RESPONSE OF HALOPHILIC FUNGI TO HIGH SALT CONCENTRATION AND HEAT STRESS

A Thesis submitted to Goa University for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

MICROBIOLOGY

By

SHWETA S. NAYAK

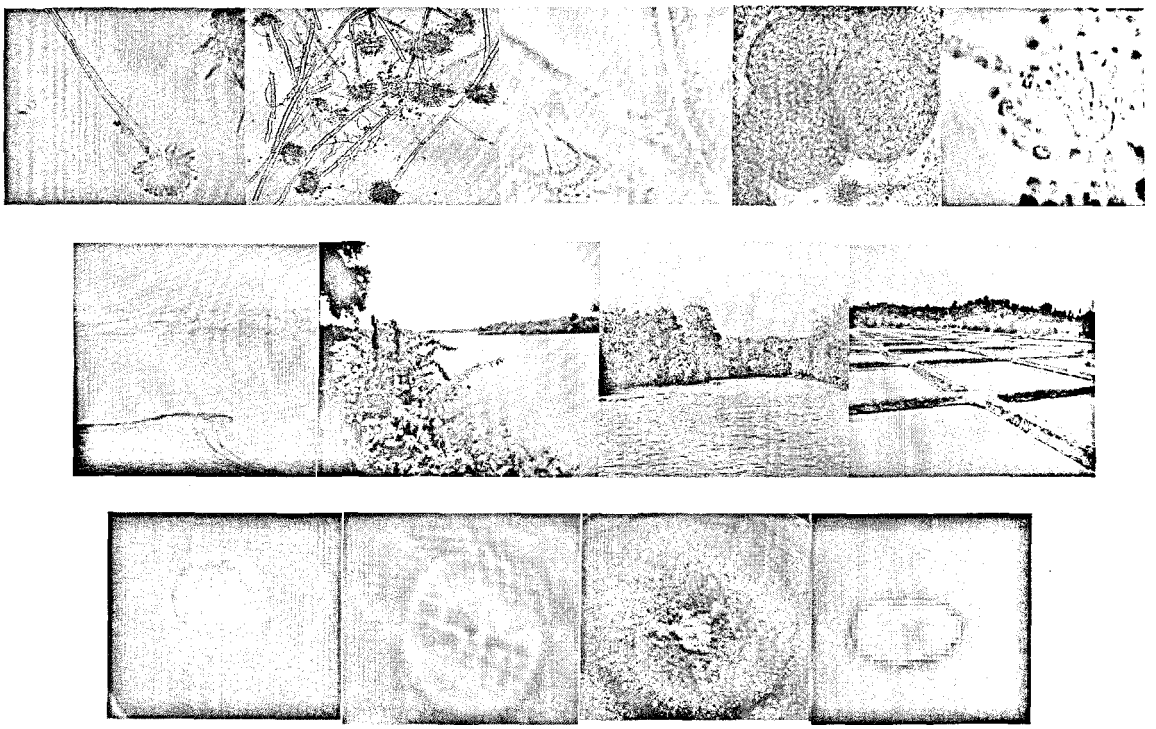
Research Guide

Dr. Sarita Nazareth

Goa University,
Taleigao, Goa

2012

RESPONSE OF HALOPHILIC FUNGI TO HIGH SALT CONCENTRATION AND HEAT STRESS



Ph.D. Thesis

by

Ms. Shweta. S. Nayak

Department of Microbiology

Goa University

Goa- 403206

2012

DECLARATION

I declare that the present thesis entitled "**Response of halophilic fungi to high salt concentration and heat stress**" is a consolidation of an original work which has been carried out by me under the supervision and guidance of Dr Nazareth at the Department of Microbiology Goa University, and that the same has not been submitted to any other university or Institution for the award of any other degree, diploma or other such title.

The literature review related to the research problem investigated, the facilities availed of and financial support received as "Research studentship" provided by Goa University have been duly acknowledged

SHWETA S. NAYAK

CERTIFICATE

This is to certify that Ms Shweta S. Nayak has worked on the thesis entitled "**Response of halophilic fungi to high salt concentration and heat stress**" under my guidance. This thesis being submitted to Goa University Taleigao Plateau,Goa for award of the degree of Doctor of Philosophy in Microbiology is a record of an original work carried out by the candidate herself and has not been submitted for the award of any other Degree, Diploma Associateship or Fellowship of this or any other University

S Nazareth

(Dr. Sarita Nazareth)
Research Guide
Associate Professor
Department of Microbiology
Goa University,Goa-403206

Corrections Suggested by the Examiners have been carried out by the candidate

S Nazareth
15/01/2013

Raaman
15/1/2013

Acknowledgement

I would like to thank and express my indebtness to my guide Dr. Sarita Nazareth, with whom I have spent countless hours in discussing various problems in the work. I have been deeply influenced by her basic concept with which she has tackled the problems. I wish to thank her for her esteemed guidance and her constructive criticism.

