grown in 0.5 M NaCl chemically defined medium in the presence of 20 mM betaine. —o— Control. —Δ— 50 μM CCCP.

4.2.4 Betaine Uptake by *Halomonas* Halo in The Presence or Absence of CCCP in 3 M NaCl Chemically Defined Medium.

The experiments were extended to look at uptake of betaine in cells grown at 3 M NaCl. Figure 4.5 shows that *Halomonas* Halo takes up betaine when grown at 3 M NaCl in the absence of cold betaine. However, the uptake of betaine at 3 M NaCl was much lower that the uptake by cells grown at 0.5 M NaCl in the absence of cold betaine (Figure 4.3). This is surprising since the cells should need more betaine to grow well at 3 M NaCl and the stimulation of growth at 3 M NaCl by betaine is highly significant (Table 4.2). On the other hand the inhibitor CCCP stopped the uptake of betaine by 3 M NaCl grown cells in a similar way to 0.5 M NaCl grown cells, although for 3 M NaCl grown cells the inhibition was not complete (Figure 4.5).

Figure 4.6 shows the rate of betaine uptake for 3 M NaCl grown *Halomonas* Halo cells grown in the presence of cold betaine. The uptake rate is very slow and appears to consist of an initial uptake, followed by a plateau and then another uptake towards the end of the 5 minute measurement perioid. The effect of CCCP is much less significant than in the other experiments in this series (Figure 4.6).

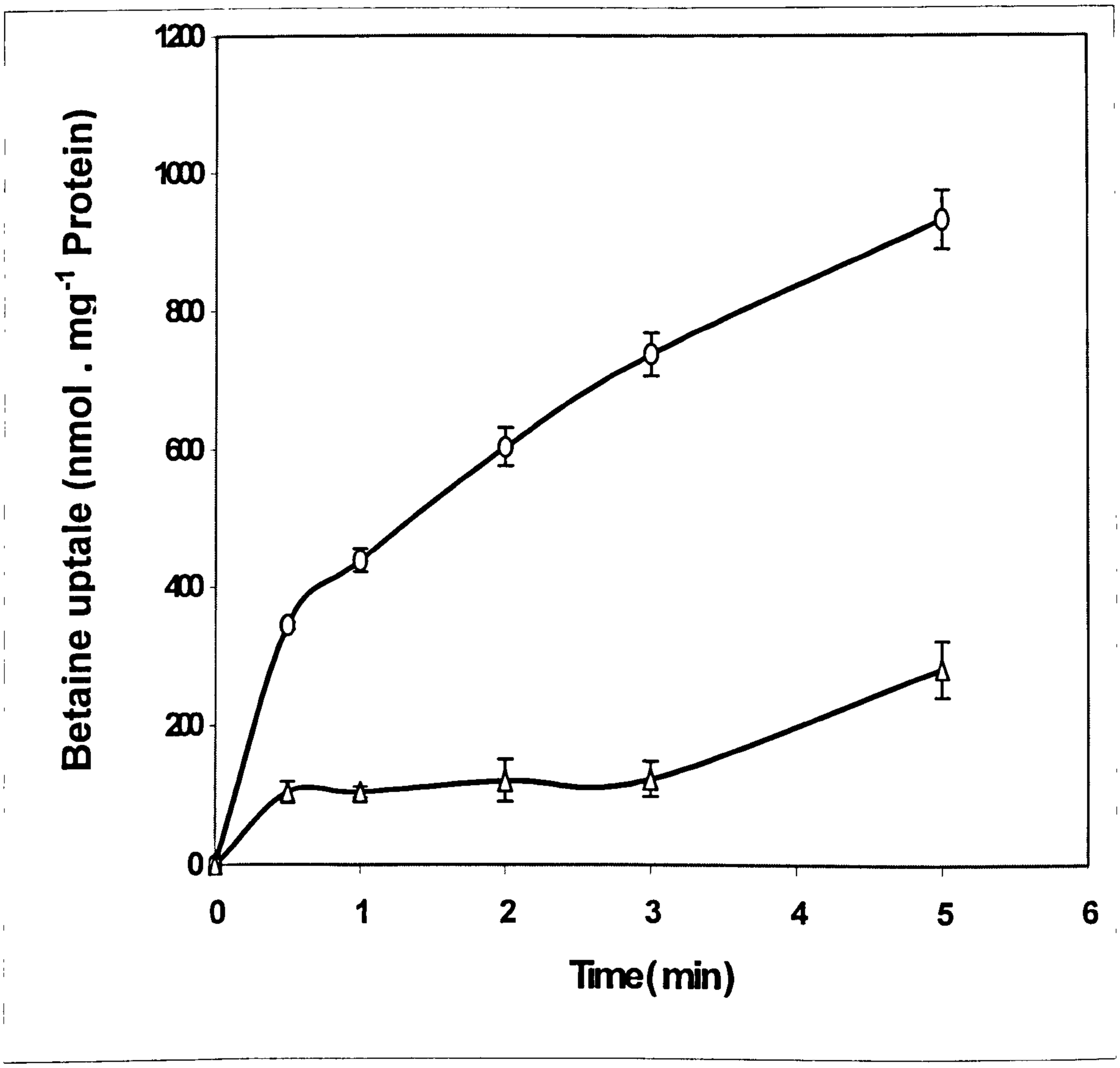


Fig: (4.5) Betaine uptake by *Halomonas* Halo in the presence or absence of CCCP. The cells were grown in 3 M NaCl chemically defined medium in the absence of betaine. —o— Control. —Δ— 50 µM CCCP.

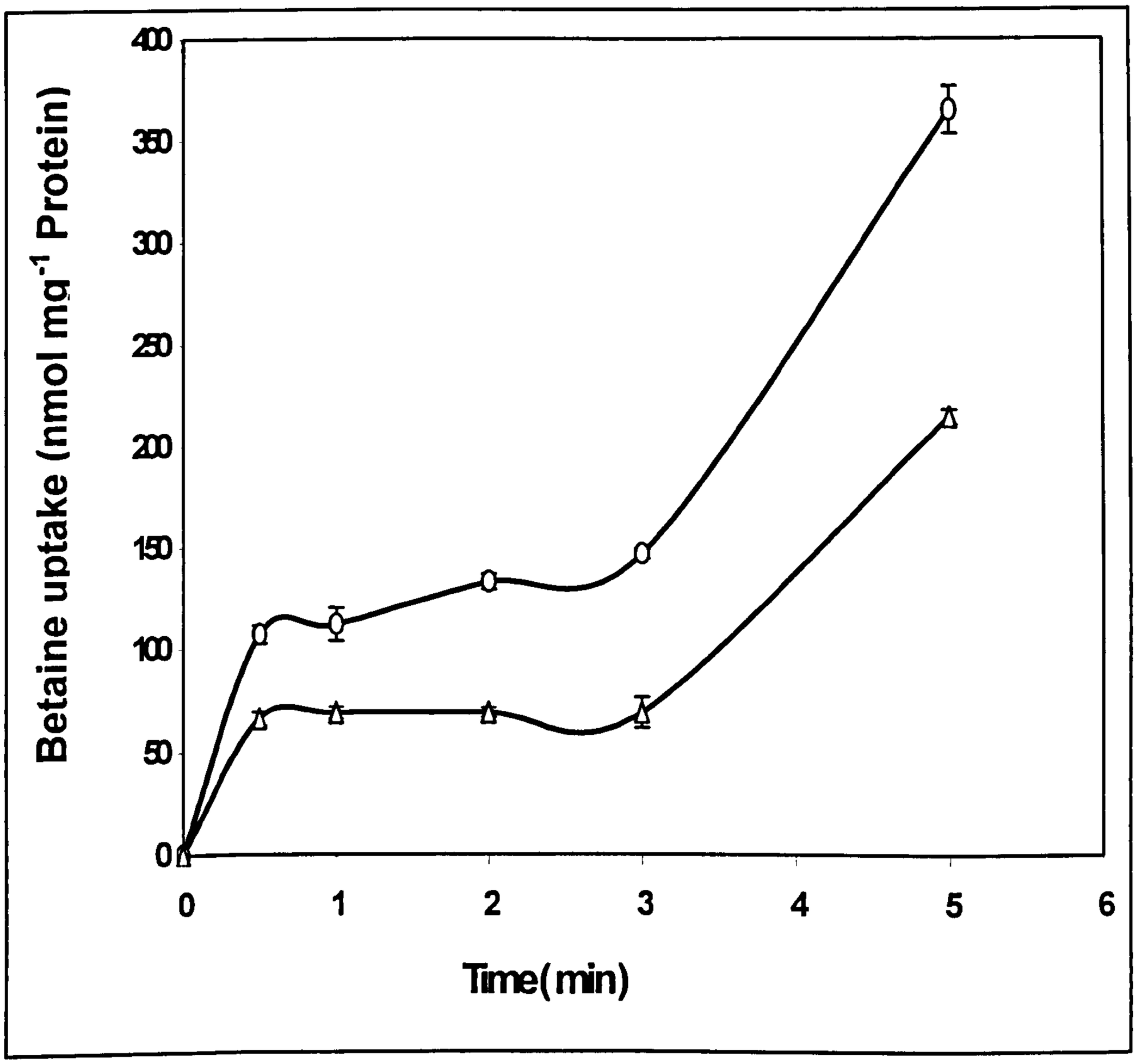


Fig: (4.6) Betaine uptake by *Halomonas* Halo in the presence or absence of CCCP. The cells were grown in 3 M NaCl chemically defined medium in the presence of 20 mM betaine. —o— Control. —Δ— 50 µM CCCP.

4.2.5 Betaine Uptake by *S. xylosus* in The Presence or Absence of CCCP in 0.5 M NaCl Chemically Defined Medium.

Figure 4.7 shows that *S. xylosus* cells grown at the relatively low salinity of 0.5 M took up radioactive betaine at a fast rate. This process is clearly energy dependent because was it was completely inhibited by the protonophore CCCP. This experiment was repeated using *S. xylosus* cells grown at 0.5 M NaCl, but this time 20 mM cold betaine was present in the medium. Figure 4.8 shows that the rate of betaine transport was diminished by the presence of cold betaine, but it was still clearly present. CCCP also inhibited the process in the presence of cold betaine, although the inhibition was not as complete as in the absence of cold betaine (Figures 4.7 and 4.8).

4.2.6 Betaine Uptake by *S. xylosus* in The Presence or Absence of CCCP in 3 M NaCl Chemically Defined Medium

The experiments were extended to look at *S. xylosus* grown at 3 M NaCl, at this salinity the presence of betaine greatly increased the growth rate (Table 4.1). In contrast to *Halomonas* Halo (Figure 4.5), the rate of betaine uptake by 3 M NaCl grown *S. xylosus* cells was not diminished by the higher salinity, although the uptake by 3 M NaCl grown cells was not linear and appeared to consist of two phases (Figures 4.7 and 4.9). This confirms that betaine uptake is crucial to the growth of *S. xylosus* at 3 M NaCl. The inhibitor CCCP again stopped uptake in cells grown at 3 M NaCl (Figure 4.9). Figure 4.10 confirms the trend of results for betaine uptake by *S. xylosus* grown at 3 M NaCl, because even in the presence of 20 mM cold betaine, the rate of

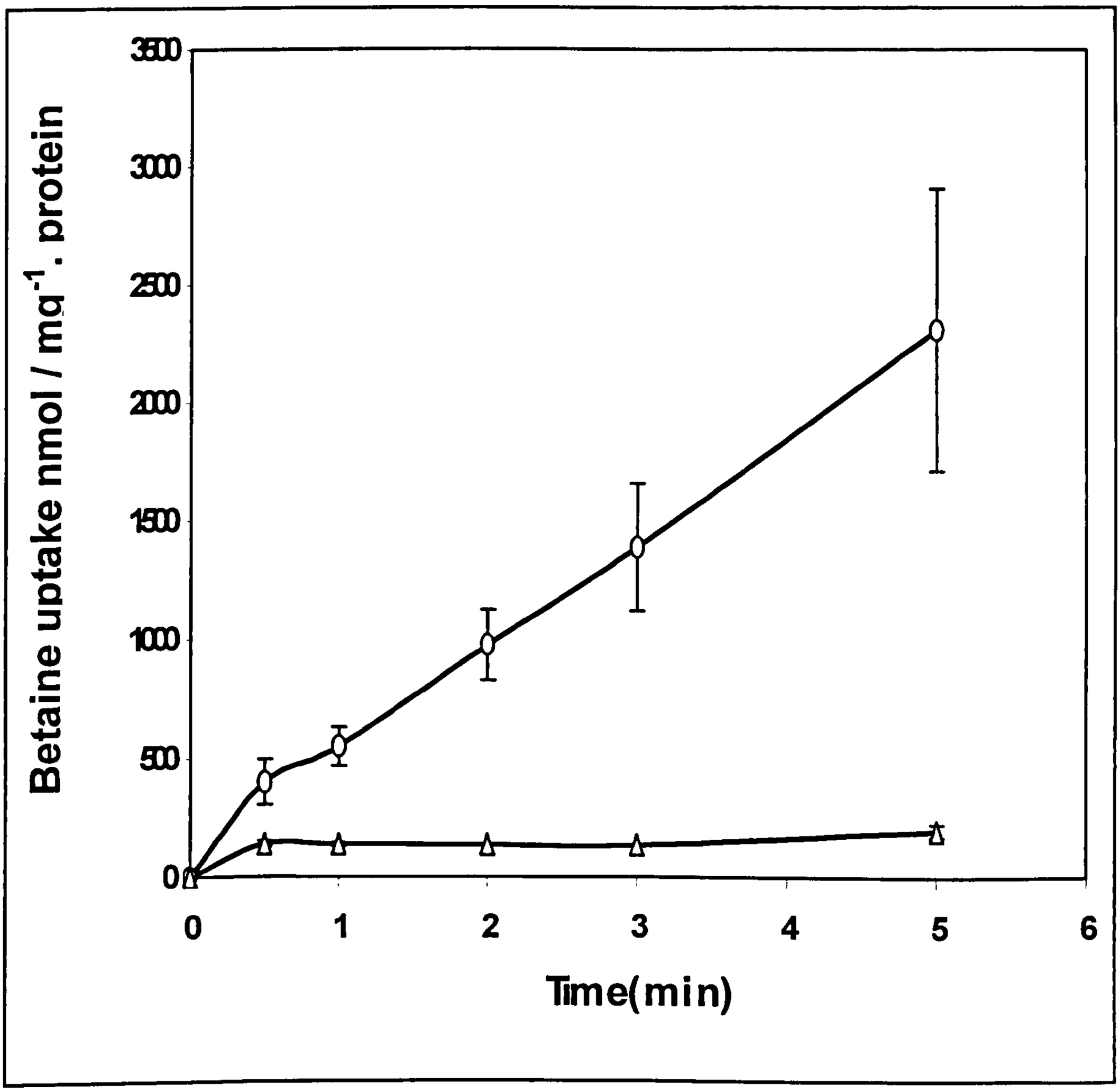


Fig: (4.7) Betaine uptake by *Staphylococcus xylosus* in the presence or absence of CCCP. The cells were grown in 0.5 M NaCl chemically defined medium in the absence of betaine. —o— Control. —Δ— 50 μM CCCP.

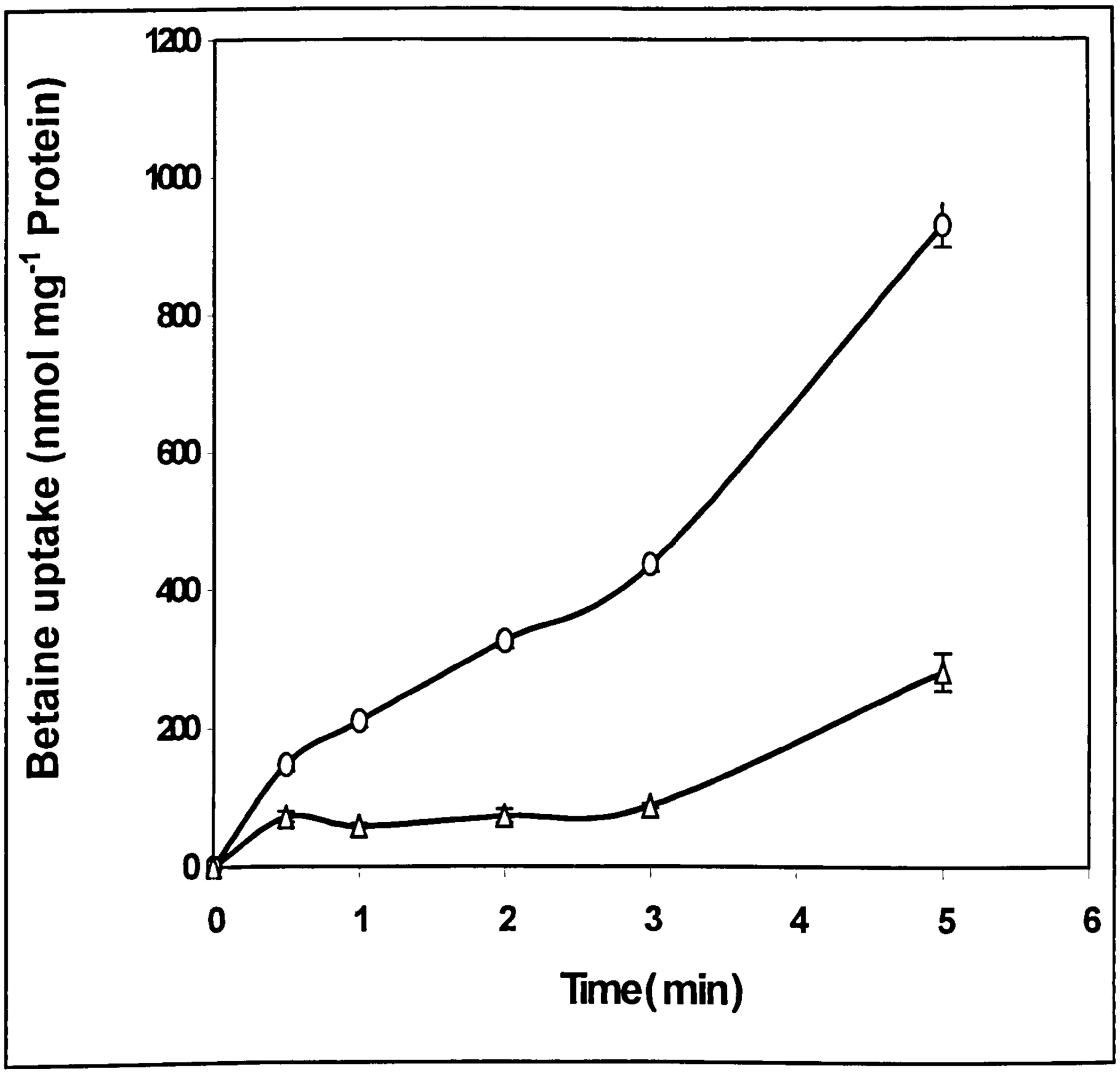


Fig:(4.8) Betaine uptake by *Staphylococcus xylosus* in the presence or absence of CCCP. The cells were grown in 0.5 M NaCl chemically defined medium in the presence of 20 mM betaine. —o— Control. —Δ— 50 µM CCCP.

uptake was undiminished. The two phase uptake seen for 3 M NaCl grown *S. xylosus* cells in the absence of cold betaine (Figure 4.9) was still present when cold betaine was added, but it was less pronounced (Figure 4.10). CCCP severely reduced the uptake of betaine by 3 M NaCl grown *S. xylosus* cells, but the inhibition was not total (Figures 4.9 and 4.10).

4.2.7 Proline Content of *S. xylosus*

Several species of *Staphylococcus* have been shown to accumulate proline in respond to increases in external salinity (da Costa *et al.*, 1998; Townsend and Wilkinson, 1992). Therefore, the proline content of *S. xylosus* cells grown in 0.5 and 2 M NaCl was measured. Table 4.3 shows that the intracellular concentration of proline did not increase in 2 M NaCl grown cells and in fact if anything there was less proline in the high salinity grown *S. xylosus* cells.

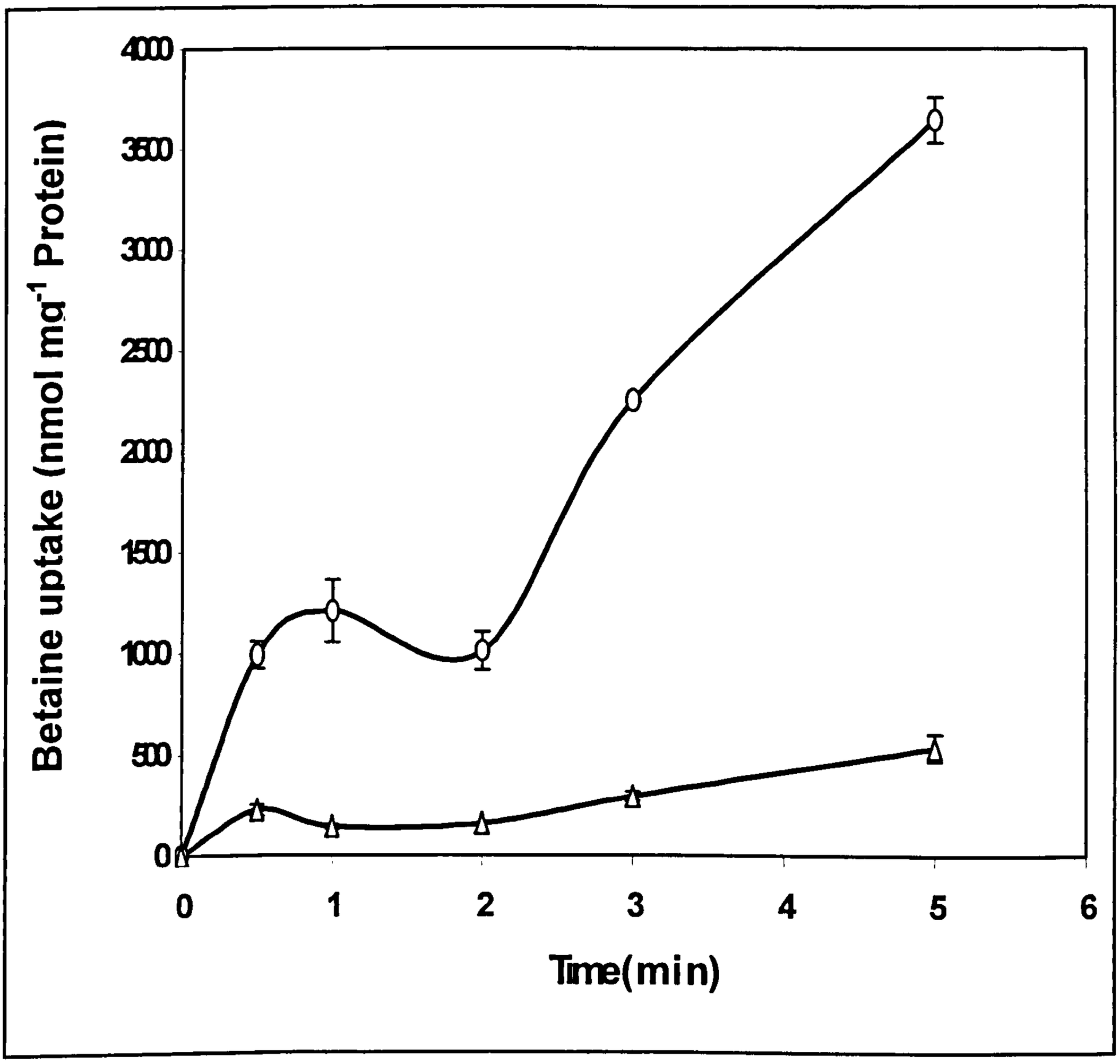


Fig:(4.9) Betaine uptake by *Staphylococcus xylosus* in the presence or absence of CCCP. The cells were grown in 3 M NaCl chemically defined medium in the absence of betaine. —o— Control. —Δ— 50 µM CCCP.