I thank all the members of the F.R.C committee for their abiding faith in me and the support they provided while undertaking my research work, Prof. G. N. Nayak, Dean and chairperson of the F.R.C, Dr. S. Nazareth, Research Guide, Dr. S. Dubey, Head of Department, Dr. S. Bhosle V C.s nominee for providing the invaluable suggestion and her critical reviews. I would like to express my deep gratitude towards to the Head of the Department, Microbiology ever since I joined here, Dr. Santosh Dubey and Dr. Saroj Bhosle for providing the necessary facilities which helped me to complete my work. In addition, I sincerely thank all the lecturers Dr. Irene Furtado, Dr. Santosh Dubey, Dr. Sandeep Garg.

I am also grateful for the financial support as 'Research studentship' by Goa University.

Special thanks to Dr. Sushma Gaikwad, Division of biochemical sciences, NCl Pune for welcoming me in her laboratory and helping me in my HPLC analysis. I thank the researchers from NCl Pune, Madhurima, Pallavi, Poornima, Avinash, Ashutosh, Gyan for helping me in my work and making my stay in Pune successful and Ansari for helping me in statistical analysis.

I am indeed grateful to Dr. Lingappa, Dr. Sonapnavar, for welcoming me in their laboratory and allowing the use of image capture microscope.

I sincerely thank Dr. Siddapur, Sonapnavar, Mahesh, Varsha, Satish, Ashish, Anant individually for providing valuable assistance, help and support.

I thank my parents for the courage, support and cooperation they have provided. I cannot forget the efforts and sacrifices of my beloved Dad and Mom. I thank my Dad for his support, sacrifices, advices and patience; and you mom for your prayers, strength, daily encouragement, moral support, valuable advices which kept me going all these years. Thank you both, for being beside me and having faith in me.

I sincerely thank my friend Naveen Pawar for his support, advice, and encouragement. I am grateful to Mufida for her help in identification. I thank my friends Valerie and Akshaya individually for helping me whenever asked for. I also thank my other friends Teja, Trelita, Sanika, Brenda, Anju, Vidya, Cristabell, Milind, Pramoda, Lakshangy, Swapnil, Meghnath, Krishna, Dnyanada, Bianca, Hemant, Sherryanne, Subhojit, Sushama, Amrita.

My experimental attempts in the laboratory would have never been a smooth affair without the help of the non-teaching staffs. I am grateful to the librarians of Goa University for allowing me to access the books whenever needed.

I thank the almighty for good health, strength, patience, tolerance and perseverance in completing my work.

Last but not the least I would like to thank my brother, relatives for their advices and encouragement, close friends and all others who helped and supported me either directly or indirectly.

Shweta Nayak

DEDICATED

TO

MY

BELOVED

PARENTS

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

LIST OF TABLES (contd)

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

LIST OF FIGURES (contd)

CHAPTER 3

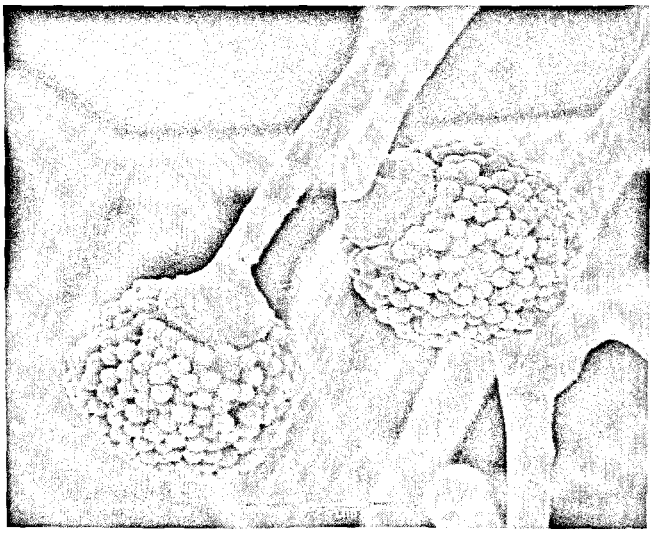
CHAPTER 4

LIST OF FIGURES (contd)