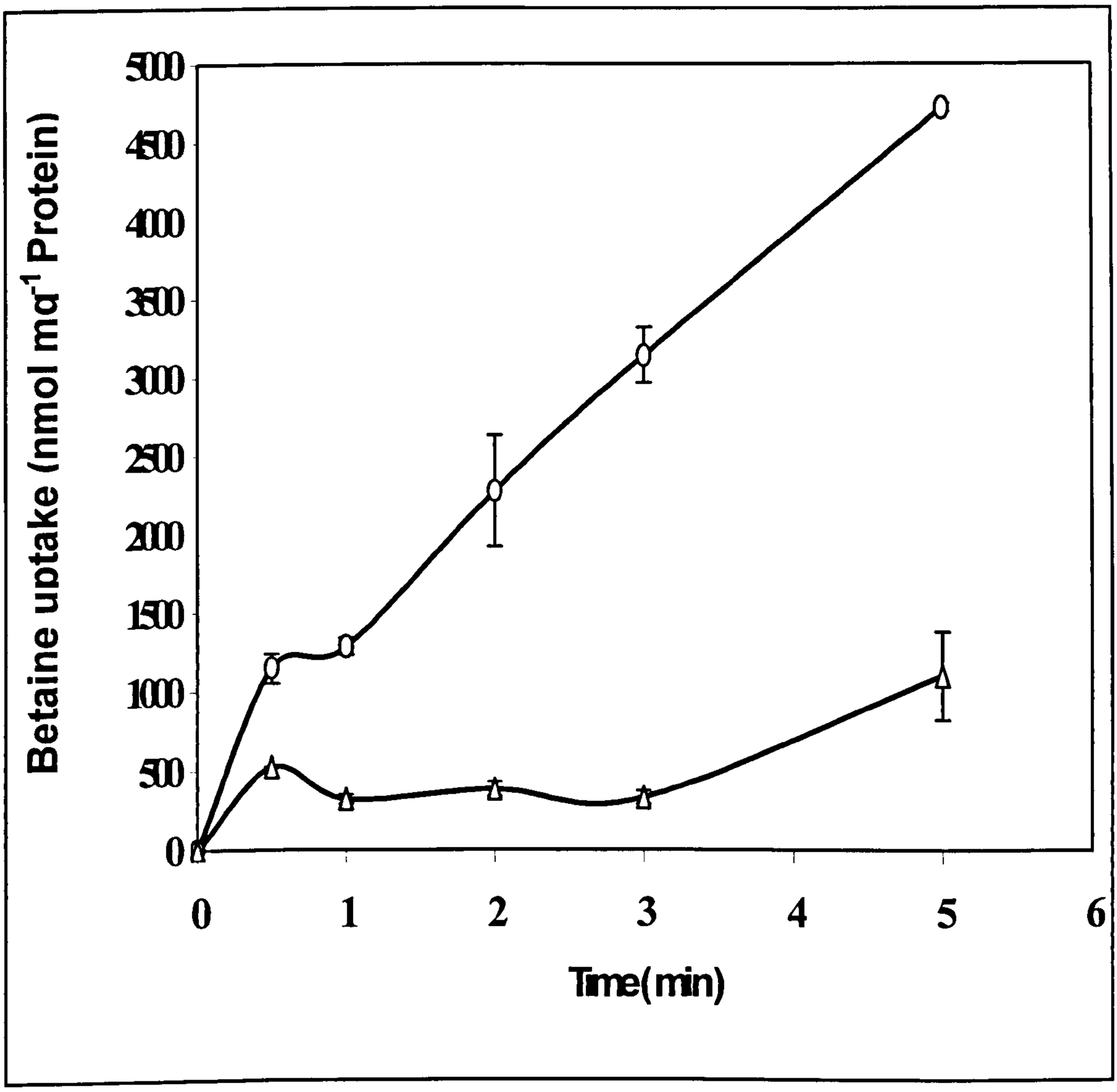


Fig:(4.10) Betaine uptake by *Staphylococcus xylosus* in the presence or absence of CCCP. The cells were grown in 3 M NaCl chemically defined medium in the presence of 20 mM betaine. —o— Control. —Δ— 50 µM CCCP.

Table (4.3) Proline content of *Staphylococcus xylosus* cells grown at 0.5 or 2 M NaCl chemically defined medium

Amount of initial sample	0.5 M NaCl *S. xylosus* (µg proline ml^{-1} cell extract)	2 M NaCl *S. xylosus* (µg proline ml^{-1} cell extract)
100 µl	92 ±4.3	54.7 ±8.8
200 µl	79.3 ±5.1	82.8 ±1.8
300 µl	98.1 ±2	88.8 ±2.9

Different amounts of cell extract were taken to ensure that at least some fell within the range of the proline concentration curve.

4.3 DISCUSSION

The composition of the growth medium can have a profound effect on the halotolerance of bacteria, because the presence in rich medium of supplements such as yeast extract provide a ready supply of compatible solutes or their precursors (Galinski, 1995). Since in this chapter, the role of compatible solutes was to be investigated, it was no longer possible to use the HDM + 1g l^{-1} yeast extract medium that had been used for the work described in Chapter 3 and a chemically defined medium was used instead (Table 2.3). A comparison of the growth of *S. xylosus* and *Halomonas* Halo on the two media (Figures 4.1 and 3.2, and 4.2 and 3.1 respectively) showed that *S. xylosus* grew much less well at 3 M NaCl on the defined medium, but that this was not true for *Halomonas* Halo. This would suggest that *S. xylosus* depended on the presence of yeast extract to grow at 3 M NaCl. Galinski (1995) subjected yeast extract to analysis by nuclear magnetic resonance and found that it contained a wide range of amino acids and betaine. Vijaranakul *et al.* (1997) showed that betaine could rescue a NaCl-sensitive mutant of *S. aureus*, therefore it is likely that the active "ingredient" in yeast extract is betaine.

Nevertheless, it was decided to add a range of compatible solutes to chemically defined medium containing 3 M NaCl to see their effect on growth of *S. xylosus* and *Halomonas* Halo. Table 4.1 confirmed the expectation that betaine was an effective compatible solute for *S. xylosus* increasing growth by just over 100 %. The only other positive effect on growth was induced by choline, but this latter effect was less than betaine and slower to act. It is

well known that betaine can be synthesised from choline by an enzymatically catalysed two step oxidation (Le Rudulier *et al.*, 1984; Strom *et al.*, 1986). The results in Table 4.1 would suggest that this pathway is also operating in *S. xylosus*. Essentially, identical results were found for *Halomonas* Halo i.e. both betaine and choline significantly increased growth at 3 M NaCl in chemically defined medium (Table 4.2). However, in the case of *Halomonas* Halo, both compatible solutes were more effective than in *S. xylosus*, and this was particularly true for choline. Canovas *et al.* (1998) clearly demonstrated the oxidation of choline to betaine in *H. elongata* and the results in Table 4.2 show that this is almost certainly true for *Halomonas* Halo. However, the fact that choline is a less effective compatible solute than betaine for *S. xylosus* means that further work is required to be certain that choline acts as a direct precursor of betaine in *S. xylosus*.

Tables 4.1 and 4.2 clearly demonstrate that exogenous betaine can increase the growth rate of both *S. xylosus* and *Halomonas* Halo at 3 M NaCl in chemically defined medium. Therefore, a series of experiments was conducted to examine transport of ^{14}C-betaine into *S. xylosus* and *Halomonas* Halo cells. Figures 4.3 and 4.4 show that betaine is taken up by *Halomonas* Halo cells at the relatively low salinity of 0.5 M NaCl. The presence of 20 mM cold betaine in the growth medium decreased the uptake presumably due to more betaine inside the cells. The protonophore, CCCP, completely abolished the uptake in the absence of cold betaine (Figure 4.3) and dramatically decreased it in the presence of cold betaine (Figure 4.4). CCCP works by making the cytoplasmic membrane permeable to protons

(Nicholls and Ferguson, 1992), which means that the proton motive force is abolished and no ATP can be produced (see Chapter 5). Therefore, the uptake of betaine is an active process, which requires either the proton motive force or a supply of ATP. For 3 M NaCl grown *Halomonas* Halo cells the rate of betaine transport was slower than for 0.5 M NaCl grown cells (Figures 4.5 and 4.6). This was surprisingly since it would be expected that more intracellular betaine is required to compensate for 3 M NaCl in the medium. However, it should be kept in mind that the rate of growth at 3 M NaCl in the presence of betaine is still significantly slower than the growth rate at 0.5 M NaCl. The OD_{660} after 24 hours at 0.5 M NaCl = 2.044 (see Figure 4.2), whereas the OD_{660} after 24 hours at 3 M NaCl in the presence of 20 mM betaine is 0.823 (see Table 4.2). This may explain this apparent discrepancy.

Figures 4.7 – 4.10 show the parallel set of ^{14}C-betaine uptake experiments for *S. xylosus*. The results are similar with betaine being transported into the cells at both 0.5 and 3 M NaCl and the uptake is sensitive to CCCP. However, in the case of *S. xylosus* uptake is more efficient at the higher salinity, which may reflect the absolute requirement for betaine to allow growth at 3 M NaCl. Although transport systems for choline (Kaenjak *et al.*, 1993) and proline (Townsend and Wilkinson, 1992) have been characterised in *S. aureus*, this is the first report of betaine transport into stapylocooci. This is probably because ^{14}C-betaine is not commercially available and has to be synthesised from ^{14}C-choline (section 2.9.1). It also means that a direct comparison is impossible between the betaine transport system of *S. xylosus*

and the transport systems of *S. aureus.* However, it is worthwhile noting that the maximum uptake achieved by the choline transport system of *S. aureus* was about 2800 nmol mg^{-1} dry weight (Kaenjak *et al.*, 1993), which is similar to the figures for the *S. xylosus* betaine system found in the present work (1000–5000 nmol mg^{-1} protein).

The final experiment in this chapter investigated whether or not proline acts as a compatible solute for *S. xylosus* (Table 4.3). It appears that proline is present at significant levels in cells grown in 0.5 or 2 M NaCl, but that it does not increase at the higher salinity. Further experiments using Nuclear Magnetic Resonance (NMR) are required to identify the compatible solutes used by *S. xylosus*.

The importance of the proton motive force for the uptake of betaine by both *S. xylosus* and *Halomonas* Halo has been demonstrated. In the next chapter, the role of the proton motive force in allowing both organisms to grow at different levels of pH will be investigated.

CHAPTER 5

RESPONSE OF *HALOMONAS* HALO AND *STAPHYLOCOCCUS XYLOSUS* TO DIFFERENT pH LEVELS

5.1 Introduction

In the previous two chapters, the effect of increased salinity on the growth of *Halomonas* Halo and *S. xylosus* was examined. In this chapter, another environmental stress will be examined i.e. growth of *Halomonas* Halo and *S. xylosus* at different pH values.

When considering the effect that changing the external pH level might have on the growth of bacteria, it is important to remember that, due to the absence of internal organelles in prokaryotic cells, the cytoplasmic membrane is the site of energy generation. Figure 5.1 shows that electron transport takes place within the cytoplasmic membrane leading to the transport of protons (H^+) across the membrane setting up both a ΔpH (alkaline inside) and a membrane potential ($\Delta\Psi$ - negative inside). Since the membrane is impermeable to H^+, the ΔpH and $\Delta\Psi$ together make up a form of potential energy called the proton motive force (Δp) (Neidhardt *et al.*, 1990). The key steps in proton motive force formation involve the activities of flavoproteins, quinones, and cytochromes (Figure 5.1). The Δp can then be used to drive ATP synthesis by the passage of H^+ through membrane bound

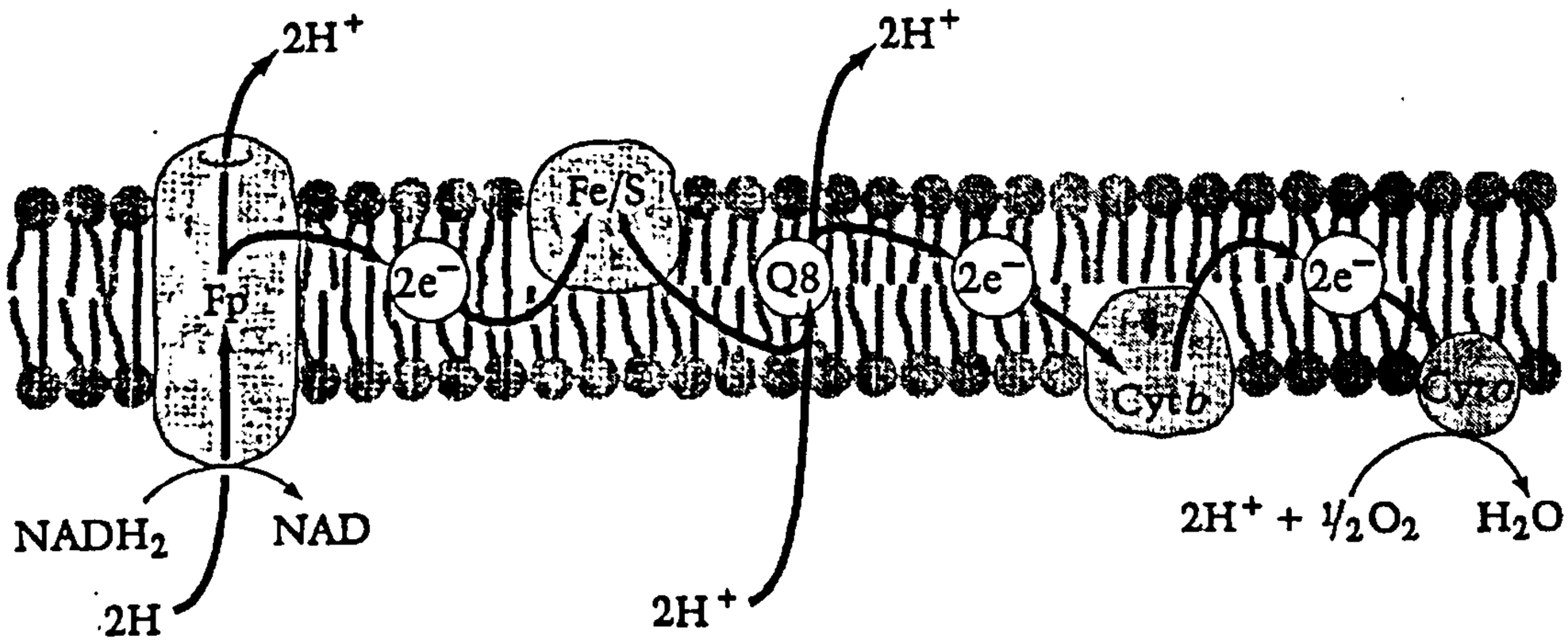


Fig: (5.1) Functional organization of one of the electron transport chains in *E. coli*. This chain, which functions in environments with high pO_2, is composed of hydrogen carriers (flavoprotein, Fp; ubiquinone, Q8) and electron carriers (iron-sulfur protein, Fe/S; cytochrome b; cyt b). Cytochrome o (cyt o) transfers electrons to O_2, thereby reducing it. e^-, electron; H^+, proton (Neidhardt *et al.*, 1990).

ATPases, ion fluxes via symports and antiports and the uptake of nutrients e.g. amino acids (Neidhardt *et al.*, 1990).

The Δp, in mV, can be calculated using the Nernst equation (Kashket, 1985):

$$\Delta p = \Delta\Psi - Z\,\Delta pH$$

Where,

Z = 2.303 RT/nF (= 59 at 25 °C or 65 at 60 °C)

R = gas constant (1.9869 cal mol^{-1} K^{-1} or 8.314 J mol^{-1} K^{-1})

T = absolute temperature (K)

n = charge of the transported species = 1

F = Faraday's constant (23.06 cal mV^{-1} eq^{-1} or 96.519 J mV^{-1} eq^{-1}).

The factor Z is used to convert pH units into mV, allowing the Δp to be measured in mV. In most bacteria the membrane potential is negative inside and the interior of the cytoplasmic membrane is alkaline compared to the exterior i.e. internal pH (pHi) is higher than the external pH (pHo). By convention the ΔpH is calculated by pHi minus pHo which gives a positive value. When this is put into the Nernst equation the $\Delta\Psi$ and ΔpH values added together give a negative value and under these conditions the Δp tends to drive H^+ into the cells (Lowe and Jones, 1984). It is clear that any change in the external pH (pHo) will directly affect the magnitude of Δp and thus potentially affect the production of ATP. Kashket (1985) and Nicholls and Ferguson (1992) list a range of measurements of Δp for bacteria and

most fall in the range of 150 - 250 mV. To investigate the relationship between ATP synthesis and the magnitude of the Δp, the phosphorylation potential (calculated from the intracellular concentrations of ADP, ATP and inorganic phosphate) must be measured and this was found to be typically 10.5 kcal mol^{-1}, which is equivalent to 450 mV (Quirk *et al.*, 1991). If the stoichiometry of H^+/ATP is assumed to be about 3 (see Nicholls and Ferguson,1992), then a Δp of around 150 mV is required for efficient ATP synthesis. Therefore, bacterial cells must maintain a suitable level of Δp in order to grow at different pH values.

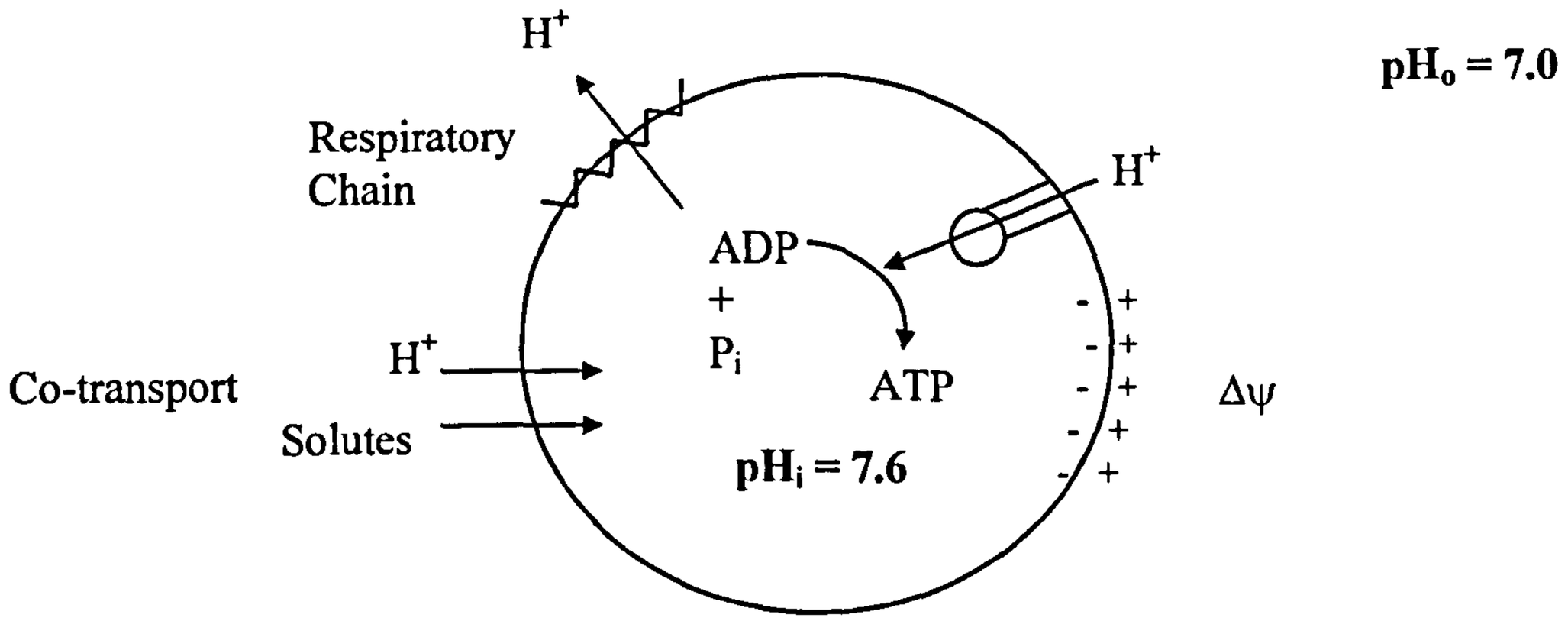
The bioenergetics of bacteria that grow at extreme pH values provide the biggest challenge to the chemiosmotic hypothesis as outlined above (Garland, 1977). Such bacteria have to solve the problem of maintaining a cytoplasmic pH that is as close as possible to pH 7 whilst generating a Δp of at least 150 mV. Internal pH values vary from one group to another. In general neutrophiles show internal pH values of 7.5-8.0, acidophiles have internal pH values of 6.0–7.0, and alkalophiles exhibit intracellular pH values of 8.2–9.0 (Figure 5.2) (Harold *et a*l., 1969; Guffanti *et al.*, 1978; Kashket, 1981; Brink and Konings, 1982; Kobayashi *et al.*, 1982; Nakamura *et al.*, 1982; Ahmad and Booth, 1983; Hackstadt, 1983; Guffanti *et al.*, 1984). This emphasises the fact that some critical cell processes require the internal pH to be controlled between 6 and 9.

In neutrophiles the internal pH values are maintained more alkaline than the external pH and this is due to the activity of the respiratory chains, which

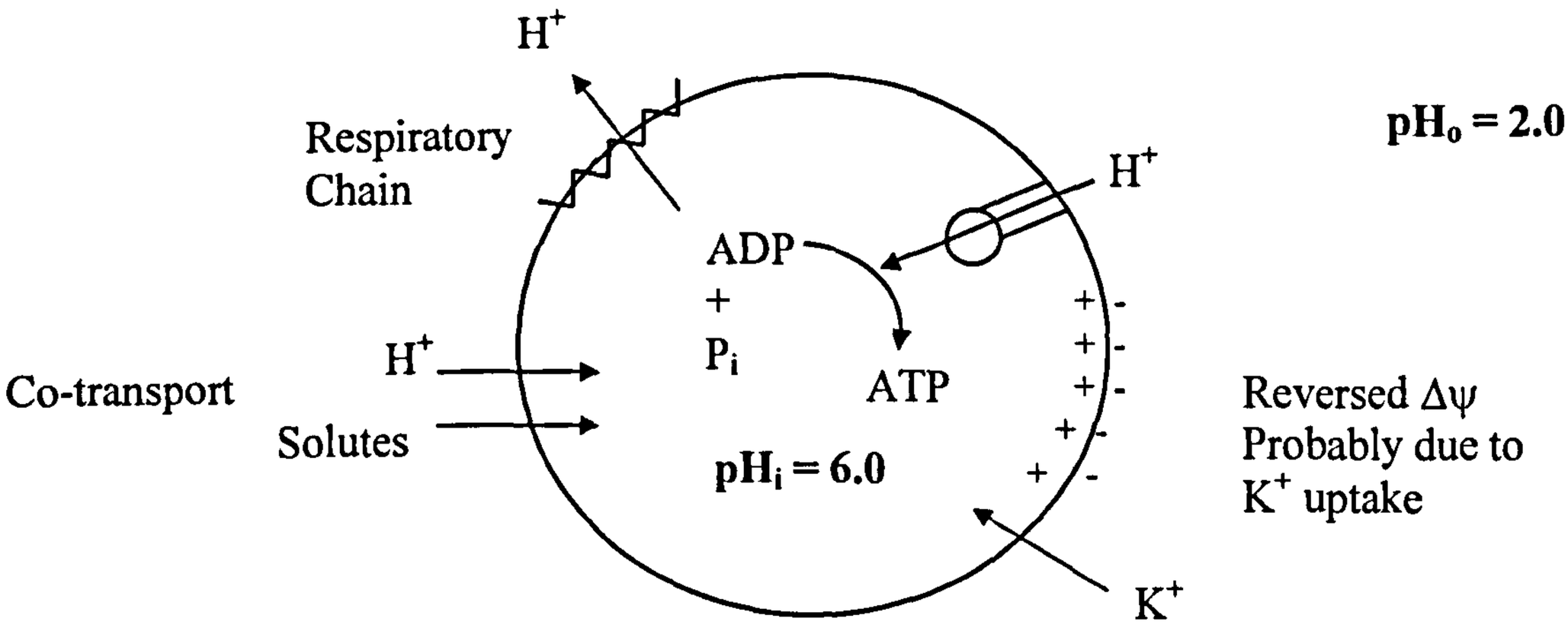
extrude protons outward to establish the proton gradient in favour of ATP synthesis (Figures 5.1 and 5.2a). Measurements of membrane potential ($\Delta\Psi$) at different values of external pH showed that an increase of $\Delta\Psi$ was observed with increasing external pH while the ΔpH decreases (Brey *et al.*, 1979; Kashket, 1982). Therefore, the magnitude of the membrane potential is dependent on the external pH (Padan *et al.*, 1979; Kroll and Booth, 1981). In neutrophiles, it was suggested that the internal pH is controlled by either K^+/H^+ or Na^+/H^+ antiporters or by both, which acidify the cytoplasm at neutral and alkaline pH values (Padan *et al.*, 1981; Booth, 1985).

Figure 5.2b shows that extreme acidophiles maintain a large ΔpH, acid out, several aerobic acidophiles that grow optimally between pH 2 and 3.5 maintain a cytoplasmic pH of at least 5.5 (Krulwich *et al.*, 1981; Booth, 1985). The cytoplasmic pH of acidophiles is somewhat below neutral during growth. This is lower than that of neutrophiles or alkaliphiles, both of which maintain internal pH in the moderately alkaline range (Krulwich and Ivey, 1990). Although the ΔpH is in the correct direction to drive ATP synthesis, the overall magnitude of the Δp must be controlled to prevent excessive H^+ influx which would acidify the cytoplasm. Acidophiles utilize a reversed $\Delta\Psi$ (i.e. inside positive) to offset the large ΔpH (Krulwich and Ivey, 1990). The reversed $\Delta\Psi$ appears to be due to K^+ transport into the cell (Figure 5.2b), probably via the Kdp transport system (Krulwich and Ivey, 1990).

a) <u>Neutralophile</u>



b) <u>Acidophile</u>



c) <u>Alkaliphile</u>

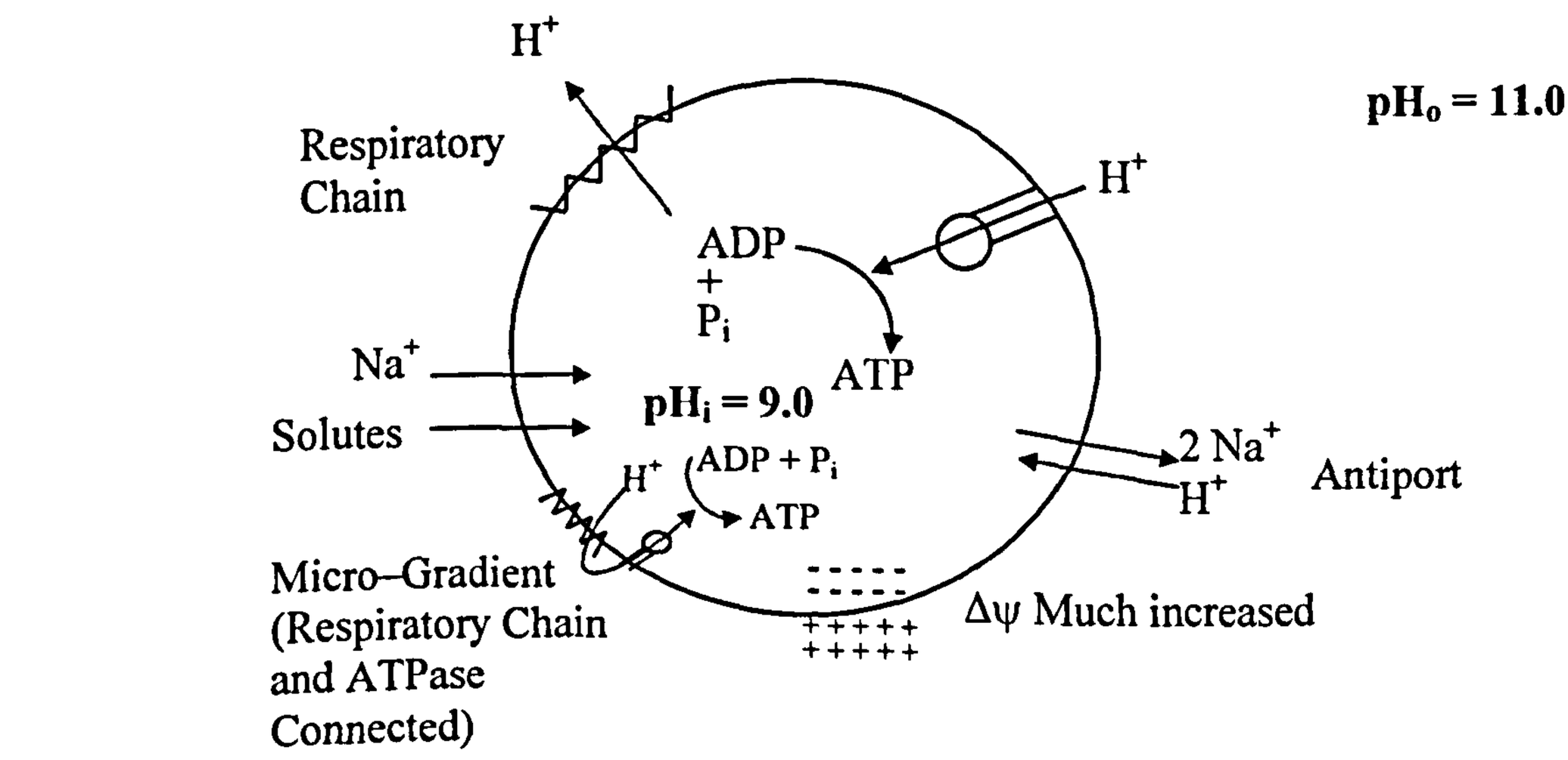


Fig: (5.2) Bioenergetic problems for acidophiles and alkalophiles.

Figure 5.2c shows that aerobic alkaliphiles that grow optimally at pH 10 – 11, maintain a cytoplasmic pH that is generally at least 2 pH units more acidic than the external pH, which means that their ΔpH is in the wrong direction to support ATP synthesis (Krulwich and Ivey, 1990). To generate a Δp in the correct direction, alkaliphiles greatly increase their $\Delta\Psi$ by utilising a Na^+/H^+ antiport (Figure 5.2c). However, the magnitude of the resulting Δp is still only around 50-70 mV, which is less than that required to synthesise ATP (Krulwich and Ivey, 1990). This has lead to speculation that the bulk gradient of protons does not drive ATP synthesis in alkaliphiles and that other factors are involved e.g. the possibility that a micro-gradient is produced by extruded protons being transferred directly to the ATPase by moving rapidly along the surface of the membrane (Figure 5.2c and Krulwich *et al.*, 1998). In the same review it was also noted that the structure of the cytochrome caa_3-type oxidase found in alkaliphiles differs from that found in neutrophiles. The low Δp also means that Na^+ is used to drive solute transport into the cells and the spinning of the flagella of motile cells, which conserves protons for ATP synthesis.

One common method used to study the relationship between Δp and growth is to utilise inhibitors. The protonophore or uncoupler, CCCP, is used to decrease the Δp because it makes the cytoplasmic membrane permeable to protons (Nicholls and Ferguson,1992). However, MacLeod *et al.* (1988) showed that as the external pH increased from 7 to 8.5, the inhibitory effect of CCCP decreased. Also, strains of *E. coli* and *Bacillus firmus* have been reported to show resistance to CCCP inhibition (Kinoshita *et al.*, 1984 and

Quirk *et al.*, 1991). This suggests that CCCP data need to be interpretated carefully.

A second common inhibitor used in bioenergetic studies is monensin, which collapses Na^+ gradients by making the cytoplasmic membrane permeable to Na^+ (Peddie *et al.*, 1999). It is clear from the discussion above that Na^+ gradients (in the form of Na^+/H^+ antiporters) are essential for the growth of alkaliphiles at pH 10 – 11. It is also well known that Na^+/H^+ antiporters play important roles in the bioenergetics of moderately halophilic bacteria (Hamaide *et al.*, 1983 and Unemoto *et al.*, 1992).

In the present chapter, *Halomonas* Halo and *S. xylosus* were grown at three different pH values (5.5, 7.0 and 8.5) and $\Delta\Psi$, ΔpH and Δp values were measured in the presence or absence of CCCP and monensin.

5.2 Results

5.2.1 Effect of different pH values on the growth of *S. xylosus* and *Halomonas* Halo.

Figure 5.3 shows that *S. xylosus* grows well at pH values 5.5, 7.0 and 8.5 with only a very slight decrease in initial growth rate at pH 8.5. *S. xylosus* did not grow at all in pH 5.0 or 9.0 (data not shown). In contrast, *Halomonas* Halo grows best at pH 7.0, because lag phases are significantly extended in media of pH 5.5 and 8.5. After 24 hours growth, cells in pH 8.5 achieve a higher OD than those cells grown at pH 5.5 (Figure 5.4). There was no growth of *Halomonas* Halo at pH 5.0 or 9.0. It appears that *S. xylosus* is more tolerant to changes in the external pH than *Halomonas* Halo.

5.2.2 Determination of the Δp as a function of external pH in *S. xylosus* and *Halomonas* Halo

Table 5.1 shows that for *S. xylosus* grown in 0.5 M NaCl Δp is highest at pH 5.5 (172 mV) and declines by just over 50 mV to 121 mV at pH 8.5. $\Delta\Psi$ increases as the external pH is increased from 5.5 to 8.5, but it only partially offsets the decline in ΔpH and therefore the overall Δp decreases. The internal pH increases from 6.7 in cells grown at pH 5.5 to 7.5 in cells grown in pH 8.5. Since growth of *S. xylosus* is good at pH 8.5 (Figure 5.3), the results suggest that a Δp of 121 mV is sufficient to drive ATP synthesis (Table 5.1).

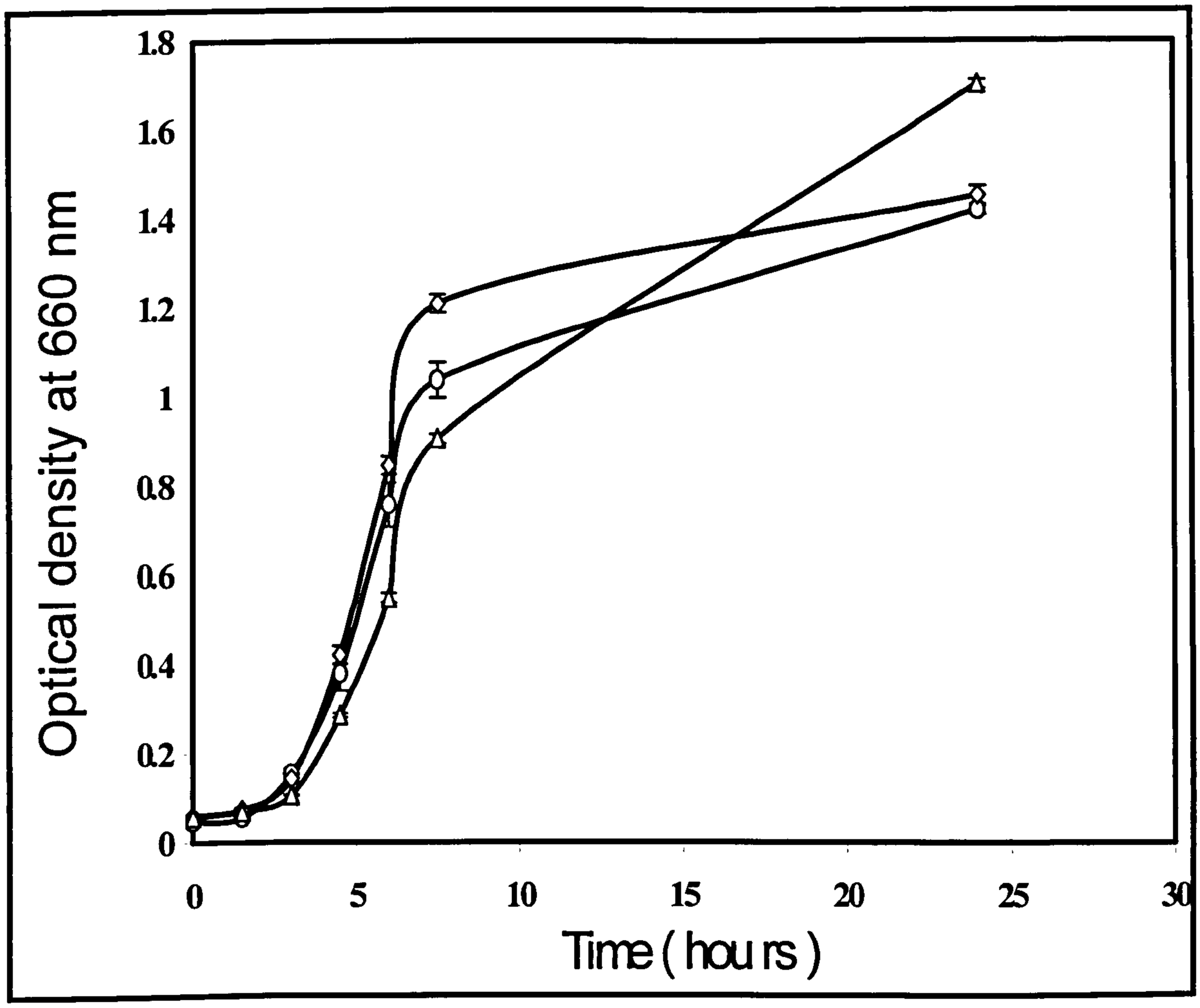


Fig: (5.3) Effect of different pH values on the growth of *Staphylococcus xylosus* at 0.5 M NaCl in *Halomonas* defined medium + 1 g l^{-1} yeast extract. —O—pH 5.5,— ◊—pH 7.0, —Δ—pH 8.5

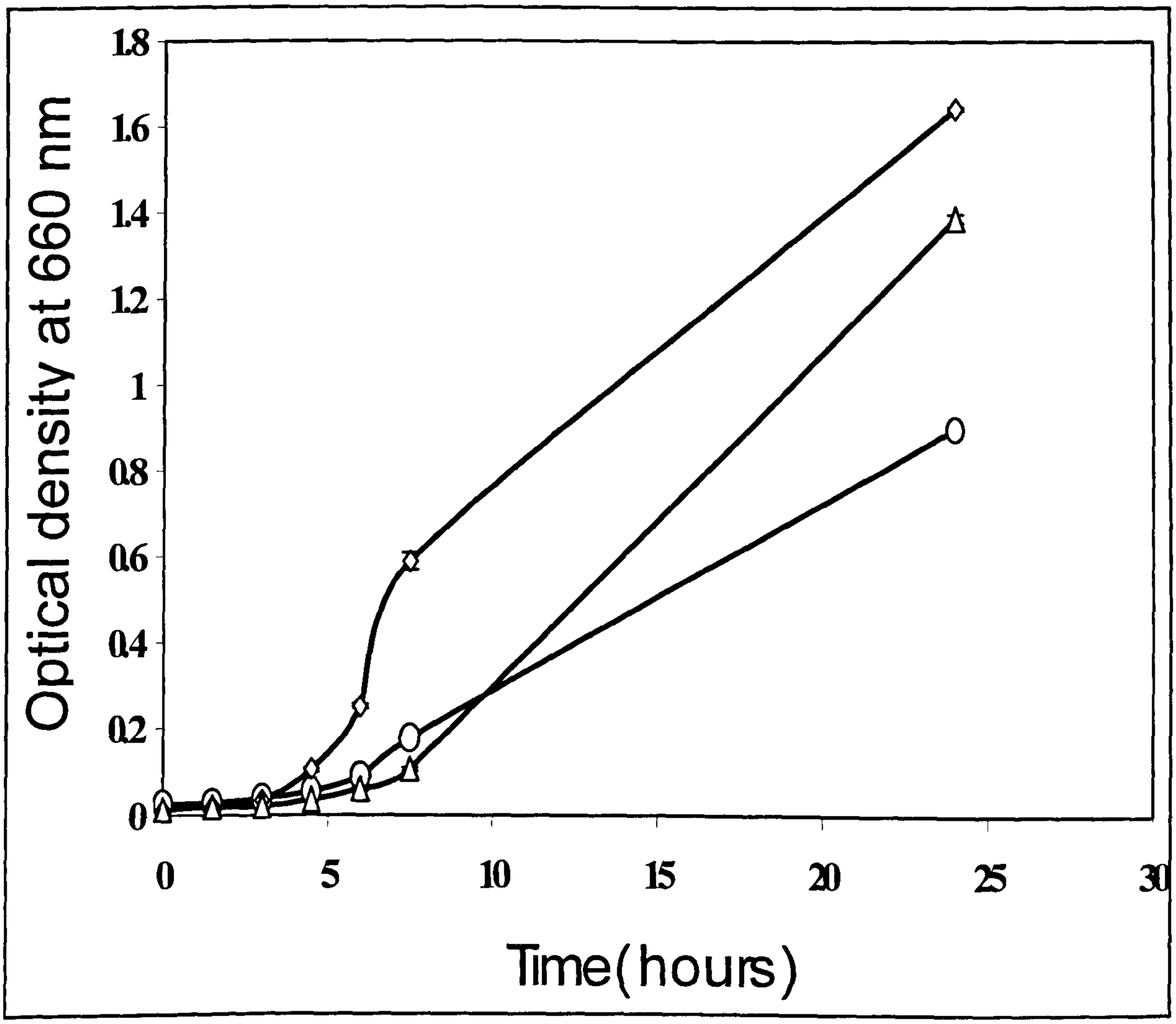


Fig: (5.4) Effect of different pH values on the growth of *Halomonas* Halo in 0.5 M NaCl *Halomonas* defined medium + 1 g l^{-1} yeast extract.

—o—pH 5.5, —◊—pH 7.0, —Δ—pH 8.5

Table (5.1): Determination of Proton Motive Force (Δp) and ΔΨ, ΔpH and pHi as a function of external pH in *Staphylococcus xylosus* in 0.5 M NaCl *Halomonas* defined medium + 1g l^{-1} yeast extract in batch culture. The experiment was repeated three times

pHo	pHi	ΔΨ (mV)	ΔpH	Δp (mV)
5.5	6.7 ± 0.1	-102.5 ± 3	1.2 ± 0.1	-172.3 ± 4.3
7	7.2 ± 0.07	-128.2 ± 0.87	0.2 ± 0.07	-138.3 ± 3.7
8.5	7.52 ± 0.02	-178.5 ± 1.3	- 0.98 ± 0.02	-120.6 ± 1.45

Table 5.2 shows that for *Halomonas* Halo grown in 0.5 M NaCl Δp is highest at pH 7.0 which agrees with the growth data (Figure 5.4) showing optimum growth at pH 7.0. However, this relationship between Δp and growth rate breaks down at the other pH values tested, because Δp remains almost unchanged at pH 5.5 and growth rate is poor and the opposite is true at pH 8.5 (Figure 5.4 and Table 5.2). A Δp of 79 mV was found at pH 8.5 for *Halomonas* Halo, this is an extremely low value, but growth at a reduced rate was still possible. The internal pH increased from 6.3 at an external pH of 5.5 to 7.8 at an external pH of 8.5 (Table 5.2).

5.2.3 Effect of CCCP and Monensin on the growth of *Staphylococcus xylosus* and *Halomonas* Halo

Figure 5.5 shows that growth of *S. xylosus* in 0.5 M NaCl pH 7.0 medium was completely inhibited by both monensin and CCCP. On the other hand in the same medium CCCP completely inhibited the growth of *Halomonas* Halo, but monensin showed only a partial inhibition (Figure 5.6).

Table (5.2): Determination of Proton Motive Force (Δp) and ΔΨ, ΔpH and pHi, as a function of external pH in *Halomonas* Halo in 0.5 M NaCl *Halomonas* defined medium + 1g l^{-1} yeast extract in batch culture. The experiment was repeated three times.

pHo	pHi	ΔΨ (mV)	ΔpH	Δp (mV)
5.5	6.3 ± 0.02	-99.3 ± 0.93	0.8 ± 0.02	-146.1 ± 1.93
7	7.14 ± 0.05	-145 ± 1.96	0.14 ± 0.05	-153.5 ± 4.98
8.5	7.83 ± 0.02	-118.5 ± 2.76	-0.67 ± 0.02	-78.9 ± 1.71

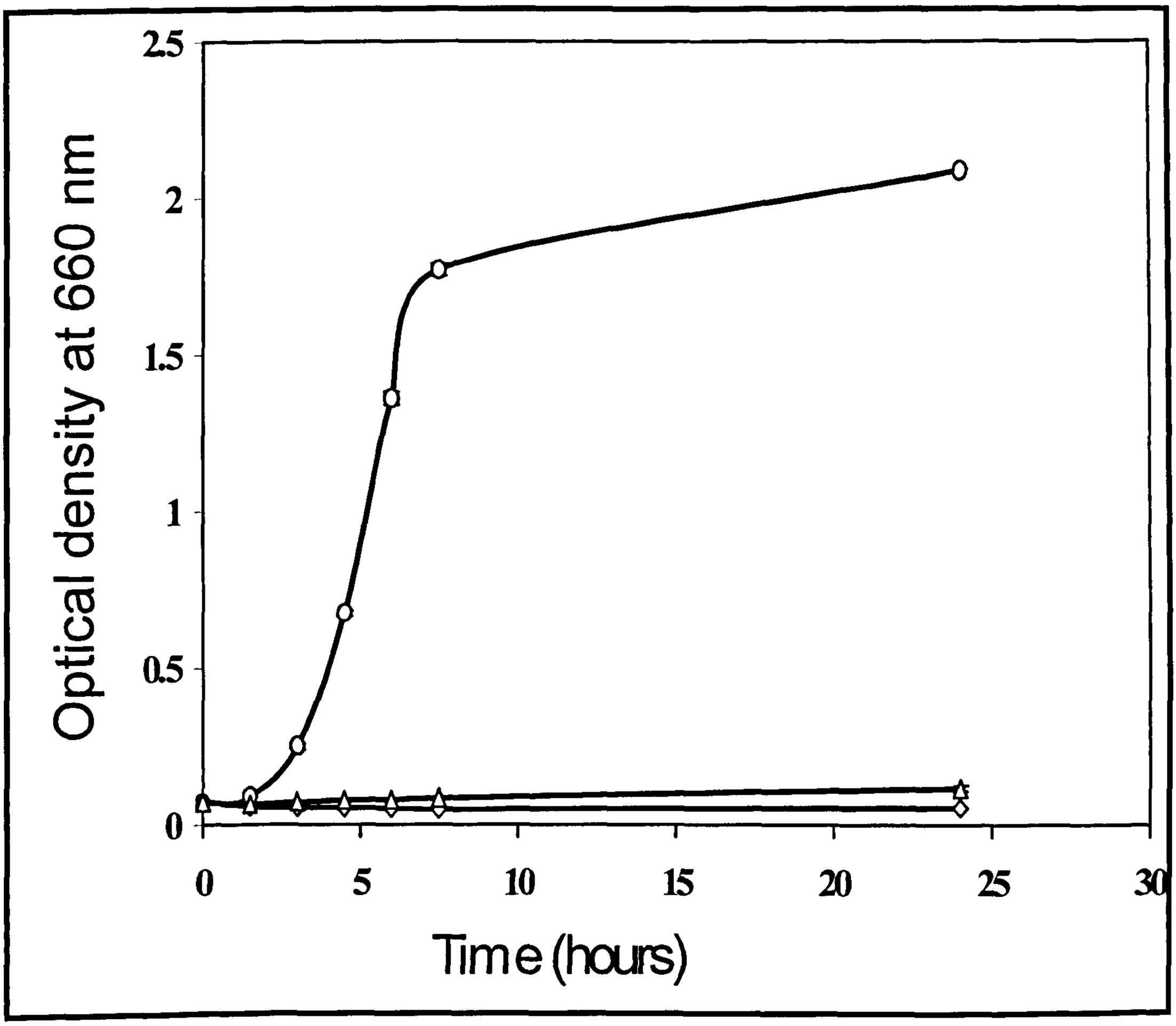


Fig: (5.5) Effect of inhibitors on the growth of *Staphylococcus xylosus* in 0.5 M NaCl *Halomonas* defined medium + 1 g l^{-1} yeast extract, pH 7.0.

—o—Control —◊— 50 µM CCCP —Δ— 50 µM Monensin

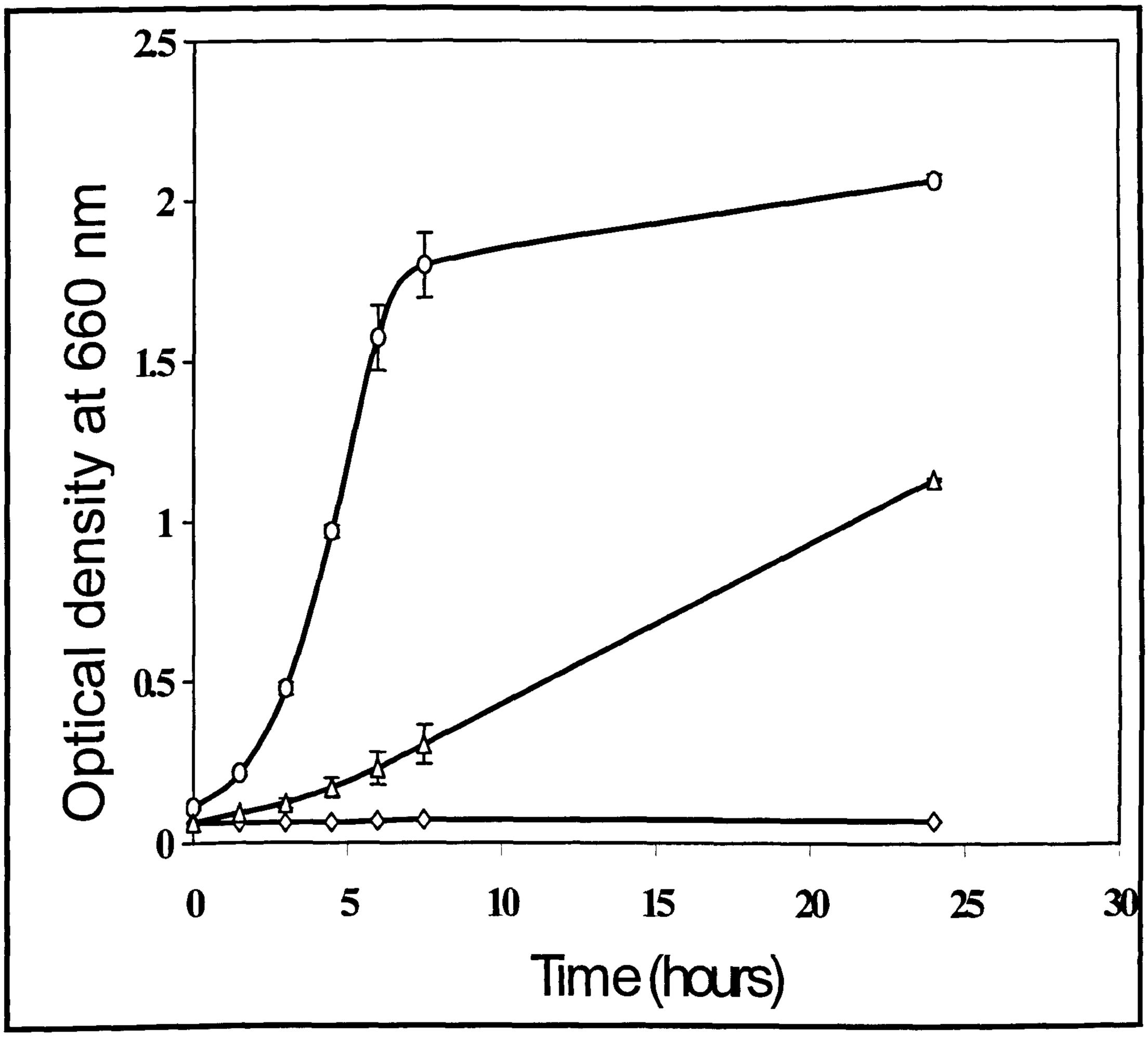


Fig: (5.6) Effect of inhibitors on the growth of *Halomonas* Halo in 0.5 M NaCl *Halomonas* defined medium + 1 g l^{-1} yeast extract, pH 7.0.

—o—control, —◊— 50 µM CCCP,—Δ— 50 µM Monensin

5.2.4 Effect of CCCP and Monensin on Δp of *S. xylosus* and *Halomonas* Halo

To further investigate the effects of CCCP and monensin on *S. xylosus* and *Halomonas* Halo their effects on the ΔΨ, ΔpH and Δp levels were investigated. Table 5.3 shows that for *S. xylosus* cells grown in 0.5 M NaCl at the three levels of pH the Δp is significantly reduced by CCCP with the level of Δp in the presence of CCCP being around 50-90 mV. Interesingly, the percentage inhibition of Δp by CCCP (comparing data in Tables 5.1 and 5.3) was very similar at all three pH values being 48-56 %. At pH 7.0, the Δp was 70 mV, but no growth was possible (Figure 5.5). In the presence of CCCP it would be expected that the internal pH would be similar to the external pH, because CCCP makes the cytoplasmic membrane permeable to protons. This is seen at pH 5.5, because the internal pH is 5.8. However, this effect is not apparent at either pH 7 or 8.5 (Table 5.3).

Monensin showed less effect on the Δp of *S. xylosus* cells than CCCP with values between 90-125 mV (Table 5.3). The percentage inhibition values were significantly different at each pH level, being 36 % at pH 5.5, 10 % at pH 7.0 and 28 % at pH 8.5 (Tables 5.1 and 5.3). The results show that monensin had little effect on the Δp at pH 7.0 and yet growth was completely inhibited by monensin at pH 7 (Figure 5.5). Interestingly, monensin also reduced the internal pH at pH 5.5, which raises the question as to whether this effect is specific to CCCP (Table 5.3).

Table (5.3): Effect of CCCP and Monensin on Proton Motive Force (Δp) and ΔΨ, ΔpH, and pHi, as a function of external pH in *Staphylococcus xylosus* in 0.5 M NaCl *Halomonas* defined medium + 1g l^{-1} yeast extract in batch culture. Inhibitors were added 30 min before doing the experiment. The experiment was repeated three times.

Inhibitors	pHo	pHi	ΔΨ (mV)	ΔpH	Δp (mV)
CCCP (50 μM)	5.5	5.8 ± 0.07	-76.1 ± 1.2	0.29 ± 0.06	-89.7 ± 4.3
	7	6.71 ± 0.02	-87.4 ± 1.46	-0.29 ± 0.01	-70.1 ± 0.73
	8.5	7.64 ± 0.02	-103.1 ± 0.81	-0.86 ± 0.03	-52.6 ± 0.75
Monensin (50 μM)	5.5	5.82 ± 0.05	-91.9 ± 3.5	0.316 ± 0.05	-110.5 ± 2.4
	7	6.84 ± 0.01	-133.4 ± 1.89	-0.155 ± 0.01	-124.3 ± 2
	8.5	7.42 ± 0.01	-150.6 ± 2.98	-1.08 ± 0.01	-87.2 ± 2.3

Table (5.4): Effect of CCCP and Monensin on Proton Motive Force (Δp) and ΔΨ, ΔpH, and pHi, as a function of external pH in *Halomonas* Halo in 0.5 M NaCl *Halomonas* defined medium + 1g l^{-1} yeast extract in batch culture. Inhibitors were added 30 min before doing the experiment. The experiment was repeated three times.

Inhibitors	pHo	pHi	ΔΨ (mV)	ΔpH	Δp (mV)
CCCP (50 µm)	5.5	5.59 ± 0.04	-76.1 ± 2.40	0.09 ± 0.04	-81.2 ± 4.94
	7	6.05 ± 0.09	-128.4 ± 5.12	-0.95 ± 0.09	-72.8 ± 1.97
	8.5	7.96 ± 0.05	-107.67 ± 0.79	-0.54 ± 0.05	-76.02 ± 0.78
Monensin (50 µM)	5.5	5.85 ± 0.04	-81 ± 6.05	0.35 ±	-101.7 ± 7.50
	7	6.84 ± 0.18	-141.6 ± 6.26	-0.16 ± 0.15	-132.4 ± 11.99
	8.5	8.06 ± 0.03	-104.6 ± 3.11	-0.44 ± 0.03	-78.46 ± 1.62

Table 5.4 shows that CCCP significantly reduced the Δp of *Halomonas* Halo at the three external pH values to around 70-80 mV. Comparing the results to those in Table 5.2 it is clear that the percentage inhibition at pH 5.5 and 7.0 is large (44 and 53 % respectively), but it is low at pH 8.5 (4 %). Of course Δp at pH 8.5 was already very low (79 mV) in the absence of the inhibitors. Nevertheless the low values of Δp for *Halomonas* Halo in the presence of CCCP agree well with the complete inhibition of growth by CCCP (Figure 5.6).

Table 5.4 also shows that monensin had less effect on the Δp of *Halomonas* Halo than CCCP giving percentage inhibitions of 30, 14 and 0.05 % at pH 5.5, 7.0 and 8.5 respectively (Table 5.2). The small effect of monensin on Δp of *Halomonas* Halo at pH 7.0 agrees well with the fact that *Halomonas* Halo could grow (albeit at a reduced rate) in the presence of monensin (Figure 5.6).

5.2.5 Effect of CCCP and Monensin on the respiration rate of *Halomonas* Halo and *S. xylosus*

Tables 5.5 and 5.6 show that there is a large inhibitory effect of CCCP on the respiration rates of both organisms. The level of inhibition was essentially unchanged at around 70-80 % when the concentration of CCCP was varied from 125 - 500 μM. At these concentrations, CCCP appears not to be working as an uncoupler, but instead is inhibiting respiratory electron flow.

Table (5.5): Effect of CCCP and Monensin on the respiration rate of *Halomonas* Halo. Cells were grown in 0.5 M NaCl *Halomonas* defined medium + 1 g l^{-1} yeast extract, pH 7.0 harvested by centrifugation at 5000 *g* for 10 minutes, washed once with and resuspended in the same medium. 2 ml samples were used each time to measure the respiration rate as described in section 2.4. The respiration rate was monitored for a few minutes until stable. Inhibitors were added to give the final concentrations indicated in the table and the respiration rate was measured immediately.

Inhibitors	Amount	Concentration (µM)	O_2 uptake (µmole.mg^{-1} protein.h^{-1})				Standard Error	Inhibition (%)
			1	2	3	Means		
Ethanol	50 µl	---	8.5	14.9	14.5	12.6	2.1	00
Ethanol	100 µl	---	---	12.8	13.1	12.95	---	00
CCCP	50 µl	125	3.2	2.9	2.7	2.9	0.15	77 %
CCCP	50 µl	250	3.1	2.7	2.9	2.9	0.12	77 %
CCCP	50 µl	500	2.1	2.5	2.5	2.4	0.13	81 %
Monensin	50 µl	125	10.4	---	13.3	11.9	--	5.6 %
Monensin	100 µl	250	12	11.6	12.4	12	0.23	7.3 %

Table (5.6): Effect of CCCP and Monensin on the respiration rate of *Staphylococcus xylosus*. Cells were grown in 0.5 M NaCl *Halomonas* defined medium + 1 g l^{-1} yeast extract, pH 7.0 harvested by centrifugation at 5000 *g* for 10 minutes, washed once with and resuspended in the same medium. 2 ml samples were used each time to measure the respiration rate as described in section 2.4. The respiration rate was monitored for a few minutes until stable. Inhibitors were added to give the final concentrations indicated in the table and the respiration rate was measured immediately.

Inhibitors	Amount	Concentration (μM)	O_2 uptake (μ mole.mg^{-1} protein.h^{-1})				Standard Error	Inhibition (%)
			1	2	3	Means		
Ethanol	50 μl	---	4.9	4.9	4.6	4.8	0.1	00
Ethanol	100 μl	---	4.4	2.5	4.1	3.8	0.45	00
CCCP	50 μl	125	1.0	1.2	1.0	1.05	0.1	77.1 %
CCCP	50 μl	250	0.7	1.0	2.2	1.3	0.46	72.9 %
CCCP	50 μl	500	1.5	1.2	1.5	1.4	0.1	70.8 %
Monensin	100 μl	250	3.9	4.3	2.2	3.6	0.7	5.3 %

A true uncoupler actually increases the respiration rate in the short term because it removes the back pressure caused by a build up of Δp since ATPases are the rate limiting step.

However, 250 μM monensin only causes a small inhibition of the respiration rates of both organisms (Tables 5.5 and 5.6). Monensin affects the Na^+ permeability across the cell membrane, so if Na^+ was important for cell respiration we would expect to see a large inhibition, but we do not see this. So Na^+ is possibly not required for cell respiration in *S. xylosus* or *Halomonas* Halo.

Table 5.7 shows that there is a significant inhibitory effect of 50 μM CCCP on the respiration rate of *Halomonas* Halo at pH 8.5, but that it takes 3 hours to reach the level of inhibition induced by 125 μM CCCP after 5 minutes at pH 7 (Table 5.5). Interestingly, 50 μM CCCP does appear to act as an uncoupler immediately after its addition to *Halomonas* Halo cells, because the respiration rate increases from 7.7 to 8.7 μmoles mg^{-1} protein h^{-1} (Table 5.7).

Table (5.7): Effect of CCCP on the respiration rate of *Halomonas* Halo at pH 8.5. Cells were grown in 0.5 M NaCl *Halomonas* defined medium + 1 g l^{-1} yeast extract, harvested by centrifugation at 5000 *g* for 10 minutes, washed once with and resuspended in the same medium. Cells were concentrated 10 fold. 2 ml samples were used each time to measure the respiration rate as described in section 2.4. The respiration rate was monitored for a few minutes until stable. Inhibitors were added to give the final concentrations indicated in the table and the respiration rate was measured immediately.

			O_2 uptake (µmole.mg^{-1} protein.h^{-1})					
Inhibitors	**concentration**	**Time**	**1**	**2**	**3**	**Means**	**Standard Error**	**Inhibition (%)**
Control Ethanol	**———**	**0.00 h**	**6.9**	**7.3**	**8.9**	**7.7**	**0.61**	**------**
		1.00 h	**7.1**	**7.6**	**6.9**	**7.2**	**0.21**	**------**
		2.00 h	**5.9**	**6.2**	**4.8**	**5.6**	**0.43**	**------**
		3.00 h	**6.2**	**5.7**	**5.9**	**5.9**	**0.15**	**------**
CCCP	**50 µM**	**0.00 h**	**7.8**	**8.5**	**9.8**	**8.7**	**0.59**	**00**
		1.00 h	**4.3**	**3.6**	**3.0**	**3.6**	**0.38**	**50 %**
		2.00 h	**2.5**	**3.0**	**2.7**	**2.7**	**0.15**	**51.8 %**
		3.00 h	**1.8**	**1.3**	**1.1**	**1.4**	**0.21**	**76.3**

5.2.6 Determination of Δp as a function of external pH in *S. xylosus* grown at 1.5 M NaCl

Table 5.8 shows Δp values for *S. xylosus* grown at 1.5 M NaCl, all the results previously reported in this chapter have been for cells grown at 0.5 M NaCl. At 1.5 M NaCl the Δp values were between 150 and 190 mV at the three external pH levels. This means that Δp is higher in 1.5 M NaCl grown cells than in 0.5 M NaCl grown cells and this is particularly true for external pH values of 5.5 and 8.5 (Tables 5.1 and 5.8). The internal pH values were identical for *S. xylosus* cells grown at 0.5 and 1.5 M NaCl with external pH levels of 5.5 and 7.0. There was a small but significant increase in internal pH in *S.xylosus* cells grown at 1.5 M NaCl, pH 8.5 (Tables 5.1 and 5.8).

5.2.7 Determination of Δp as a function of external pH in *S. xylosus* grown in continuous culture

All of the results reported thus far in this chapter have been for cells growing in batch culture. To see if growing the cells in continuous culture affected the Δp, *S.xylosus* was grown in continuously with a dilution rate (D) = 0.056 h^{-1}. Table 5.9 shows that Δp values were between 120 and 155 mV and that the values of Δp at external pH values of 5.5 and 7.0 were lower than the equivalent values in batch culture (Table 5.1). However, the Δp at an external pH of 8.5 was higher in the cells grown in continuous culture (Tables 5.1 and 5.9). *S. xylosus* cells grown in continuous culture showed lower internal pH values at pH 5.5 and 7.0 than cells grown in batch culture.

Table (5.8): Determination of Proton Motive Force (Δp) and ΔΨ, ΔpH, and pHi, as a function of external pH in *Staphylococcus xylosus* in 1.5 M NaCl *Halomonas* defined medium + 1g l^{-1} yeast extract in batch culture. The experiment was repeated three times.

pH outside	pH inside	ΔΨ (mV)	ΔpH	Δp (mV)
5.5	6.6 ± 0.03	-127.9 ± 2.6	1.07 ± 0.04	-191 ± 4.8
7	7.2 ± 0.02	-137.9 ± 0.5	0.17 ± 0.02	-147.9 ± 0.9
8.5	7.85 ± 0.03	-191.5 ± 2.4	-0.64 ± 0.03	-153.9 ± 0.5

Table (5.9): Determination of Proton Motive Force (Δp) and ΔΨ, ΔpH, and pHi, as a function of external pH in *Staphylococcus xylosus* in 0.5 M NaCl *Halomonas* defined medium + 1 g l^{-1} yeast extract in continuous culture. The experiment was repeated three times.

pHo	pHi	ΔΨ (mV)	ΔpH	Δp (mV)
5.5	6.5 ± 0.03	-97.5 ± 1.7	0.99 ± 0.03	-155.3 ± 1.77
7	6.8 ± 0.05	-131.7 ± 3.7	-0.19 ± 0.05	-120.2 ± 0.9
8.5	7.7 ± 0.17	-180.9 ± 7.7	-0.82 ± 0.17	-132.9 ± 2.15

However, the situation was reversed at pH 8.5, where the internal pH was higher in cells grown in continuous culture (Tables 5.1 and 5.9).

Finally, *S. xylosus* cells were grown at 1.5 M NaCl in continuous culture (D= 0.056 h^{-1}) and the Δp values were between 160 and 210 mV for the three values of external pH (Table 5.10). These are the highest values of Δp measured for *S. xylosus* in the present work. The internal pH values were similar to those found for cells grown at different pH levels in batch and continuous culture (Tables 5.1, 5.7, 5.8 and 5.9).

Table (5.10): Determination of Proton Motive Force (Δp) and ΔΨ, ΔpH, and pHi, as a function of external pH in *Staphylococcus xylosus* in 1.5 M NaCl *Halomonas* defined medium + 1 g l^{-1} yeast extract in continuous culture. The experiment was repeated three times.

pHo	pHi	ΔΨ (mV)	ΔpH	Δp (mV)
5.5	6.7 ± 0.12	-141.6 ± 3.9	1.21 ± 0.13	-212.8 ± 11.2
7	7.1 ± 0.02	-151.1 ± 2.24	0.114 ± 0.02	-157.9 ± 2.1
8.5	7.5 ± 0.26	-221.6 ± 1.3	-0.99 ± 0.02	-164 ± 3.1

5.3 Discussion

The original description of *S. xylosus* by Schleifer and Kloos (1975) did not give information on the pH range for growth. The physiological studies by McMeekin *et al.* (1987), Rodriguez *et al.* (1996) and Vilhelmsson *et al.* (1997) and the molecular biological investigations by Fiegler *et al.* (1999) all grew *S. xylosus* at pH 7.0 - 7.2. Therefore, the present study appears to be the first investigation of the pH tolerance of S. *xylosus.* Figure 5.3 showed that *S. xylosus* grew well at pH values of 5.5, 7.0 and 8.5 indicating a fairly broad tolerance to pH. However, pH values outside this range quickly became inhibitory and no growth was found at pH 5 or 9.0.

In contrast, *Halomonas* Halo showed significant decreases in growth rate at pH 5.5 and 8.5, indicating a narrow pH optimum around pH 7.0 (Figure 5.4). The original description of the type species of *Halomonas, H. elongata,* by Vreeland *et al.* (1980) stated that growth was possible over the pH range 5 - 9, but that no growth was found outside of this range. The optimum pH for growth of *H. elongata* was not stated, nevertheless, it is clear that *Halomonas* Halo has a similar pH tolerance to *H. elongata*. However, not all members of the genus *Halomonas* are neutrophiles, recently two species of haloakaliphilic bacteria have been added to the genus. *H. desiderata* was isolated from a sewage treatment plant in Germany by Berendes *et al.* (1996). It has a pH range for growth of 7 - 11 and optimum growth is found betwen pH 9 and 10. *H. desiderata* is capable of dissimilatory nitrate reduction at alkaline pH levels and it can reduce nitrate to N_2. The second species was assigned to the *Halomonas* genus on the basis of 16S RNA

sequencing, it was isolated from an alkaline lake in Oregon, USA, which had been contaminated with 2,4-dichlorophenoxyacetic acid (2,4-D) (Maltseva *et al.* (1996). It could grow over a wide pH range from 6.5 - 10.5, with optimum growth between pH 8.4 and 9.4. In minimal medium, the pH range for growth was reduced to 7.4 - 9.8. In the present work, both *Halomonas* Halo and *S. xylosus* were grown in the presence of yeast extract when their pH tolerance was determined (Figures 5.3 and 5.4). Therefore, on minimal medium their degree of tolerance may be reduced.

A key element in the ability of a bacterium to grow over a range of pH values is the generation of a proton motive force (Δp) across the cytoplasmic membrane (section 5.1). Since *S. xylosus* grows equally well at the three pH values tested, it would be expected that the magnitude of the Δp would be similar at each external pH. However, the results shown in Table 5.1 do not support this expectation, because the Δp varies by 50 mV between cells grown in pH 5.5 and 8.5. This would imply that the lower level of Δp was sufficient to support a near maximum growth rate (Figure 5.3). The lower level of Δp for *S. xylosus* was 121 mV found in cells grown at pH 8.5 (Table 5.1). This value is less than the theoretical minimum level of Δp required to drive ATP synthesis i.e. 150 mV (section 5.1; Quirk *et al.*, 1991; Nicholls and Ferguson, 1992). A survey of the literature found two publications in which the Δp of *S. aureus* was determined, in both cases the measurements were made at external pH levels of 6–7 and the Δp varied from 180 mV at pH 7 to about 250 mV at pH 6 (Collins and Hamilton, 1976; Kashket, 1981). These values are some 50 mV higher than the measured values for *S. xylosus*

found in the present study (Table 5.1). There are no measurements of Δp in the literature for staphylococci growing at pH 8.5, but the results of Collins and Hamilton (1976) and Kashket (1981) may suggest that the Δp values for *S. xylosus* in the present study have been underestimated. If this is the case then the Δp for *S. xylosus* would be above the theoretical 150 mV requirement.

However, the Δp for *Halomonas* Halo cells grown at pH 8.5 is only 79 mV, which is barely half of the theoretical requirement of 150 mV (Table 5.2). In the case of *Halomonas* Halo growth is reduced at pH 8.5, but after 24 hours the final OD achieved was only slightly less than the final OD for cells grown at pH 7.0 (Figure 5.4). The main effect of growing *Halomonas* Halo at pH 8.5 is the lengthening of the initial lag phase and once growth starts after about 7 hours the growth rate is similar to that found for cells growing at pH 7.0 (Figure 5.4). This suggests that a period of adaptation takes place before growth can commence at pH 8.5. It may be that this adaptation involves processes which increase the Δp above the threshold of 150 mV. It must be kept in mind that the Δp value of 79 mV was measured for cells that had been grown overnight, concentrated and then incubated with the isotopes for 30 minutes to measure $\Delta\Psi$ and ΔpH. It does not measure possible changes to the level of Δp which may take place as the growth curve progresses and more experiments are required to clarify this point. There appears to have been no previous measurements of Δp in *Halomonas* species. However, Kashket (1985) summarised a range of Δp values for other Gram negative bacteria such as *E. coli, Salmonella and Vibrio* species. In general, the Δp

measurements for other Gram negative bacteria were higher than *Halomonas* Halo when grown at pH 7.0 (150 - 200 mV), however when *Vibrio alginolyticus* was grown at pH 8.0 the Δp was reduced to 125 mV. Nevertheless, as with *S. xylosus,* the measurements of Δp for *Halomonas* Halo in the present work are on the low side (Table 5.2).

The next step in the investigation was to examine the effect of inhibitors on the growth and Δp of *S. xylosus* and *Halomonas* Halo. Two inhibitors were chosen, firstly CCCP was used to increase the permeability of the cytoplasmic membrane to protons, which should abolish the Δp and make the internal pH the same as the external pH (Nicholls and Ferguson, 1992). Secondly, monensin was used to make the cytoplasmic membrane permeable to Na^+, which disrupts the activity of the Na^+/H^+ antiporter (Peddie *et al.*, 1999).

Both inhibitors were found to completely stop the growth of *S. xylosus* at pH 7.0 (Figure 5.5). At pH 7.0, in the presence of CCCP, the Δp was reduced to 70 mV from 138 mV (Tables 5.1 and 5.3). The residual Δp of 70 mV may appear quite large, but it should be noted that fixed charges on the cell wall and membrane phospholipids of bacteria give rise to a Donnan potential of about 50 mV (Nicholls and Ferguson, 1992). Therefore, almost all of the actively generated Δp had been abolished by CCCP. The effect of CCCP on Δp of *S. xylosus* at pH 5.5 and 8.5 was also found to be significant, particularly at pH 8.5 where it was reduced to 53 mV. MacLeod *et al.* (1988) found that CCCP was less effective at pH 8.5, but this was not the case for *S.*

xylosus cells in the present work (Table 5.3). The results demonstrate that the reduction in Δp is sufficient to account for the inhibition of growth by CCCP (Figure 5.3).

Monensin also completely inhibits growth of *S. xylosus* (Figure 5.5), but its effect on the Δp is significantly less than CCCP's effect (Table 5.3). It is possible that the reduction in Δp is sufficient to account for the complete inhibition of growth of *S. xylosus* by monensin. However, it may be that the effect that monensin has on the activity of Na^+/H^+ antiporter may be the main cause of its inhibitory effect. The Na^+/H^+ antiporter has an important role to play in the regulation of internal pH when cells are exposed to high levels of external pH (Figure 5.2c; Krulwich *et al.*, 1998). However, there is not normally a role for this antiporter at pH 7.0, therefore, the inhibition of the growth of *S. xylosus* by monensin at pH 7.0 is not easily explained.

When *Halomonas* Halo was exposed to CCCP and monensin, the results were different. Although, CCCP completely inhibited growth, monensin was much less effective causing an approximately 50 % decrease (Figure 5.6). However, the effect of monensin on the Δp of *Halomonas* Halo was almost identical to its effect on the Δp of *S. xylosus* (Tables 5.3 and 5.4). This confirms the suggestions made above that the main target of monensin's inhibitory effect is not the Δp directly.

The experiments described in section 5.2.5 were carried out quite early in the project and as a consequence higher concentrations of CCCP and monensin

were used to maximise the inhibitory effect on the respiration rates of *S. xylosus* and *Halomonas* Halo (Tables 5.5 and 5.6). With hindsight lower concentrations of the inhibitors should have been used to allow comparisons with their effects on growth and Δp. The important conclusion that can be drawn from Tables 5.5 and 5.6 is that CCCP inhibits the respiration rate of both *S. xylosus* and *Halomonas* Halo at concentrations of 125 μM and above. However, similar concentrations of monensin had very little inhibitory effect. This adds weight to the suggestions made above that the effect of monensin is indirect and it does not act on the respiratory chain.

When CCCP is acting as an uncoupler, it should actually increase the respiration rate over a short period of time. This is because the passage of H^+ ions through the ATPase is the rate limiting step (Figure 5.1; Nicholls and Ferguson, 1992) and bypassing this step should transiently increase electron transport and hence oxygen uptake. There is no increase in respiration rate evident at the high concentrations of CCCP used in the experiments reported in Tables 5.5 and 5.6. However, when a lower concentration of CCCP was used (50 μM) with *Halomonas* Halo cells grown at pH 8.5, a transient increase in respiration rate was seen (Table 5.7). More experiments are required to characterise this stimulatory effect.

The final set of experiments described in this chapter related to *S. xylosus* only and involved measuring the Δp under a variety of different growth conditions (Tables 5.8 - 5.10). Increasing the salinity from 0.5 to 1.5 M NaCl led to an increase in Δp at all three levels of external pH (Tables 5.1 and 5.8).

This is probably due to a greater demand for energy to transport choline into the cells and to convert it to glycine betaine to allow osmoadaptation at the higher salinity (Oren, 1999). Growing the cells in continuous culture at 0.5 M NaCl led to a small, but significant decrease in the Δp when compared with batch culture grown cells at 0.5 M NaCl (Tables 5.1 and 5.9). In contrast when the cells were grown in continuous culture at 1.5 M NaCl there was an increase in the levels of Δp, which again presumably reflects the increased requirement for compatible solute accumulation (Table 5.10). These preliminary experiments using continuous culture suggest that it would be fruitful to continue them in a systematic way to allow the calculation of yield values and maintenance energies when grown at different salinities and pH values (Neidhardt *et al.*, 1990).

CHAPTER 6

Effect of Temperature on the Activities of Enzymes Extracted from *Staphylococcus xylosus* and *Halomonas* Halo Cells Grown at Different Salinities

6.1 Introduction

In the prevoius chapters, tolerance of *S. xylosus* and *Halomonas* Halo to salinity and pH stress has been examined. In this final results chapter, the effect of another environmental stress factor, temperature, will be examined. In contrast to salinity or pH stress, microorganisms cannot avoid temperature stress by keeping their cytoplasm at a higher or lower temperature than ambient (Brock, 1986). However, it is possible that some protective benefit can be found by accumulating certain compounds. The presence of compatible solutes, synthesised in response to salinity increases, can protect against a range of other stresses such as high temperature (Lippert and Galinski, 1992), low temperature and freezing (Carpenter *et al.,* 1986), and desiccation (Lippert and Galinski, 1992).

In the present chapter, the possibility that compatible solutes may protect intracellular enzymes found in *S. xylosus* or *Halomonas* Halo was examined by growing the organisms at 0.5 and 2.0 M NaCl. Higher levels of compatible solutes will be found in the 2.0 M NaCl grown cells (Galinski, 1995). Cell-free extracts were then prepared and the activities of fumarase, malate

dehydrogenase, acid phosphatase and β-galactosidase were measured over a range of temperatures.

6.2 Results

6.2.1 Fumarase (E.C 4.2.1.2)

This enzyme is one of the tricarboxylic acid (TCA) cycle enzymes and it catalyses the following hydration reaction.

Fumarate + H_2O ⟶ L - malate

Fumarase activity was measured in cell-free extracts prepared from *Halomonas* Halo and *S. xylosus* cells grown in 0.5 and 2 M NaCl HDM + 1 g l^{-1} yeast extract at pH 7. The activity of fumarase from *Halomonas* Halo increased as the temperature was increased from 30-50 °C (Figure 6.1). There was then a plateau in the activity at 60 °C and then it decreased back to the 30 °C level when the extract was exposed to the highest temperature tested 70 °C. There was no significant difference between the response of fumarase from 2.0 M NaCl grown cells to that of fumarase from 0.5 M NaCl grown cells (Figure 6.1).

On the other hand, Figure 6.2 shows that the fumarase activity of cell extracts from *S. xylosus* grown at 2.0 M NaCl was higher than that of cell extracts from 0.5 M NaCl grown cells at all temperatures from 30 – 60 °C. Surprisingly, at the highest temperature tested, 70 °C, the reverse was true and the higher activity was found in 0.5 M NaCl grown cells. In contrast to fumarase from *Halomonas* Halo, the activity of the *S. xylosus* fumarase

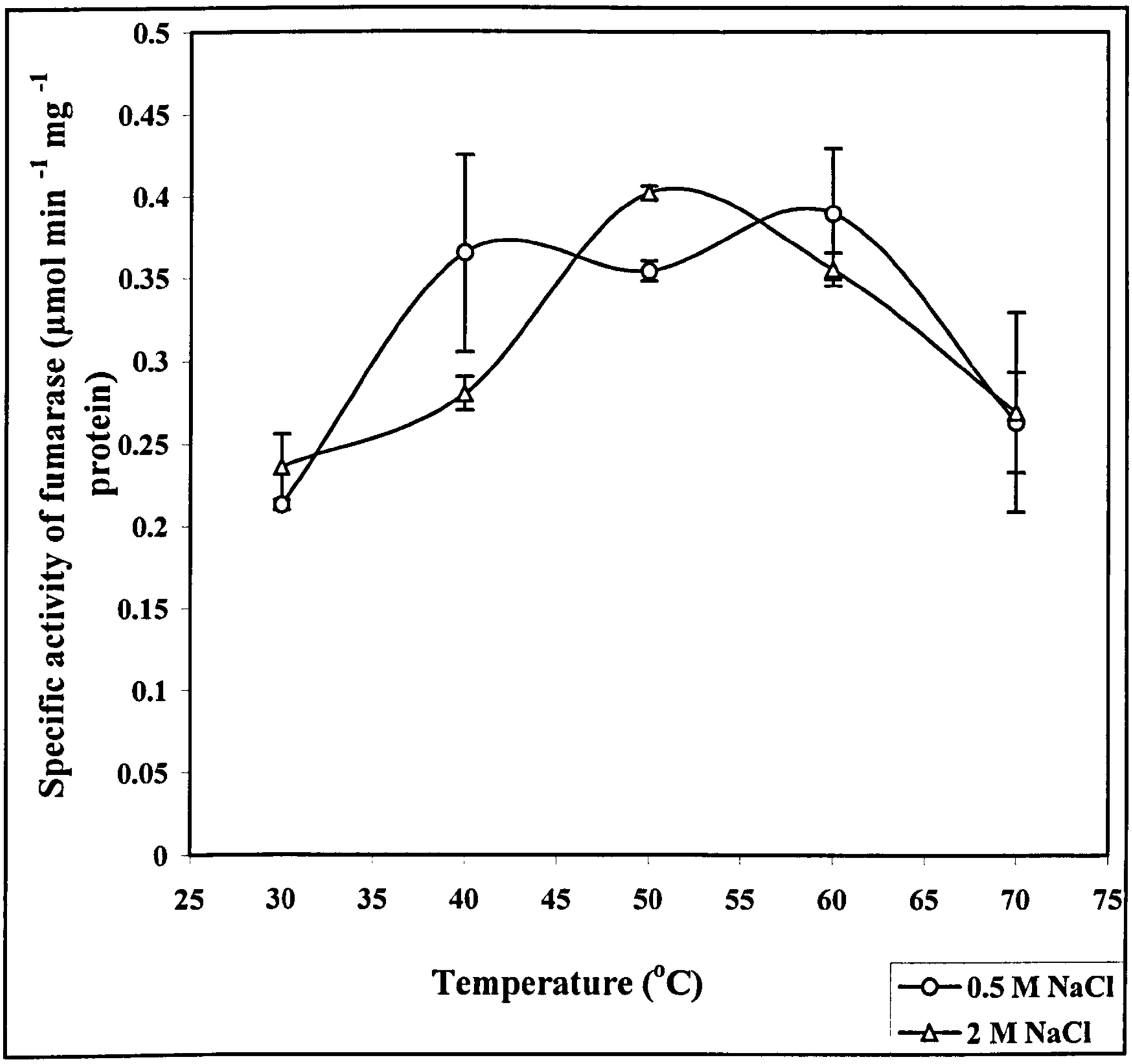


Fig: (6.1) Fumarase activity in cell - free extracts from *Halomonas* Halo. Cells were grown in 0.5 or 2 M NaCl HDM + 1 g l^{-1} yeast extract at pH 7. The enzyme extraction and the assay methods were described in sections 2.11.1 and 2.11.3.

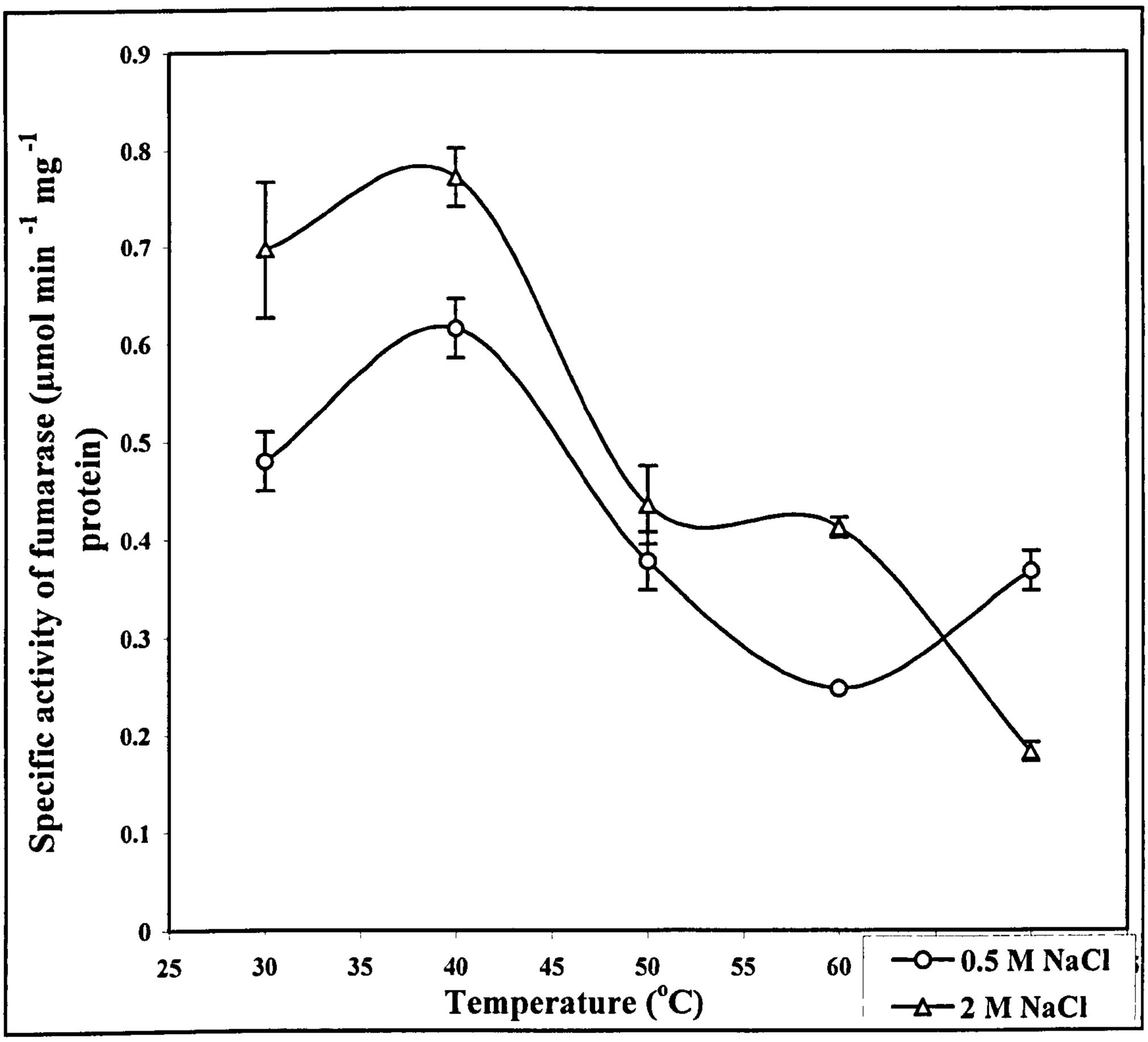


Fig: (6.2) Fumarase activity in cell - free extracts from *S. xylosus*.

Cells were grown in 0.5 or 2 M NaCl HDM + 1 g l^{-1} yeast extract at pH 7. The enzyme extraction and the assay methods were described in sections 2.11.1 and 2.11.3.

decreased significantly with increasing temperature (Figures 6.1 and 6.2). However, it should be noted that the initial activity of the *S. xylosus* fumarase at 30 °C was significantly higher than the *Halomonas* Halo enzyme.

6.2.2 Malate dehydrogenase (E.C 1.1.1.37)

This enzyme is also found in the TCA cycle and it catalyse the following reaction.

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

The equilibrium of the reaction lies far to the left and therefore the reaction is studied by measuring the oxidation of NADH. Malate dehdrogenase was measured in cell-free extracts from *Halomonas* Halo cells grown at 0.5 and 2 M NaCl. Figure 6.3 shows that the activity was much higher in the cell-free extracts from 2 M NaCl grown cells at 30, 40 and 50 °C, but that no activity was found at 60 or 70 °C for either the high or low salinity grown cells. It appears that malate dehydrogenase from *Halomonas* Halo is more susceptible to denaturation at high temperatures than fumarase from *Halomonas* Halo (Figures 6.1 and 6.3).

However, Figure 6.4 shows that the activity of malate dehydrogenase from *S. xylosus* cells grown at 0.5 M NaCl was higher than the activity from 2.0 M NaCl grown cells across the full range of temperatures tested (30 – 70 °C). This contradicts the findings for the same enzyme extracted from *Halomonas* Halo, but it should be noted that the activity of the *S. xylosus* malate dehydrogenase was very low compared to the activity found for the *Halomonas* Halo enzyme (Figures 6.3 and 6.4).

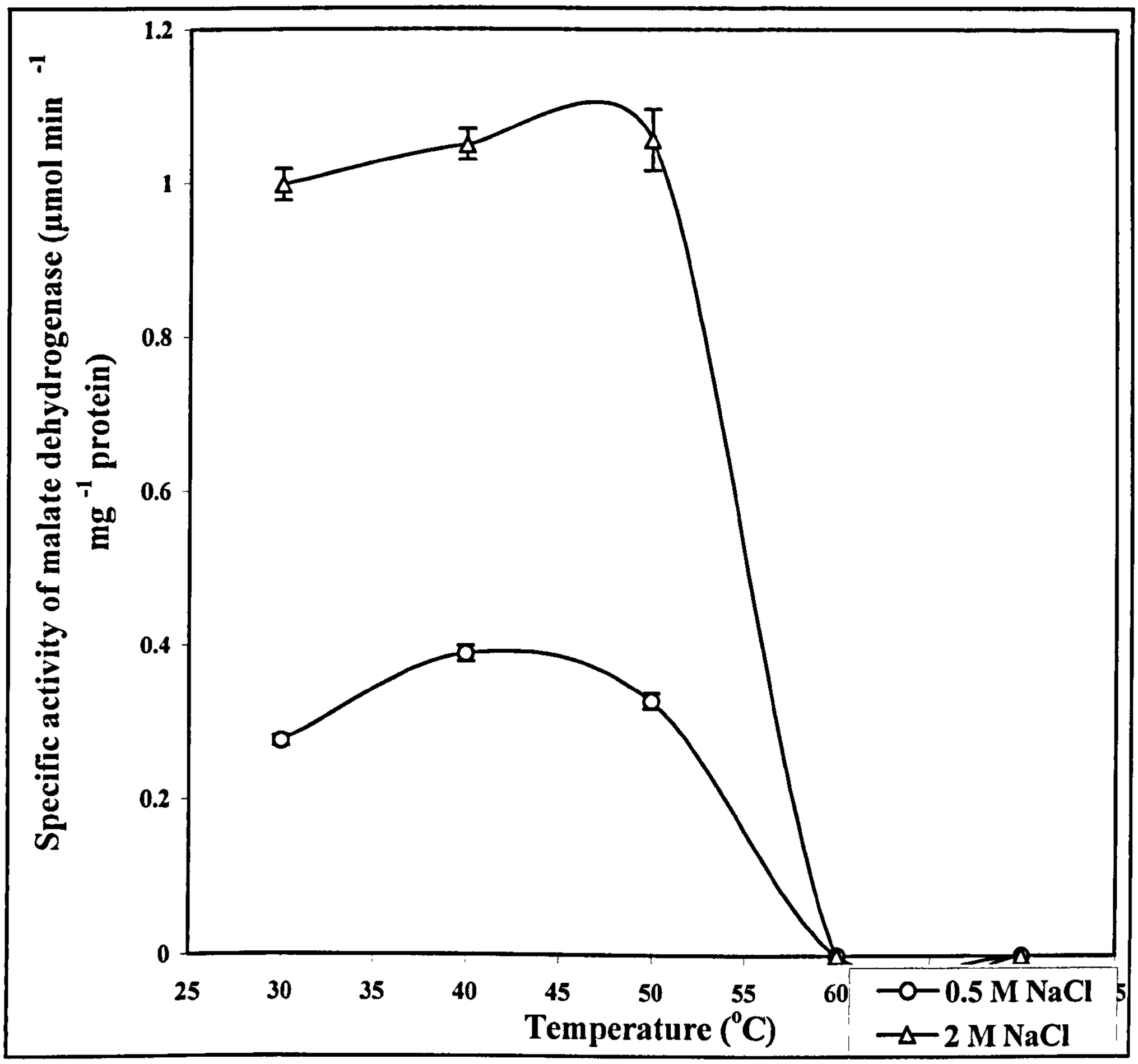


Fig: (6.3) Malate dehydrogenase activity in cell - free extracts from *Halomonas* Halo. Cells were grown in 0.5 or 2 M NaCl HDM + 1 g l^{-1} yeast extract at pH 7. The enzyme extraction and the assay methods were described in sections 2.11.1 and 2.11.4.

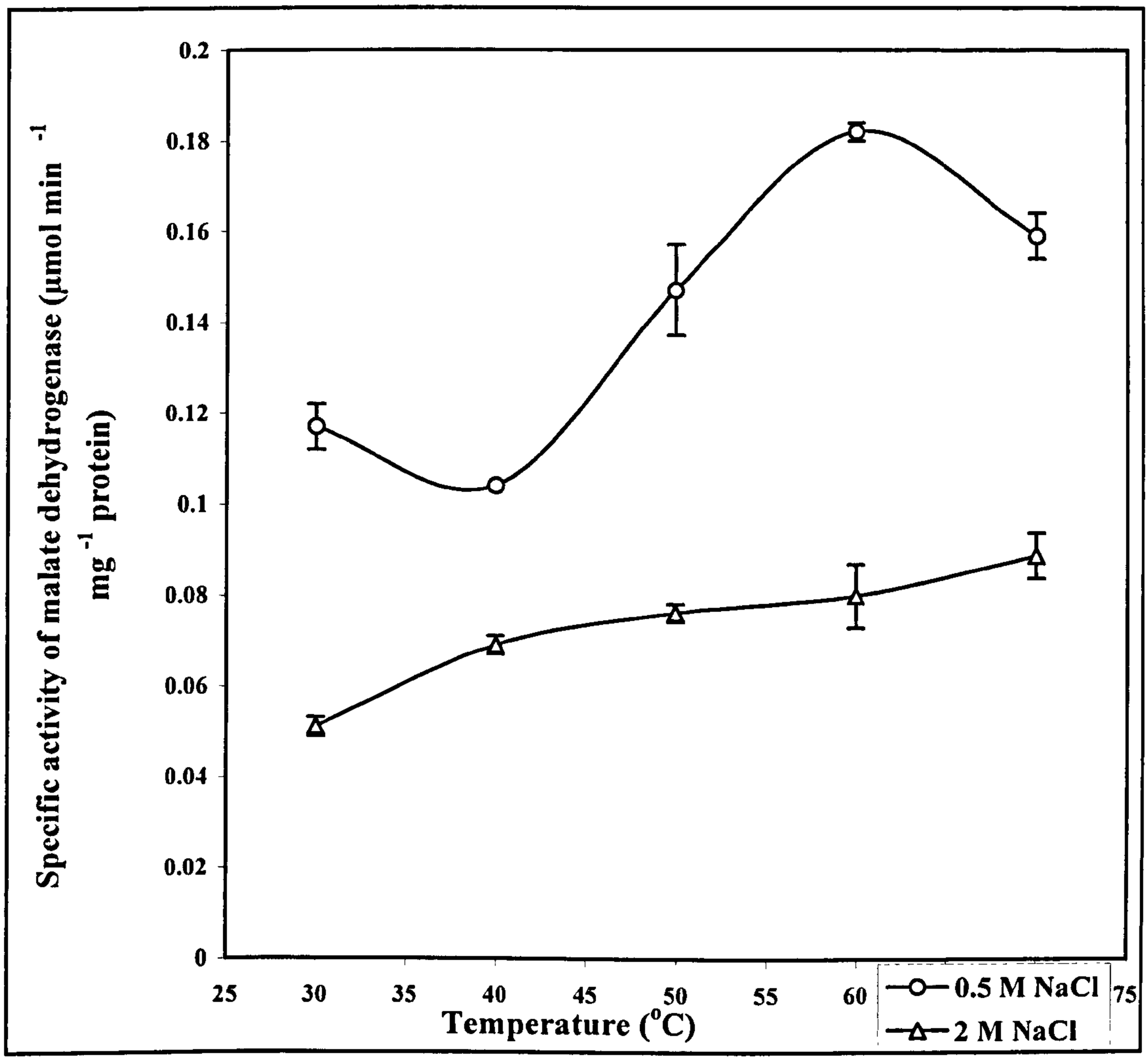


Fig: (6.4) Malate dehydrogenase activity in cell - free extracts from *S. xylosus*. Cells were grown in 0.5 or 2 M NaCl HDM + 1 g l^{-1} yeast extract at pH 7. The enzyme extraction and the assay methods were described in sections 2.11.1 and 2.11.4.

6.2.3 Acid phosphatase

The reaction used to measure acid phosphatase activity is shown below.

4-Nitrophenyl phosphate + H_2O → 4-nitrophenol + Pi (pH 4.8)

Acid phosphatase activity was measured in cell-free extracts from *Halomonas* Halo cells grown at 0.5 and 2.0 M NaCl. Figure 6.5 shows that there was a distinct maximum in the acid phosphatase activity at 40°C for both high and low salinity grown cells. The activity then declined sharply and was very low at 60 °C. At temperatures of 30, 40 and 50 °C, the low salinity grown cells show a slightly higher level of activity than 2.0 M NaCl grown cells (Figure 6.5).

The response of acid phosphatase extracted from *S. xylosus* cells was very different (Figure 6.6). Cell extracts from 0.5 M NaCl grown cells showed a similar level of acid phosphatase activity across the temperature range of 30 - 60 °C, with only a relatively small decrease in activity at 60 °C. In contrast, acid phosphatase activity from the high salinity grown cells increased to a peak at 50 °C before declining sharply at 60°C. However, again it should be noted that the acid phosphatase activity found in cell extracts from *S. xylosus* was much less than the activity in *Halomonas* cell extracts (Figures 6.5 and 6.6).

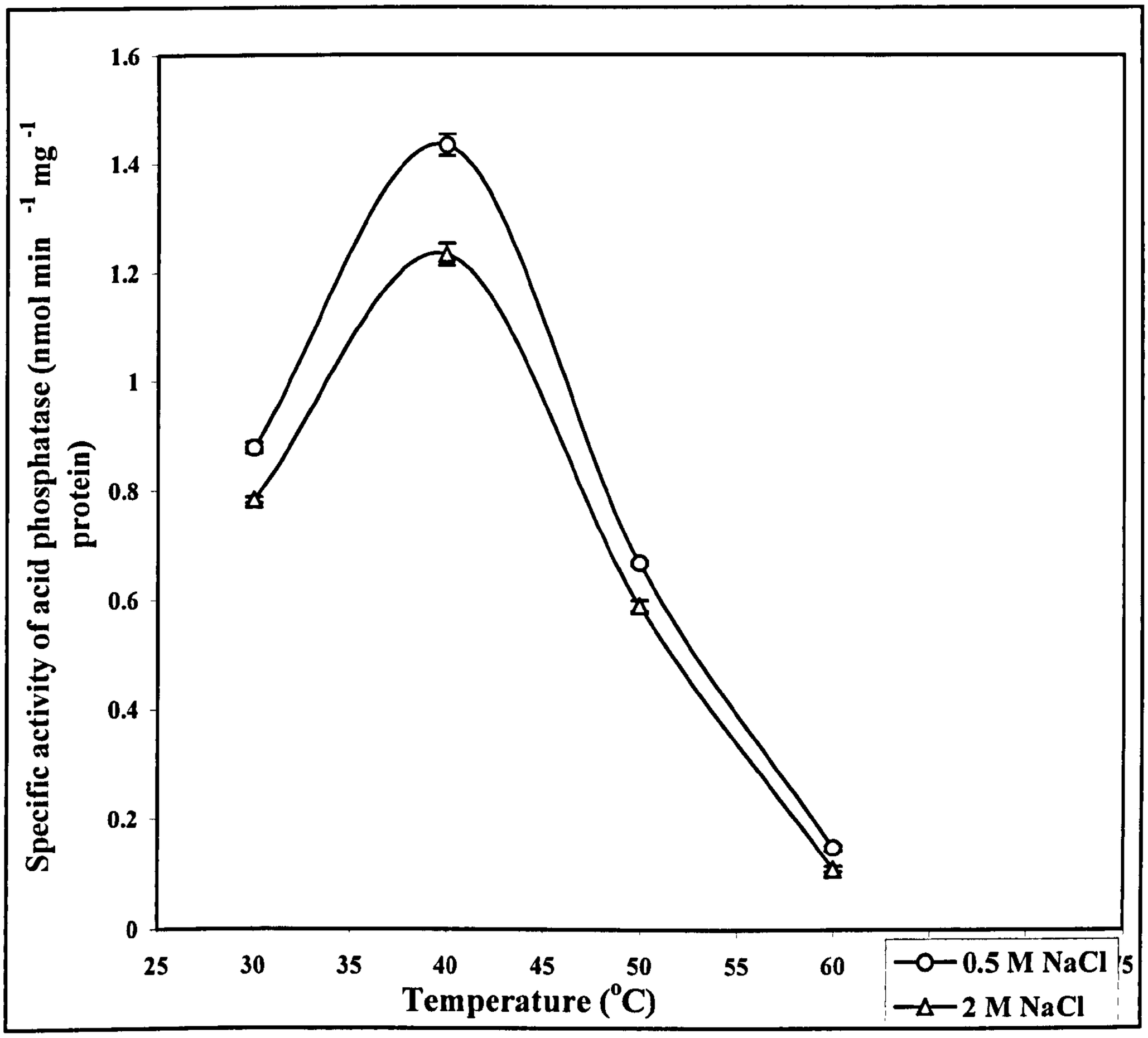


Fig: (6.5) Acid phosphatase activity in cell – free extracts from *Halomonas* Halo. Cells were grown in 0.5 or 2 M NaCl HDM + 1 g l^{-1} yeast extract at pH 7. The enzyme extraction and the assay methods were described in sections 2.11.1 and 2.11.5.

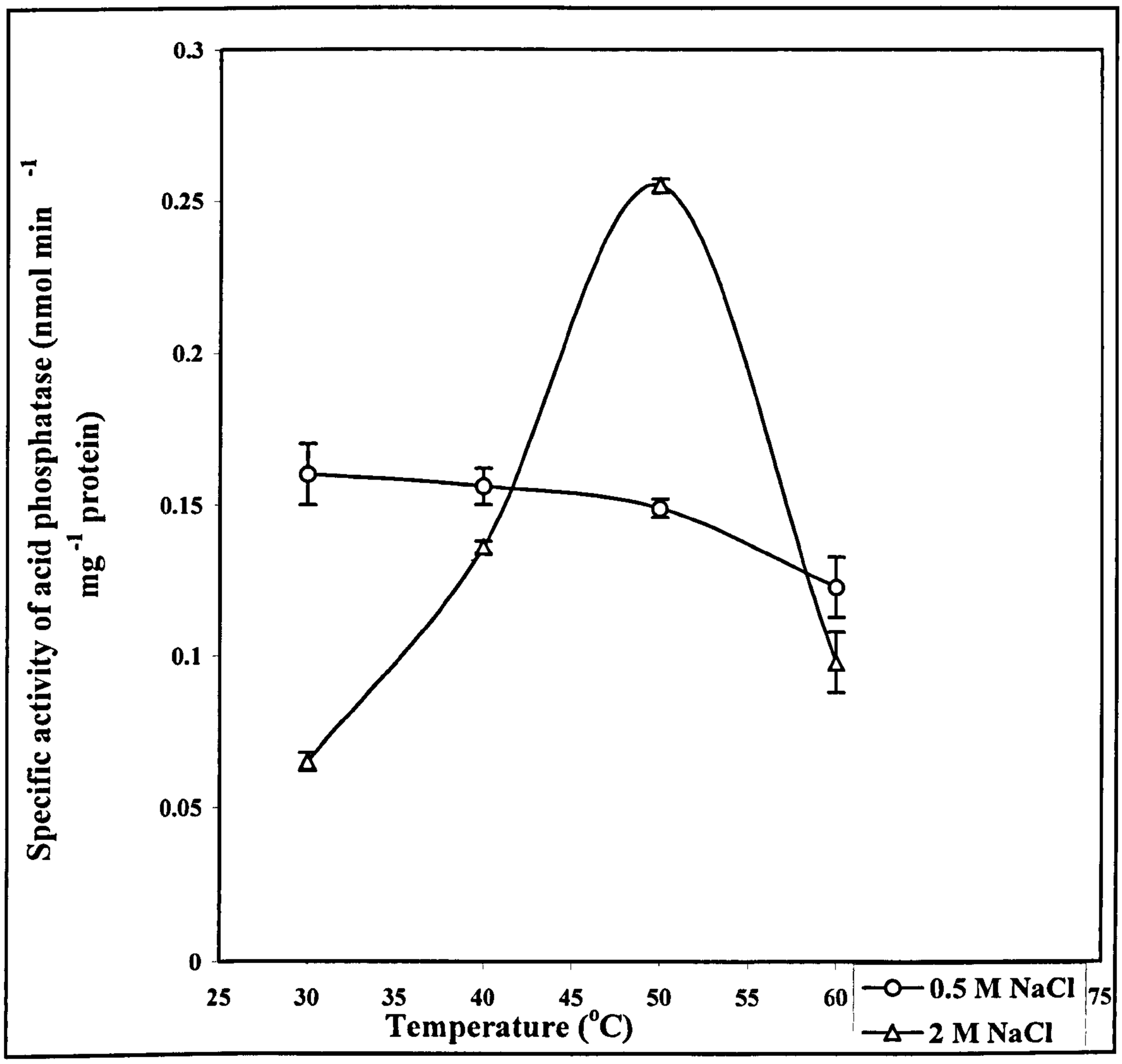


Fig: (6.6) Acid phosphatase activity in cell - free extracts from *S. xylosus*. Cells were grown in 0.5 or 2 M NaCl HDM + 1 g l^{-1} yeast extract at pH 7. The enzyme extraction and the assay methods were described in section 2.11.1 and 2.11.5.

6.2.4 β-Galactosidase (EC 3.2.1.23)

The reaction catalysed by β-galactosidase is shown below.

Lactose + H_2O ⟶ β - D – galactose + D – glucose

To induce the production of β-galactosidase, glucose was removed from the HDM medium + 1 g l^{-1} yeast extract and replaced with 20 mM lactose. *Halomonas* Halo did not grow under these conditions so β-galactosidase activity was measured in *S. xylosus* cell extracts only. Figure 6.7 shows that β-galactosidase was less resistant to high temperature than the other enzymes investigated. No activity was found at 47.5 °C or above, but there was a very significant difference in activity between high and low salinity grown cells at temperatures below 47.5 °C. At 30 and 40 °C the activity was much higher in the low salinity grown cells and at 45 °C while there was significant activity in low salinity grown cells, there was no β-galactosidase activity in high salinity grown cells (Figure 6.7).

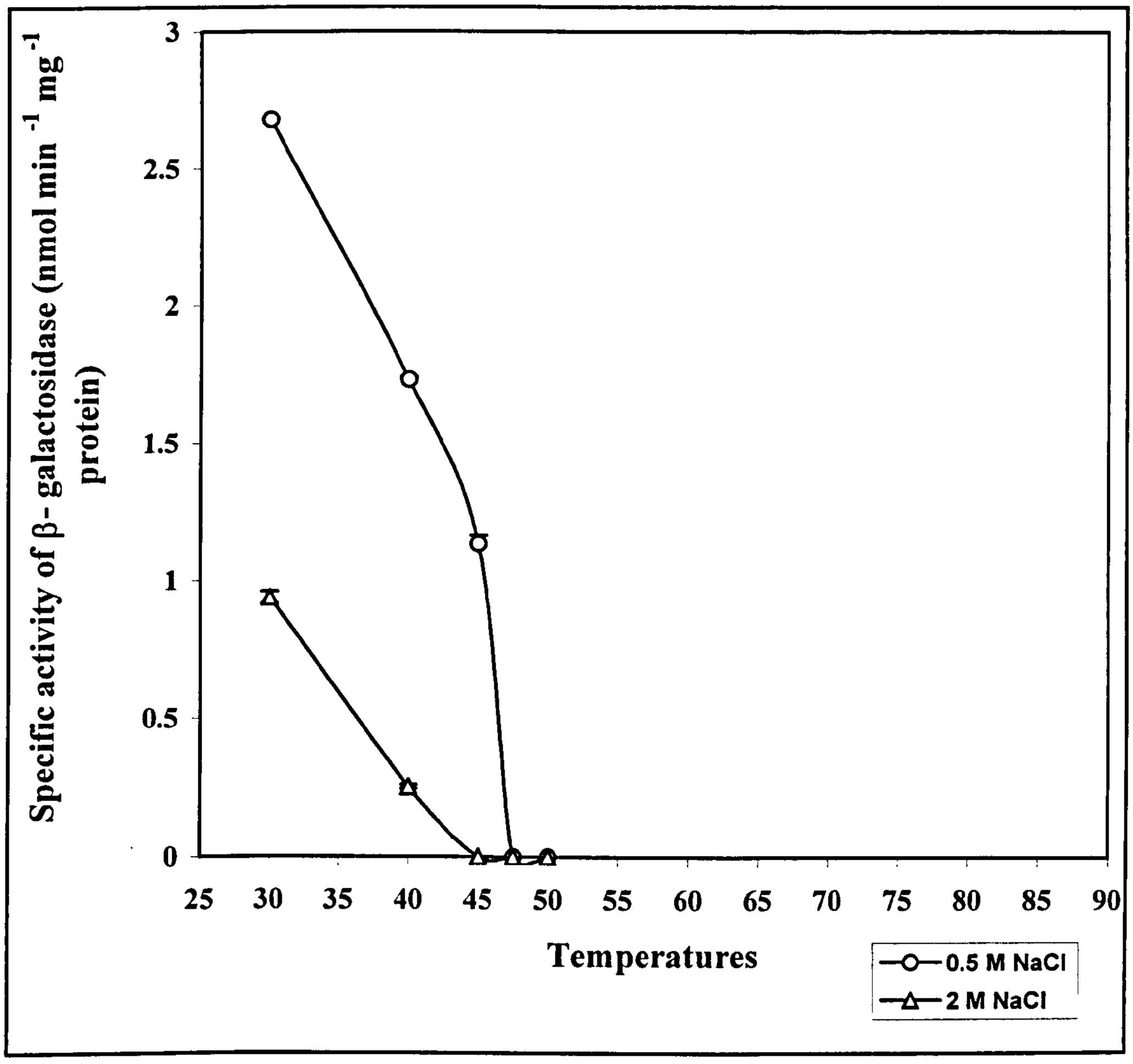


Fig: (6.7) β-galactosidase activity in cell - free extracts from *S. xylosus*.

Cells were grown in 0.5 or 2 M NaCl HDM + 1 g l^{-1} yeast extract, containing 20 mM lactose instead of glucose at pH 7. The enzyme extraction and the assay methods were described in section 2.11.1 and 2.11.6.

6.3 Discussion

The hypothesis being tested in this chapter is that enzymes from cells grown at higher salinities will be more resistant to higher temperatures due to the presence of intracellular compatible solutes (da Costa *et al.,* 1998). This was shown to be true for fumarase from *S. xylosus* (except at 70 °C, Figure 6.2) and malate dehydrogenase from *Halomonas* Halo (Figure 6.3). There was little or no support for the hypothesis from the other enzyme activities measured in *S. xylosus* and *Halomonas* Halo cell extracts. The fumarase activity from *Halomonas* (Figure 6.1) and the acid phosphatase activity from *S. xylosus* (Figure 6.6) did not show any significant effect of growth at high salinity on the enzyme's resistance to high temperature. Indeed higher activities in low salinity grown cells were found for acid phosphatase from *Halomonas* Halo (Figure 6.5) and for malate dehydogenase and β-galactosidase from *S. xylosus* (Figures 6.4 and 6.7).

It is difficult to draw definite conclusions from these data. However, it is important to consider the effect of the method of preparing the cell free extract on the results (section 2.11.1). Essentially the cells were broken open while suspended in Tris buffer. This will immediately dilute the concentration of compatible solutes in the cell-free extract and presumably reduce their effectiveness in protecting the enzymes. In addition, the cell-free extract is further diluted when it is added to the reaction mixture (sections 2.11.3 - 2.11.6). Therefore, during the measurement of activity the concentration of compatible solutes will be much reduced and the enzymes may not be effectively protected. This may well account for the variable

effect of the presence of compatible solutes on enzyme activity found in the present work.

To continue this work an alternative approach is required e.g. measurement of metabolic activity of intact cells when exposed to high temperatures. This could be achieved by measuring the utilisation of a radioactively labelled compound. Since these measurements would be made on intact cells, the intracellular concentration of compatible solutes would not be affected.

CHAPTER 7

GENERAL DISCUSSION

The major aim of the work described in this thesis was to compare the mechanisms of salt tolerance in Gram-positive and Gram-negative bacteria. To this end the well-studied Gram-negative bacterium *Halomonas* Halo was compared to a newly isolated Gram-positive coccus, which was identified as *Staphylococcus xylosus.* The work described in Chapter 3 showed that they both grew over a range of salinities from 0.1 – 3.0 M NaCl, with optimum growth at 0.5 M NaCl (for *Halomonas* Halo) and 0.1 M NaCl (for *S. xylosus)* (Figures 3.1 and 3.2). In the rich medium used in these experiments (HDM + 1 g l^{-1} yeast extract), *S. xylosus* grew much better at 3.0 M NaCl than *Halomonas* Halo. The salinity range tolerated by a bacterium can be reduced by using minimal medium (Canovas *et al.*, 1996) and at the beginning of Chapter 4 experiments were reported that looked at growth of *S. xylosus* and *Halomonas* Halo in a chemically defined medium (Figures 4.1 and 4.2). The main difference for *S. xylosus* was that growth at 3.0 M NaCl was much reduced in the absence of yeast extract, but this decrease in growth in 3.0 M NaCl minimal medium was much less apparent for *Halomonas* Halo. Taken together with the different optimum salinity for growth, these initial growth results confirm that *Halomonas* Halo was a

moderate halophile and that *S. xylosus* was a halotolerant bacterium (section 1.6 and Kushner, 1985).

To follow up the growth experiments, the requirement of Na^+ for growth of *Halomonas* Halo and *S. xylosus* was investigated (Chapter 3). It is problematical to completely remove Na^+ from the medium so the approach was to look at the effect of Na^+ on respiration rate (Figure 3.12). The results clearly showed a stimulation of respiration rate by Na^+ for *Halomonas* Halo, but not for *S. xylosus*. Another approach described in Chapter 3 was to look at growth of *Halomonas* Halo and *S. xylosus* in the presence of large amounts of KCl, good growth was found for both organisms up to 2 M KCl, but *S. xylosus* grew better at 3 M KCl than *Halomonas* Halo (Figures 3.8 and 3.9). When both organisms were subjected to 1.5 M NaCl + 1.5 M KCl, *Halomonas Halo* grew better (Figures 3.10 and 3.11). The likely conclusion is that *Halomonas* Halo does require Na^+ for growth, although mM levels are probably sufficient (Vreeland, 1987; Cummings and Gilmour, 1995). However, it appears that *S. xylosus* probably does not require Na^+, although further experiments are required to demonstrate this by careful elimination of Na^+ from the growth medium.

Previous work on *Halomonas* spp has shown that species of this genus can synthesise the compatible solute ectoine *de novo* (Galinski, 1995). It is also known that if choline or betaine are available in the medium, they will be transported into the *Halomonas* Halo cells and used in preference to ectoine (Cummings and Gilmour, 1995; Canovas *et al.*, 1996). This happens

because the transport of compatible solutes is energetically more favourable than their *de novo* synthesis (Oren, 1999). Results in Chapter 4 demonstrated that *Halomonas* Halo transports betaine into the cell when grown in 0.5 or 2.0 M NaCl in an energy dependent process (Figures 4.3 – 4.6). The presence of betaine or choline greatly stimulated the growth of *Halomonas* Halo at 3 M NaCl (Table 4.2), indicating that this strain is responding in the same way as other *Halomonas* strains. *S. xylosus* cells were also capable of betaine transport when grown at 0.5 or 3.0 M NaCl and again the process was energy dependent (Figures 4.7 – 4.10). Due to the fact that ^{14}C-betaine is not commercially available, this is the first demonstration of betaine transport in a *Staphylococcus* species. The presence of betaine also stimulated growth of *S. xylosus* at 3.0 M NaCl, although the effect was not as strong as for *Halomonas* Halo (Tables 4.1 and 4.2). In addition, the stimulatory effect of choline added to 3.0 M NaCl medium was much less for *S. xylosus* (Table 4.1). Choline does not act directly as a compatible solute; instead it is converted into betaine by a two step pathway (Figure 1.2). The enzymes involved, choline oxidase and betaine aldehyde dehydrogenase, were first identified by Landfald and Strom (1986) in *E. coli* and future experiments should look for these enzymes in *S. xylosus*.

However, it is clear *S. xylosus* can utilise external compatible solutes for growth at high salinities, but there is some evidence that this process may not be as efficient as it is in *Halomonas* Halo. If this is true the lack of efficiency is not due to the transport process, which appeared at least as

good if not better than the equivalent system in *Halomonas* Halo (Figures 4.3 – 4.10). What is presently unknown is which compatible solutes are synthesised *de novo* by *S. xylosus* in minimal medium. Proline is known to be synthesised by some *Staphylococcus* species (da Costa *et al.*, 1998), however no increase in proline content of *S. xylosus* was seen when cells were grown in 0.5 and 2.0 M NaCl (Table 4.3). It is possible that proline is always present at fairly high values in *S. xylosus* and that it has a role like trehalose in *E. coli* i.e. it accumulates in response to any stress situation (da Costa *et al.*,1998). Clearly, future work on *S. xylosus* must include investigations into the compatible solutes used in minimal medium.

The second part of the present project turned to other environmental stresses i.e. pH in Chapter 5 and temperature in Chapter 6. Both organisms were shown to tolerate a pH range from 5.5 – 8.5, but *S. xylosus* showed optimum growth across the full range whereas *Halomonas* Halo showed a distinct optimum at pH 7 (Figures 5.3 and 5.4). Most of the rest of Chapter 5 was devoted to an examination of the proton motive force (Δp) of both organisms across the pH range tolerated (5.5 – 8.5). Theoretical considerations described in section 5.1 suggest that a minimum Δp of 150 mV (inside negative) is required for ATP synthesis and thus growth. However, *S. xylosus* and *Halomonas* Halo did not always achieve this value, particularly at pH 8.5 (Tables 5.1 and 5.2). Since growth was unquestionably possible at pH 8.5, there is a paradox; either the theoretical calculations are wrong or the values of Δp are underestimated in the present study. It should also be noted

that the values in this study are low compared to a range of Δp values for other bacteria listed by Kashket (1985).

Are there any reasons why Δp may have been underestimated in this study? Perhaps the most likely explanation is that the cells were not actively metabolising during the period that the components of Δp, ΔΨ and ΔpH were being determined (see sections 2.10.2 and 2.10.5). During measurements the cells were in a dense suspension in a 1.5 ml Eppendorf tube with no shaking, therefore the suspension may have become anaerobic with a concomitant decrease in Δp. This possibility needs to be investigated in future experiments. Also, the cells were grown overnight before being concentrated for the Δp experiments which means that no attempt was made to determine Δp at different times during the batch culture growth curve. Harvesting cells at different points during growth should also be tried in the future.

It has already been mentioned that CCCP stopped the transport of betaine into cells of both organisms (Chapter 4). In Chapter 5, it was shown that CCCP completely inhibited the growth of *S. xylosus* and *Halomonas* Halo at pH 7.0 (Figures 5.5 and 5.6) and CCCP also reduced the Δp to very low levels in the range of 50 – 90 mV (Tables 5.3 and 5.4). Not all of the Δp is actively maintained, it is estimated that about 50 mV is due to the fixed negative charges on the cell wall and cytoplasmic membrane (called the Donnan Potential, see Nicholls and Ferguson, 1992). This means that CCCP

is abolishing almost all of the actively generated Δp, which accounts for the lack of growth in the presence of CCCP.

MacLeod *et al* (1988) started controversy over the efficiency of CCCP inhibition at alkaline pH. In the present study, CCCP had little effect on Δp of *Halomonas* Halo at pH 8.5, but this was probably because it was already very low in the absence of CCCP (Tables 5.2 and 5.4). In contrast, CCCP was very effective at reducing the Δp of *S. xylosus* from 121 mV to 53 mV at pH 8.5 (Tables 5.1 and 5.3). Therefore, CCCP appears to function well at pH 8.5.

Monensin completely inhibited growth of *S. xylosus,* but was only 50 % effective against *Halomonas* Halo (Figures 5.5 and 5.6). However, in both cases the Δp was reduced by similar amounts and the reductions were much less than the CCCP induced Δp reductions (Tables 5.3 and 5.4). Monensin is known to increase the permeability of membranes to Na^+ (Peddie *et al.*, 1999), but it appears that this has a relatively small effect on the magnitude of Δp in *S. xylosus* and *Halomonas* Halo. The relative insensitivity of *Halomonas* Halo to monensin is perhaps surprising, because the results in Chapter 3 suggested that Na^+ was required for growth. More experiments are required to look at the possible role of Na^+, probably via Na^+/H^+ antiporters, in internal pH regulation in both organisms.

High concentrations of CCCP and monensin (125 μM and above) completely inhibited respiration in both organisms (Tables 5.5 and 5.6). However, it was

interesting to note that a more normal concentration of CCCP (50 μM) caused an initial increase in oxygen uptake in *Halomonas* Halo cells grown at pH 8.5 (Table 5.7). These data suggest that at 50 μM, CCCP acts as a true uncoupler i.e. it transiently allows electron transport to increase because it allows the ATPases to be bypassed (section 5.3 and Nicholls and Ferguson, 1992). However, at higher concentrations, it is probably acting as a general metabolic poison.

In the final part of Chapter 5, the Δp of *S. xylosus* was also measured in continuous culture and in cells grown in 1.5 M NaCl. These preliminary experiments should be followed up by a systematic series of experiments using continuous culture techniques to determine yield values and maintenance energies at a range of salinities and pH values.

Finally, Chapter 6 examined the hypothesis that compatible solutes may protect enzymes from denaturation by high temperatures. The concentration of compatible solutes was increased by growing both organisms at 0.5 and 2.0 M NaCl HDM + 1 g l^{-1} yeast extract. In the presence of yeast extract it is likely that betaine was the major compatible solute inside both *S. xylosus* and *Halomonas* Halo cells (Cummings and Gilmour, 1995; Galinski, 1995; da Costa *et al.*, 1998). The results were inconclusive and as pointed out in section 6.3, there are flaws in the methods used, because the level of intracellular compatible solutes would be diluted during the preparation of the cell-free extracts. Clearly, more work is required to overcome these problems. It may be more fruitful to measure metabolic activities of intact

cells or known compatible solutes could be included in the enzyme reaction mixtures.

The work described in this thesis shows that the Gram-positive bacterium, *Staphylococcus xylosus*, responds to environmental stress in broadly similar ways to the Gram-negative bacterium *Halomonas* Halo.

Reference List

Ahmad, S. and Booth, I.R. (1983) The effect of β-galactosidase on the proton motive force and growth of *Escherichia coli*. *Journal of General Microbiology* **129**, 2521-2529.

Allen, R.D. and Baumann, P. (1971) Structure and arrangement of flagella in species of the genus *Beneckea*, and *Photobacterium fischeri*. *Journal of Bacteriololgy* **107**, 295-302.

Arakawa, T. and Timasheff, S.N. (1985) The stabilization of proteins by osmolytes. *Biophysical Journal* **47**, 411-414.

Ashbolt, N.J. (1991) Biotechnology in the antarctic - a unique source of microorganisms. *Genetic Engineer And Biotechnologist* **11**, 19-22.

Bae, J.H. and Miller, K.J. (1992) Identification of 2 proline transport-systems in *Staphylococcus aureus* and their possible roles in osmoregulation. *Applied and Environmental Microbiology* **58**, 471-475.

Bates, I.S., Waldren, R.P. and Teare, I.D. (1973) Rapid determination of free proline for water-stress studies. *Plant and Soil* **29**, 205-207.

Berendes, F., Gottschalk, G., HeineDobbernack, E., Moore, E.R.B. and Tindall, B.J. (1996) *Halomonas desiderata sp nov*, a new alkaliphilic, halotolerant and denitrifying bacterium isolated from a municipal sewage works. *Systematic And Applied Microbiology* **19**, 158-167.

Booth, I.R. (1985) Regulation of cytoplasmic pH in bacteria. *Microbiological Reviews* **49**, 359-378.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.

Brey, R.N., Rosen, B.P. and Sorensen, E.N. (1979) Cation/proton antiport system in *Escherichia coli*. *Journal Of Biological Chemistry* **255**, 39-44.

Brink, B. and Konings, W.N. (1982) Electrochemical proton gradient and lactate concentration in *Streptococcus cremoris* cells grown in batch culture. *Journal of Bacteriology* **152**, 682-686.

Brock, T.D. (1979) Ecology of Saline Lakes. In: Shilo, M., (Ed.) *Strategies of Microbial Life in Extreme Environments.*, pp. 29-47. Berlin: Dahlem Konferenzen.

Brock, T.D. (1986) *Thermophiles, General, Molecular and Applied Microbiology.*, New York: Wiley.

Brown, A.D., Mackenzie, K.F. and Singh, K.K. (1986) Selected aspects of microbial osmoregulation. *FEMS Microbiology Reviews* **39**, 31-36.

Brown, A.D. (1990) *Microbial Water Stress Physiology: Principles and Perspectives.*, UK: John Wiely & Sons.

Canovas, D., Vargas, C., Csonka, L.N., Ventosa, A. and Nieto, J.J. (1996) Osmoprotectants in *Halomonas elongata*: High-affinity betaine transport system and choline-betaine pathway. *Journal Of Bacteriology* **178**, 7221-7226.

Canovas, D., Vargas, C., IglesiasGuerra, F., Csonka, L.N., Rhodes, D., Ventosa, A. and Nieto, J.J. (1997) Isolation and characterization of salt-sensitive mutants of the moderate halophile *Halomonas elongata* and cloning of the ectoine synthesis genes. *Journal Of Biological Chemistry* **272**, 25794-25801.

Canovas, D., Vargas, C., Csonka, L.N., Ventosa, A. and Nieto, J.J. (1998) Synthesis of glycine betaine from exogenous choline in the moderately halophilic bacterium *Halomonas elongata*. *Applied and Environmental Microbiology* **64**, 4095-4097.

Cappuccino, J.G. and Sherman, N. (1983) *Microbiology: A Laboratory Manual.*, London: Addison-Wesley.

Carpenter, J.F., Hand, S.C., Crowe, L.M. and Crowe, J.H. (1986) Cryoprotection of phosphofructokinase with organic solutes: characterization of enhanced protection in the presence of divalent cations. *Archives Biochemistry Biophysics* **250**, 505-512.

Cayley, S., Lewis, B.A. and Record, M.T. (1992) Origins of the osmoprotective properties of betaine and proline in *Escherichia coli* K-12. *Journal of Bacteriology* **174**, 1586-1595.

Collins, C.H. and Lyne, P.M. (1985) *Microbiological Methods.*, London: Butterworths.

Collins, S.H. and Hamilton, W.A. (1976) Magnitude of the protonmotive force in respiring *Staphylococcus aureus* and *Escherichia coli*. *Journal of Bacteriology* **126**, 1224-1231.

Crogham, P.C. (1958) The survival of *Artemia salina* (L) in various media. *Journal of Experimental Biology.* **35**, 213-219.

Csonka, L.N. (1989) Physiological and genetic responses of bacteria to osmotic stress. *Microbiological Reviews* **53**, 121-147.

Cummings, S.P. and Gilmour, D.J. (1995) The effect of NaCl on the growth of a *Halomonas* species - accumulation and utilization of compatible solutes. *Microbiology* **141**, 1413-1418.

da Costa, M.S., Santos, H. and Galinski, E.A. (1998) An overview of the role and diversity of compatible solutes in bacteria and archaea. *Advances in Biochemical Engineering Biotechnology* **61**, 117-153.

Delieu, T. and Walker, D.A. (1972) An improved cathode for the measurement of photosynthetic oxygen evolution by isolated chloroplasts. *New Photologist.* **71**, 201-225.

del Moral, A., Valderrama, M.J., Ferrer, M.R., Peran, F., Quesada, E. and Ramos-Cormenzana, A. (1991) Effect of external salinity changes on cellular composition of some ions and amino-acids in *Deleya halophila*. *Research In Microbiology* **142**, 103-107.

del Moral, A., Severin, J., Ramos-Cormenzana, A., Truper, H.G. and Galinski, E.A. (1994) Compatible solutes in new moderately halophilic isolates. *FEMS Microbiology Letters* **122**, 165-172.

Deutch, C.E. (1994) Characterization of a novel salt-tolerant *Bacillus* sp from the nasal cavities of desert iguanas. *FEMS Microbiology Letters* **121**, 55-60.

Dobson, S.J., James, S.R., Franzmann, P.D. and McMeekin, T.A. (1991) A numerical taxonomic study of some pigmented bacteria isolated from Organic Lake, an antarctic hypersaline lake. *Archives of Microbiology* **156**, 56-61.

Dsouzaault, M.R., Smith, L.T. and Smith, G.M. (1993) Roles of N-acetylglutaminylglutamine amide and glycine betaine in adaptation of *Pseudomonas aeruginosa* to osmotic-stress. *Applied and Environmental Microbiology* **59**, 473-478.

Epstein, W. (1986) Osmoregulation by potassium-transport in *Escherichia-coli*. *FEMS Microbiology Reviews* **39**, 73-78.

Erlich, A. and Dor, I. (1985) Photosynthetic microorganisms of the Gavish Sabkha. In: Friedman, G.M., (Ed.) *Hypersaline Ecosystems.*, pp. 296-321.

Fiegler, H., Bassias, J., Jankovic, I. and Brückner, R. (1999) Identification of a gene in *Staphylococcus xylosus* encoding a novel glucose uptake protein. *Journal of Bacteriology* **181**, 4929-4936.

Foissy, H. (1974) Examination of *Brevibacterium linens* by an electrophoretic zymogram technique. *Jounal of General Microbiology* **80**, 197-207.

Franzmann, P.D., Burton, H.R. and McMeekin, T.A. (1987) *Halomonas subglaciescola,* a new species of halotolerant bacteria isolated from antarctica. *International Journal of Systematic Bacteriology* **37**, 27-34.

Franzmann, P.D. (1991) The microbiota of saline lakes of the Vestfold Hills, Antarctica. In: Rodriguez-Valera, F., (Ed.) *General and Applied Aspects of Halophilic Microorganisms.*, pp. 9-14. New York: Plenum Press.

Frings, E., Kunte, H.J. and Galinski, E.A. (1993) Compatible solutes in representatives of the genera *Brevibacterium* and *Corynebacterium* - occurrence of tetrahydropyrimidines and glutamine. *FEMS Microbiology Letters* **109**, 25-32.

Galinski, E.A. and Truper, H.G. (1982) Betaine, a compatible solute in the extremely halophilic phototrophic bacterium *Ectothiorhodospira halochloris*. *FEMS Letters* **13**, 357-360.

Galinski, E.A., Pfeiffer, H.P. and Truper, H.G. (1985) 1,4,5,6-Tetrahydro-2-methyl-4-pyrimidinecarboxylic acid - a novel cyclic amino acid from halophilic phototrophic bacteria of the genus *Ectothiorhodospira*. *European Journal Of Biochemistry* **149**, 135-139.

Galinski, E.A. and Lippert, K. (1991) Novel compatible solutes and their potential application as stabilisers in enzyme technology. In: Rodriguez-Valera, F., (Ed.) *General and Applied Aspects of Halophilic Microorganisms.*, pp. 351-358. New York: Plenum Press.

Galinski, E.A. (1993) Compatible solutes of halophilic eubacteria - molecular principles, water-solute interaction, stress protection. *Experientia* **49**, 487-496.

Galinski, E.A. (1995) Osmoadaptation in bacteria. *Advances in Microbial Physiology.* **37**, 273-328.

Garland, P.B. (1977) Energy transduction and transmission in microbial systms. *Symposium of the Societyof General Microbiology* **27**, 1-21.

Gauthier, M.J., Munro, P.M. and Mohajer, S. (1987) Influence of salts and sodium chloride on the recovery of *Esherichia coli* from seawater. *Current Microbiology* **15**, 5-10.

Gavish, E. (1980) Recent Sabkhas marginal to the southern coasts of Sinia Red Sea. In: Nissenbaum, A., (Ed.) *Developments in Sedimentology*, pp. 233-252. Amsterdam: Elsevier.

Gerba, C.P. and Mcleod, J.S. (1976) Effects of sediments on the survival of *Esherichia coli* in marine waters. *Applied and Environmental Microbiology* **32**, 114-120.

Gerdes, G., Krumbein, W.E. and Holtkamp. (1985a) Salinity and water activity related zonation of microbial communities and potential stromatolites of the Gavish Sabkha. In: Friedman, G.M., (Ed.) *Hypersaline Ecosystems.*, pp. 238-266. Berlin: Springer Verlag.

Gerdes, G., Spira, Y. and Dimentman, C. (1985b) The fauna of the Gavish Sabkha and the Solar Lake - A comparative study. In: Friedman, G.M., (Ed.) *Hypersaline Ecosystems.*, pp. 322-345. Berlin: Springer Varlag.

Ghoul, M., Bernard, T. and Cormier, M. (1990) Evidence that *Esherichia coli* accumulates glycine betaine from marine sediments. *Applied and Environmental Microbiology* **56**, 551-554.

Gilmour, D.J., Hipkins, M.F. and Boney, A.D. (1982) The effect of salt stress on the primary processes of photosynthesis in *Dunaliella tertiolecta*. *Plant Science Letters* **26**, 325-330.

Gilmour, D.J. (1990) Halotolerant and halophilic microorganisms. In: Edwards, C., (Ed.) *Microbiology of Extreme Environments.*, pp. 147-177. Milton Keynes: Open University Press.

Gimmler, H. and Schirling, R. (1978) Cation permeability of the halotolerant alga *Dunaliella parva*. II Cation content and glycerol concentration of the cells as dependent upon external NaCl concentration. *Zeitschrift fur Pflanzenphysiologie* **87**, 435-444.

Gimmler, H., Kaaden, R., Kirchner, U. and Weyand, A. (1984) The chloride sensitivity of *Dunaliella parva* enzymes. *Zeitschrift fur Pflanzenphysiologie* **114**, 131-150.

Ginzburg, M., Sachs, L. and Ginzburg, B.Z. (1971) Ion metabolism in *Halobacterium*. *Journal of Membrane Biology* **5**, 78-101.

Gochnauer, M.B., Leppard, G.G., Komaratat, P., Kates, M., Novitsky, T. and Kushner, D.J. (1975) Isolation and characterization of *Actinopolyspora halophila*, *gen. et sp. nov.*, an extremely halophilic actinomycete. *Canadian Journal of Microbiology* **21**, 1500-1511.

Golubic, S. (1980) Halophily and halotolerance in Cyanophytes. *Origins of Life* **10**, 169-183.

Gouesbet, G., Blanco, C., Hamelin, J. and Bernard, T. (1992) Osmotic adjustment in *Brevibacterium ammoniagenes*: pipecolic acid accumulation at elevated osmolalities. *Journal of General Microbiology* **138**, 959-965.

Graham, J.E. and Wilkinson, B.J. (1992) *Staphylococcus aureus* osmoregulation - roles for choline, glycine betaine, proline, and taurine. *Journal of Bacteriology* **174**, 2711-2716.

Grant, W.D. and Ross, H.M. (1986) The ecology and taxonomy of *Halobacteria*. *FEMS Microbiology Reviews* **39**, 9-15.

Grant, W.D. and Tindall, B.J. (1986) The alkaline saline environment. In: Herbert, R.A. and Codd, G.A., (Eds.) *Microbes in Extreme Environments.*, pp. 25-54. London: Academic Press.

Guffanti, A.A., Susman, P., Blanco, R. and Krulwich, T.A. (1978) The proton motive force and α-aminoisobutyric acid transport in an obligately alkalophilic bacterium. *Journal of Biological Chemistry* **253**, 708-715.

Guffanti, A.A., Fuchs, R.T., Schneier, M., Chiu, E. and Krulwich, T.A. (1984) A $\Delta\Psi$ generated by respiration is not equivalent to a diffusion potential of the same magnitude for ATP synthesis by *Bacillus firmus* RAB. *Journal of Biological Chemistry* **259**, 2971-2975.

Hackstadt, T. (1983) Estimation of the cytoplasmic pH of *Coxiella burnetti* and effect of substrate oxidation on proton motive force. *Journal of Bacteriology* **154**, 591-597.

Hamaide, F., Kushner, D.J. and Sprott, G.D. (1983) Proton motive force and Na^+-H^+ antiport in a moderate halophile. *Journal of Bacteriology* **156**, 537-544.

Harold, F.M., Pavlasova, E. and Baarda.J.R. (1969) A transmembrane pH gradient in *Streptococcus faecalis*. Origin and dissipation by proton conductors and N,N-dicyclohexylcarbodiamide. *Biochimica and Biophysica Acta* **196**, 235-244.

Hart, D.J. and Vreeland, R.H. (1988) Changes in the hydrophobic-hydrophilic cell surface character of *Halomonas elongata* in response to NaCl. *Journal of Bacteriology* **170**, 132-135.

Hebert, A.M. and Vreeland, R.H. (1987) Phenotypic comparison of halotolerant bacteria - *Halomonas halodurans sp-nov*, *nom rev*, *comb-nov*. *International Journal of Systematic Bacteriology* **37**, 347-350.

Hegazi, F.Z. (1984) The microbial flora of salted raw milk. *Systematic and Applied Microbiology* **5** , 527-533.

Hildebrand, H.H. (1958) Estudios biologicos preliminarios sobre la laguna madre de tamaulipas. *Ciencia.* **17**, 7-15.

Hill, R.L. and Bradshaw, R.A. (1969) Fumarase. *Methods in Enzymology* **13**, 91-99.

Hiramatsu, T., Yano, I. and Masui, M. (1980) Effect of NaCl concentration on the protein species and phospholipid composition of the outer membrane in a moderately halophilic bacterium. *FEMS Microbiology Letters* **7**, 289-292.

Hocking, A.D. and Norton, R.S. (1983) Natural-abundance C-13 nuclear magnetic-resonance studies on the internal solutes of xerophilic fungi. *Journal of General Microbiology* **129**, 2915-2925.

Hussain, M., Hastings, J.M. and White, P.J. (1991) A chemically defined medium for slime production by coagulase negative *Staphylococci*. *Journal of Medical Microbiology* **34**, 143-147.

Huval, J.H., Latta, R., Wallace, R., Kushner, D.J. and Vreeland, R.H. (1995) Description of two new species of *Halomonas*: *Halomonas israelensis sp.nov.* and *Halomonas canadensis sp.nov.* *Canadian Journal of Microbiology* **41**, 1124-1131.

Imhoff, J.F. (1986) Osmoregulation and compatible solutes in eubacteria. *FEMS Microbiology Reviews* **39**, 57-66.

Javor, B.J. (1983) Planktonic standing crop and nutrients in a saltern ecosystem. *Limnology and Oceanography* **28**, 153-159.

Javor, B.J. (1989) *Hypersaline Environments. Microbiology and Biogeochemistry.*, Berlin: Springer Verlag.

Javor, B.J. and Porta, J. (1991) Some primary and secondary metabolites of hypersaline microbial mats and associated sediments. In: Rodriguez-Valera, F., (Ed.) *General and Applied Aspects of Halophilic Microorgamisms.*, pp. 33-44. New York: Plenum Press.

Jay, J.M. (1992) Microbiological food safety. *Food Technology* **46**, 30.

Johnson, K.G. and Lanthier, P.H. (1986) Beta-lactamases from *Actinopolyspora halophila*, an extremely halophilic actinomycete. *Archives of Microbiology* **143**, 379-386.

Jorgensen, B.B. and Desmarais, D.J. (1986) Competition for sulfide among colorless and purple sulfur bacteria in cyanobacterial mats. *FEMS Microbiology Ecology* **38**, 179-186.

Juez, G. (1988) Taxonomy of extremely halophilic archaebacteria. In: Rodriguez-Valera, F., (Ed.) *Halophilic Bacteria*, pp. 3-24. Florida: CRC Press.

Kaenjak, A., Graham, J.E. and Wilkinson, B.J. (1993) Choline transport activity in *Staphylococcus aureus* induced by osmotic-stress and low phosphate concentrations. *Journal of Bacteriology* **175**, 2400-2406.

Kamekura, M. (1998) Diversity of extremely halophilic bacteria. *Extremophiles.* **2**, 289-295.

Kamekura, M. and Onishi, H. (1978) Flocculation and adsorption of enzymes during growth of a moderate halophile, *Micococcus varians var. halophilus. Canadian Journal of Microbiology* **24**, 703-709.

Kashket, E.R. (1981) Effect of aerobiosis and nitrogen sources on the proton motive force in growing *Escherichia coli* and *Klebsiella pneumoniae* cells. *Journal of Bacteriology* **146**, 377-384.

Kashket, E.R. (1982) Stoichiometry of the H^+-ATPase of growing and resting aerobic *Escherichia coli. Biochemistry* **21**, 5534-5538.

Kashket, E.R. (1985) The proton motive force in bacteria - a critical-assessment of methods. *Annual Review of Microbiology* **39**, 219-242.

Kessel, M., Cohen, V. and Walsby, A.E. (1985) Structure and physiology of square shaped and other halophilic bacteria from the Gavish Sabkha. In: Friedman, G.M., (Ed.) *Hypersaline Ecosystems.*, pp. 267-287. Berlin: Springler Verlag

Kinne, R.H. (1993) The role of organic osmolytes in osmoregulation - from bacteria to mammals. *Journal of Experimental Zoology* **265**, 346-355.

Kinoshita, N., Unemoto, T. and Kobayashi, H. (1984) Proton motive force is not obligatory for growth of *Escherichia coli*. *Journal of Bacteriology* **160**, 1074-1077.

Kirkpatrick, M.B., Middleton, R.M. and Bass, J.B. (1993) Comparison of gram stain results from directly smeared protected specimen brush samples to culture results - a retrospective analysis. *American Review Of Respiratory Disease* **147**, A39

Kobayashi, H., Murakami, N. and Unemoto, T. (1982) Regulation of the cytoplasmic pH of *Streptococcus faecalis*. *Journal of Biological Chemistry* **257**, 13246-13252.

Komaratat, P. and Kates, M. (1975) The lipid composition of a halotolerant species of *Staphylococcus epidermidis*. *Biochimica Biophysica Acta* **398**, 464-484.

Kroll, R.G. and Booth, I.R. (1981) The role of potassium transport in the generation of a pH gradient in *Escherichia coli*. *Biochemical Journal.* **198**, 691-698.

Krulwich, T.A., Guffanti, A.A. and Mandel, K.G. (1981) The role of the membrane in the bioenergetics of bacteria. In: Ghosh, B.K., (Ed.) *Membranous strucures in bacterial cells*, pp. 53-71. West Palm Beach, Florida.: CRC Press.]

Krulwich, T.A. and Ivey, D.M. (1990) Bioenergetics in extreme environments. In: Krulwich, T.A., (Ed.) *The Bacteria Volume xii*, pp. 417-447. New York: Academic Press, Inc.

Krulwich, T.A., Ito, M., Gilmour, R., Hicks, D.B. and Guffanti, A.A. (1998) Energetics of alkalophilic *Bacillus* species: physiology and molecules. *Advances in Microbial Physiology.* **40**, 401-438.

Kunin, C.M. and Rudy, J. (1991) Effect of NaCl-induced osmotic-stress on intracellular concentrations of glycine betaine and potassium in *Escherichia coli*, *Enterococcus faecalis*, and *Staphylococci*. *Journal of Laboratory and Clinical Medicine* **118**, 217-224.

Kushner, D.J. (1985) The Halobacteriaceae. In: Woese, C.R. and Wolfe, R.S., (Eds.) *The Bacteria Volume viii*, pp. 171-215. New York: Academic Press.

Lai, M.C., Sowers, K.R., Robertson, D.E., Roberts, M.F. and Gunsalus, R.P. (1991) Distribution of compatible solutes in the halophilic methanogenic archaebacteria. *Journal of Bacteriology* **173**, 5352-5358.

Landfald, B. and Storm, A.R. (1986) Choline-Glycine betaine pathway confers a high level of osmotic tolerance in *Escherichia coli*. *Journal of Bacteriology* **165**, 849-855.

Lanyi, J.K. (1974) Salt-dependent properties of proteins from extremely halophilic bacteria. *Bacteriology Reviews* **38**, 272-290.

Larher, F. (1988) Natural abundance C-13-nuclear magnetic-resonance studies on the compatible solutes of halophytic higher-plants. *Plant Physiology and Biochemistry* **26**, 35-45.

Larsen, H. (1980) Ecology of hypersaline environments. In: Nissenbaum, A., (Ed.) *Developments of Sedimentology*, pp. 23-40. Amsterdam Elsevier.

Le Rudulier, D., Strom, A.R., Dandekar, A.M., Smith, L.T. and Valentine, R.C. (1984) Molecular biology of osmoregulation. *Science* **224**, 1064-1068.

Lippert, K. and Galinski, E.A. (1992) Enzyme stabilization by ectoine-type compatible solutes - protection against heating, freezing and drying. *Applied Microbiology and Biotechnology* **37**, 61-65.

Lowe, A.G. and Jones, M.N. (1984) Proton motive force - what price Δp. *Trends In Biochemical Sciences* **9**, 11-12.

Macleod, R.A., Wisse, G.A. and Stejskal, F.L. (1988) Sensitivity of some marine bacteria, a moderate halophile, and *Escherichia coli* to uncouplers at alkaline pH. *Journal Of Bacteriology* **170**, 4330-4337.

Maltseva, O., McGowan, C., Fulthorpe, R. and Oriel, P (1996) Degradation of 2,4-dichlorophenoxyacetic acid by haloalkaliphilic bacteria. *Microbiology* **142**, 1115-1122.

Martin, E.L., Duryearice, T., Vreeland, R.H., Hilsabeck, L. and Davis, C. (1983) Effects of NaCl on the uptake of alpha-(C-14)Aminoisobutyric acid by the halotolerant bacterium *Halomonas elongata*. *Canadian Journal of Microbiology* **29**, 1424-1429.

Mathrani, I.M. and Boone, D.R. (1985) Isolation and characterization of a moderately halophilic methanogen from a solar saltern. *Applied and Environmental Microbiology* **50**, 140-143.

Mathrani, I.M., Boone, D.R., Mah, R.A., Fox, G.E. and Lau, P.P. (1988) *Methanohalophilus zhilinae sp-nov*, an alkaliphilic, halophilic, methylotrophic methanogen. *International Journal of Systematic Bacteriology* **38**, 139-142.

Mcmeekin, TA., Chandler, R.E., Doe, P.E., Garland, C.D., Olley, J., Putro, S. and Ratkowsky, D.A (1987) Model for combined effect of temperature and salt concentration water activity on the growth-rate of *Staphylococcus xylosus*. *Journal of Applied Bacteriology* **62**, 543-550.

Miller, J.H. (1972) *Experiments in molecular genetics.*, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory.

Miller, K.J. and Leschine, S. (1984) A halotolerant *Planococcus* from Antarctic dry Valleys. *Current Microbiology* **11**, 205-210.

Moss, D.W. (1984) Acid phosphatase. In: Moss, D.W., (Ed.) *Methods of Enzymatic Analysis*, pp. 92-105. Verlag Chemie, Basel.

Munro, P.M., Gauthier, M.J. and Laumond, F. (1987) Previous growth of enteric bacteria on a salted medium increases their survival in seawater. *Letters In Applied Microbiology* **4**, 121-124.

Munro, P.M., Gauthier, M.J., Breittmayer, V.A. and Bongiovanni, J. (1989) Influence of osmoregulation processes on starvation survival of *Esherichia coli* in seawater. *Applied and Environmental Microbiology* **55**, 2017-2024.

Nakamura, T., Tokuda, H. and Unemoto, T. (1982) Effect of pH and monovalent cations on the potassium ion exit from the marine bacterium, *Vibrio alginolyticus* and the manipulation of cellular cation content. *Biochimica.Biophysica.Acta* **692**, 389-396.

Neidhardt, F.C., Ingraham, J.L. and Schaechter, M. (1990) *Physiology of the Bacterial Cell*, First edn. Sunderland, Massachusetts: Sinauer Associates, Inc.

Nicholls, D.G. and Ferguson, S.J. (1992) *Bioenergetics 2*, Second edn. London: Academic Press Limited.

Noda, F., Hayashi, K. and Mizunuma, T. (1980) Antagonism between osmophilic lactic acid bacteria and yeasts in brine fermentation of soy sauce. *Applied and Environmental Microbiology* **40**, 452-457.

Novitsky, T. and Kushner, D.J. (1976) *Planococcus halophilus sp. nov.*, a facultatively halophilc coccus. *International Journal of Systematic Bacteriology* **26** , 53-57.

Onishi, H. and Hidaka, O. (1978) Purification and properties of amylase produced by a moderately halophilic *Acinetobacter sp.* *Canadian Journal of Microbiology* **24**, 1017-1023.

Oren, A. and Shilo, M. (1982) Population-dynamics of *Dunaliella-parva* in the Dead Sea. *Limnology and Oceanography* **27**, 201-211.

Oren, A., Weisburg, W.G., Kessel, M. and Woese, C.R. (1984) *Halobacteroides halobius gen-nov, sp-nov,* a moderately halophilic anaerobic bacterium from the bottom sediments of the Dead Sea. *Systematic and Applied Microbiology* **5**, 58-70.

Oren, A. (1986) The ecology and taxonomy of anaerobic halophilic eubacteria. *FEMS Microbiology Reviews* **39**, 23-29.

Oren, A., Pohla, H. and Stackebrandt, E. (1987) Transfer of *Clostridium lortetii* to a new genus *Sporohalobacter gen-nov* as *Sporohalobacter lortetii comb-nov*, and description of *Sporohalobacter marismortui sp-nov. Systematic and Applied Microbiology* **9**, 239-246.

Oren, A., Kessel, M. and Stackebrandt, E. (1989) *Ectothiorhodospira-marismortui sp-nov*, an obligately anaerobic, moderately halophilic purple sulfur bacterium from a hypersaline sulfur spring on the shore of the Dead Sea. *Archives Of Microbiology* **151**, 524-529.

Oren, A., Simon, G. and Galinski, E.A. (1991) Intracellular salt and solute concentrations in *Ectothiorhodospira marismortui* - glycine betaine and n-alpha-carbamoyl glutamineamide as osmotic solutes. *Archives of Microbiology* **156**, 350-355.

Oren, A. (1999) Bioenergetic aspects of halophilism. *Microbiology and Molecular Biology Reviews* **63**, 334-348.

Padan, E., Patel, L. and Kaback, H.R. (1979) Effect of diethylpyrocarbonate on lactose/proton symport in *Escherichia coli* membrane vesicles. *Proceedings of National Academy of Sciences of United States of America* **76**, 6221-6225.

Padan, E., Zilberstein, D. and Schuldiner, S. (1981) pH homeostasis in bacteria. *Biochimica.Biophysica.Acta* **650**, 151-166.

Paterek, J.R. and Smith, P.H. (1985) Isolation and characterization of a halophilic methanogen from Great Salt Lake. *Applied and Environmental Microbiology* **50**, 877-881.

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

Pfiffner, S.M., McInerney, M.J., Jenneman, G.E. and Knapp, R.M. (1986) Isolation of halotolerant, thermotolerant, facultative polymer producing bacteria and charachterization of the exopolymer. *Applied and Environmental Microbiology* **51**, 1224-1229.

Poolman, B. and Glaasker, E. (1998) Regulation of compatible solute accumulation in bacteria. *Molecular Microbiology* **29**, 397-407.

Post, J.F. (1977) The microbial ecology of the Great Salt Lake. *Microbial Ecology* **3**, 143-165.

Potts, M. (1980) Blue-green algae (Cyanophyta) in marine coastal environments of the Sinai Peninsula; distribution, zonation, stratification and taxonomic diversity. *Phycologia* **19** , 60-73.

Pourkomailian, B. and Booth, I.R. (1994) Glycine betaine transport by *Staphylococcus aureus* - evidence for feedback-regulation of the activity of the 2 transport-systems. *Microbiology* **140**, 3131-3138.

Purser, B. (1985) Coastal evaporite systems. In: Friedman, G.M., (Ed.) *Hypersaline Ecosystems.*, pp. 72-102. Berlin: Springer Verlag.

Quesada, E., Ventosa, A., Rodriguez-Valera, F., Megias, L. and Ramos-Cormenzana, A. (1983) Numerical taxonomy of moderately halophilic gram-negative bacteria from hypersaline soils. *Journal of General Microbiology* **129**, 2649-2657.

Quesada, E., Ventosa, A., Ruizberraquero, F. and Ramos-Cormenzana, A. (1984) *Deleya halophila*, a new species of moderately halophilic

bacteria. *International Journal of Systematic Bacteriology* **34**, 287-292.

Quirk, P.G., Guffanti, A.A., Plass, R.J., Clejan, S. and Krulwich, T.A. (1991) Protonophore-resistance and cytochrome expression in mutant strains of the facultative alkaliphile *Bacillus firmus* OF4. *Biochimica Biophysica Acta* **1058**, 131-140.

Ramos-Cormenzana, A. (1991) Halophilic organisms and the environment. In: Rodriguez-Valera, F., (Ed.) *General and Applied Aspects of Halophilic Microorganisms.*, pp. 15-24. New York.: Plenum Press.

Reeves, H.C., Rabin, R., Wegenser, W.S. and Ajl, S.J. (1971) Assays of enzymes of the tricarboxylic acid and glyoxylate cycles. *Methods in Microbiology* **6A**, 425-462.

Rengpipat, S., Lowe, S.E. and Zeikus, J.G. (1988) Effect of extreme salt concentrations on the physiology and biochemistry of *Halobacteroides-acetoethylicus*. *Journal of Bacteriology* **170**, 3065-3071.

Robertson, D.E., Noll, D., Roberts, M.F., Menaia, J.F. and Boone, D.R. (1990) Detection of the osmoregulator betaine in methanogens. *Applied and Environmental Microbiology* **56**, 563-565.

Robinson, J. and Gibbons, N. (1952) The effect of salts on the growth of *Micrococcus halodenitrificans*. *Canadian Journal of Botany.* **30**, 147-154.

Rodriguez, M., Nunez, F., Cordoba, J.J., Bermudez, E. and Asensio, M.A. (1996) Gram-positive, catalase-positive cocci from dry cured Iberian Ham and their enterotoxigenic potential. *Applied and Environmental Microbiology* **62**, 1897-1902.

Rodriguez-Valera, F., Ventosa, A., Juez, G. and Imhoff, J.F. (1985) Variation of environmental features and microbial populations with salt

concentrations in a multi-pond saltern. *Microbial Ecology* **11**, 107-115.

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

Rodriguez-Valera, F. (1988) *Halophilic Bacteria.*, Florida: CRC Press, Inc.

Rosenstein, R., FutterBryniok, D. and Gotz, F. (1999) The choline-converting pathway in *Staphylococcus xylosus* C2A: Genetic and physiological characterization. *Journal of Bacteriology* **181**, 2273-2278.

Roszak, D.B., Grimes, D.J. and Colwell, R.R. (1984) Viable but nonrecoverable stage of *Salmonella enteritidis* in aquatic systems. *Canadian Journal of Microbiology* **30**, 334-338.

Rottenberg, H. (1979) The measurement of the membrane potential and ΔpH in cells, organelles, and vesicles. *Methods In Enzymology* **55**, 547-569.

Rottenberg, H. (1989) Proton electrochemical potential gradient in vesicles, organelles, and prokaryotic cells. *Methods In Enzymology* **172**, 63-84.

Schleifer, K.H. and Kloos, W.E. (1975) Isolation and characterization of Staphylococci from human skin. *International Journal of Systematic Bacteriology* **25**, 50-61.

Schoffeniels, E. and Gilles, R. (1970) Osmoregulation in marine Mollusca. In: Florkin, M. and Scheer, B., (Eds.) *Chemical Zoology.*, pp. 343-420. London: Academic Press

Severin, J., Wohlfarth, A. and Galinski, E.A. (1992) The predominant role of recently discovered tetrahydropyrimidines for the osmoadaptation of halophilic eubacteria. *Journal of General Microbiology* **138**, 1629-1638.

Smibert, R.M. and Krieg, N.R. (1994) Phenotypic Charaterization. In: Gerhardt, P., Murray, R.G.E., Wood, W.A. and Krieg, N.R., (Eds.) *Methods for General and Molecular Biology.*, pp. 607-654. Washington: American Society for Microbiology.

Sowers, K.R. and Gunsalus, R.P. (1995) Halotolerance in *Methanosarcina* spp - role of N^{ε}-acetyl-β-lysine, α-glutamate, glycine betaine, and K^{+} as compatible solutes for osmotic adaptation. *Applied and Environmental Microbiology* **61**, 4382-4388.

Steinhorn, I. and Assaf, G. (1980) The physical structure of the Dead Sea water column. In: Nissenbaum, A., (Ed.) *Developments in Sedimentology.*, pp. 145-154. Amsterdam: Elsevier.

Stetter, K.O., Lauerer, G., Thomm, M. and Neuner, A. (1987) Isolation of extremely thermophilic sulfate reducers - evidence for a novel branch of archaebacteria. *Science* **236**, 822-824.

Stoeckenius, W., Bivin, D. and McGinnis, K. (1985) Photoactive pigments in *Halobacteria* from the Gavish Sabkha. In: Friedman, G.M., (Ed.) *Hypersaline Ecosystems.*, pp. 288-295. Berlin: Springer Verlag.

Strom, A.R., Falkenberg, P. and Landfald, B. (1986) Genetics of osmoregulation in *Escherichia coli* - uptake and biosynthesis of organic osmolytes. *FEMS Microbiology Reviews* **39**, 79-86.

Takahashi, I. and Gibbons, N. (1959) Effect of salt concentration on the morphology and chemical composition of *Micrococcus halodenitreficans*. *Canadian Journal of Microbiology* **5**, 25-35.

Tamplin, M.L. and Colwell, R.R. (1986) Effect of microorganism salinity and organic substrate concentration on production of *Vibrio cholerae* in the estuarine and marine environment. *Microbial Ecology* **40**, 386-390.

Thompson, J. and Macleod, R.A. (1973) Na^{+} and K^{+} gradients and α-aminobutyric acid transport in a marine *Pseudomonad*. *Journal of Biological Chemistry* **248**, 7106-7111.

Timasheff, S.N. (1992) Water as ligand - preferential binding and exclusion of denaturants in protein unfolding. *Biochemistry* **31**, 9857-9864.

Tindall, B.J., Ross, H.M. and Grant, W.D. (1984) *Natronobacterium gen-nov* and *Natronococcus gen-nov,* 2 new genera of haloalkaliphilic archaebacteria. *Systematic and Applied Microbiology* **5**, 41-57.

Tindall, B.J. (1988) Prokaryotic life in the alkaline saline, athalassic environment. In: Rodriguez-Valera, F., (Ed.) *Halophilic Bacteria.*, pp. 31-70. Florida.: CRC Press.

Townsend, D.E. and Wilkinson, B.J. (1992) Proline transport in *Staphylococcus-aureus* - a high-affinity system and a low-affinity system involved in osmoregulation. *Journal of Bacteriology* **174**, 2702-2710.

Unemoto, T., Akagawa, A., Mizugaki, M. and Hayashi, M. (1992) Distribution of Na^+-dependent respiration and a respiration-driven electrogenic Na^+ pump in moderately halophilic bacteria. *Journal of General Microbiology* **138**, 1999-2005.

Vanqua, D., Simidu, U. and Taga, N. (1981) Purification and some properties of halophilic protease produced by a moderately halophilic marine *Pseudomonas-sp.* *Canadian Journal of Microbiology* **27**, 505-510.

Ventosa, A., Quesada, E., Rodriguez-Valera, F., Ruizberraquero, F. and Ramos-Cormenzana, A. (1982) Numerical taxonomy of moderately halophilic gram-negative rods. *Journal of General Microbiology* **128**, 1959-1968.

Ventosa, A., Ramos-Cormenzana, A. and Kocur, M. (1983) Moderately halophilic gram-positive cocci from hypersaline environments. *Systematic and Applied Microbiology* **4**, 564-570.

Ventosa, A. (1988) Taxonomy of moderately halophilic heterotrophic eubacteria. In: Rodriguez-Valera, F., (Ed.) *Halophilic Bacteria.*, pp. 71-84. Florida: CRC Press.

Ventosa, A., Nieto, J.J. and Oren, A. (1998) Biology of moderately halophilic aerobic bacteria. *Microbiology And Molecular Biology Reviews* **62**, 504-544.

Vijaranakul, U., Nadakavukaren, M.J., Dejonge, B.M., Wilkinson, B.J. and Jayaswal, R.K. (1995) Increased cell-size and shortened peptidoglycan interpeptide bridge of NaCl-stressed *Staphylococcus aureus* and their reversal by glycine betaine. *Journal of Bacteriology* **177**, 5116-5121.

Vijaranakul, U., Nadakavukaren, M.J., Bayles, D.O., Wilkinson, B.J. and Jayaswal, R.K. (1997) Characterization of an NaCl-sensitive *Staphylococcus aureus* mutant and rescue of the NaCl-sensitive phenotype by glycine betaine but not by other compatible solutes. *Applied and Environmental Microbiology* **63**, 1889-1897.

Vilhelmsson, O., Hafsteinsson, H. and Kristjansson, J.K. (1997) Extremely halotolerant bacteria characteristic of fully cured and dried cod. *International Journal of Food Microbiology* **36**, 163-170.

Villar, M., Holgado, A.D., Sanchez, J.J., Trucco, R.E. and Oliver, G. (1985) Isolation and characterization of *Pediococcus halophilus* from salted anchovies *(Engraulis anchoita*). *Applied and Environmental Microbiology* **49**, 664-666.

Vreeland, R.H. and Martin, E.L. (1980) Growth characteristics, effects of temperature, and ion specificity of the halotolerant bacterium *Halomonas elongata. Canadian Journal of Microbiology* **26**, 746-752.

Vreeland, R.H., Litchfield, E.L., Martin, E.L. and Elliot, E. (1980) *Halomonas elongata*, a new genus and species of extremely salt-tolerant bacteria. *International Journal of Systematic Bacteriology* **30**, 485-495.

Vreeland, R.H., Mierau, B.D., Litchfield, C.D. and Martin, E.L. (1983) Relationship of the internal solute composition to the salt tolerance of *Halomonas elongata*. *Canadian Journal of Microbiology* **29**, 407-414.

Vreeland, R.H., Anderson, R. and Murray, R.E. (1984) Cell wall and phospholipid composition and their contribution to the salt tolerance of *Halomonas elongata*. *Journal of Bacteriology* **160**, 879-883.

Vreeland, R.H. (1987) Mechanisms of halotolerance in microorganisms. *CRC Critical Reviews In Microbiology* **14**, 311-356.

Vreeland, R.H. and Huval, J.H. (1991) Phenotypic characterization of halophilic bacteria from ground water sources in the United States. In: Rodriguez-Valera, F., (Ed.) *General and Applied Aspects of Halophilic Microorganisms.*, pp. 53-62. New York: Plenum Press.

Walsby, A.E. (1980) A square bacterium. *Nature* **283** 69-71.

Walsby, A.E., van Rijn, J. and Cohen, Y. (1983) The biology of a new gas-vacuolate cyanobacterium, *Dactylococcopsis salina sp-nov*, in Solar Lake. *Proceedings Of The Royal Society Of London Series B-Biological Sciences* **217**, 417-447.

Wegmann, K. (1986) Osmoregulation in eukaryotic algae. *FEMS Microbiology Reviews* **39**, 37-43.

Welsh, D.T., Reed, R.H. and Herbert, R.A. (1991) The role of trehalose in the osmoadaptation of *Escherichia coli* NCIB-9484 - interaction of trehalose, K^+ and glutamate during osmoadaptation in continuous culture. *Journal of General Microbiology* **137**, 745-750.

Wohlfarth, A., Severin, J. and Galinski, E.A. (1990) The spectrum of compatible solutes in heterotrophic halophilic eubacteria of the family *Halomonadaceae*. *Journal of General Microbiology* **136**, 705-712.

Wohlfarth, A., Severin, J. and Galinski, E.A. (1993) Identification of n-delta-acetylornithine as a novel osmolyte in some gram-positive halophilic eubacteria. *Applied Microbiology and Biotechnology* **39**, 568-573.

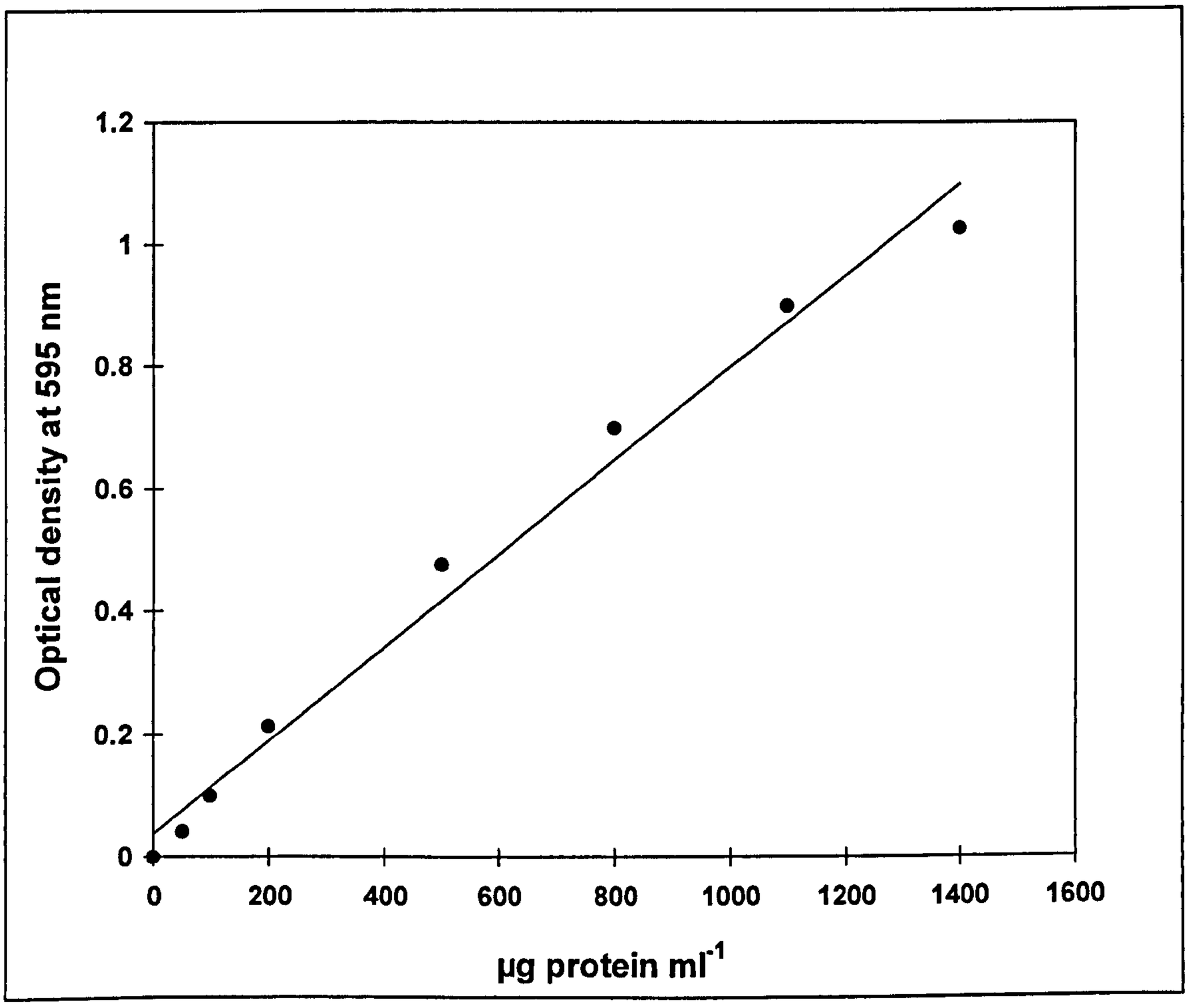
Yancey, P.H., Clark, M.E., Hand, S.C., Bowlus, R.D. and Somero, G.N. (1982) Living with water-stress - evolution of osmolyte systems. *Science* **217**, 1214-1222.

Zeikus, J.G., Hegge, P.W., Thompson, T.E., Phelps, T.J. and Langworthy, T.A. (1983) Isolation and description of *Haloanaerobium praevalens gen-nov* and *sp-nov*, an obligately anaerobic halophile common to Great Salt Lake sediments. *Current Microbiology* **9**, 225-234.

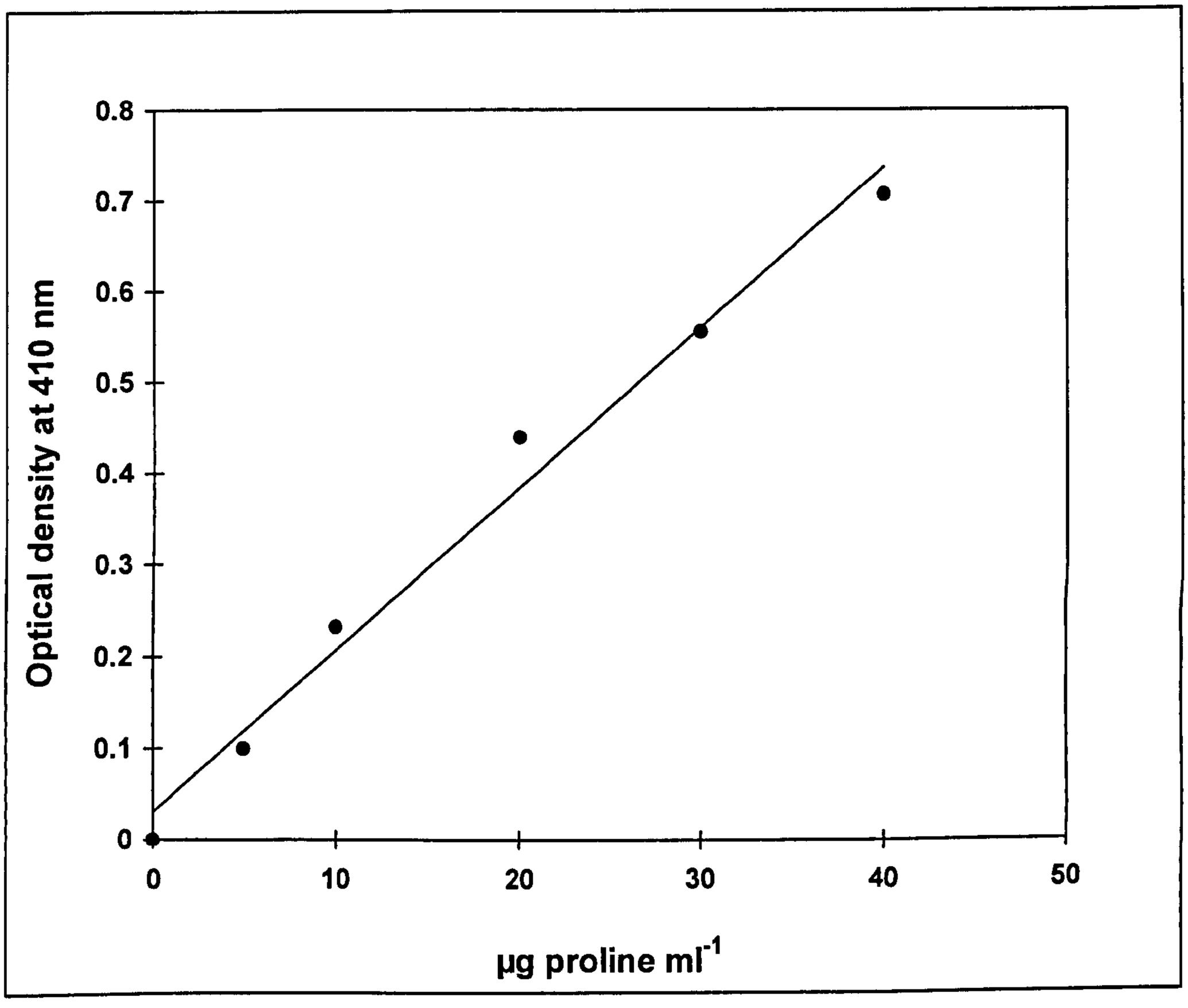
Zhilina, T.N. (1986) Methanogenic bacteria from hypersaline environments. *Systematic and Applied Microbiology* **7**, 216-222.

Zillig, W., Holz, I., Klenk, H.P., Trent, J., Wunderl, S., Janekovic, D., Imsel, E. and Haas, B. (1987) *Pyrococcus woese*i, *sp-nov*, an ultra-thermophilic marine archaebacterium, representing a novel order, Thermococcales. *Systematic and Applied Microbiology* **9**, 62-70.

Appendix (A) Standard Protein Concentration Curve.



Appendix (B) Standard Proline Concentration Curve.



Appendix (C) Standard O-nitrophenol Concentration Curve.