CHAPTER 5

CHAPTER 1

INTRODUCTION



CONTENTS

GENERAL INTRODUCTION

In living cells water serves as a medium of interactions of small and large molecules between themselves and with each other. The solutes dissolved in the cell water and the structure of this water control all the vital processes: enzyme action and regulation, assembly and disassembly of organelles, membrane structure and function. Effects of high solute concentrations may be due to the solutes themselves, or to the effect the solutes have on water activity, a_w. Everything that dissolves in a solvent is attracted to the molecules of that solvent and thereby reduces their freedom. Salt and solute- tolerant organisms are widely-distributed among the bacteria, fungi, yeasts, algae and protozoa and among the viruses.

1.1 Halophiles

Halophiles are salt-loving organisms, the term halophily referring to the ionic requirements for life at high salt concentrations. They include mainly prokaryotic and eukaryotic microorganisms, with a capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salt. Halophilic and halotolerant microorganisms are found in all three domains of life: archaea, eubacteria, and eucaryotes (Galinski 1993, Oren 1999b). Colonization of hypersaline environments such as salt lakes and of salted food products by salt-loving and/or salt-tolerant microorganisms may reach high population densities.

Hypersaline waters also termed thalassohaline (Oren 2002), are defined as having salt concentrations greater than 3.5% w/v, that of seawater (Grant *et al.* 1998, Mermelstein *et al.*1998). They include coastal salterns, saline lakes, evaporite lagoon sediments saltflats, natural lakes such as the Great Salt Lake and deep sea hypersaline basins (Javor 1989). These ponds provide a variation in the environments where different conditions of salinity,

pH, temperature, light intensity, oxygen and nutrient concentrations are found, allowing the study of different microbial communities (Gunde-Cimerman *et al.*2000, Pedros-Alio *et al.* 2000, Pedros-Alio 2004).

In their natural environment, the salt concentration may fall to low levels after rainfall, however, after longer periods of sunlight, the soil will dry out and the salinity will gradually increase from low concentrations up to concentrations at which salt begins to crystallize. Therefore, every microbe living in such an environment has to cope with changing salinities over a very wide range, from about 0–3.5M NaCl.

According to Kushner (1978), many marine organisms are slight halophiles (with 3% w/v NaCl in sea water). Moderate halophiles grow optimally at 3–15% w/v NaCl; extreme halophiles at 25% w/v NaCl (halobacteria and halococci) and borderline extreme halophiles require at least 12% w/v salt.

Halophilic organisms living in saline environments are challenged by two stress factors, the high inorganic ion concentration and the low water potential. An increase in the external salt concentration poses a serious problem to any living cell due to the permeability of membranes for water, and will lead to a loss of water and this will lead to cell death if no counter measures are taken (Brown 1976). A non-adapted organism exposed to such an environment must cope with its cytoplasmic water having a higher water potential than the water of the surrounding environment. Water always flows from a high to a low potential until the potential gradient is abolished. Thus, the cytoplasm, which is surrounded by a membrane freely permeable to water, will lose its free cytoplasmic water, resulting in cell shrinkage (Cayley *et al.* 1991). The loss of water will cause cessation of growth, possibly due to molecular crowding, and thus reduced diffusion rates of proteins and metabolites.

1.2 Mechanism of tolerance

Halophiles have developed two principal mechanisms to lower the potential of cytoplasmic water, avoiding the loss of water from the cell and achieving a cytoplasm of osmotic strength similar to that of the surrounding medium. These two mechanisms, which allow osmotic adaptation, are the salt-in-cytoplasm mechanism and the organic-osmolyte mechanism.

1.2a Salt-in-cytoplasm mechanism

Halophilic organisms maintain an osmotic balance by accumulating high concentrations of salt intracellularly. The strategies used by bacteria to survive in environments with high and changing salinities have received much attention in the last few years. The general physiological response after an upshift of the salt concentration in a medium includes three phases. First, inorganic ions usually Na^+ and Cl^- enter the cell after the turgor collapse resulting from the large difference in water potentials across the cytoplasmic membrane. Second, Na^+ is exchanged for K^+, which saves the cell metabolism from the toxic influence of high Na^+ concentrations. Stabilization of turgor by accumulation of ions is possible only in halophilic archaea and some halophilic bacteria. In the third phase solutes are usually accumulated, which allows exclusion of the inorganic ions without a further change of turgor halophilic archaea, by use of a Na^+ pump, push Na^+ ions out of the cell, while concentrating K^+ ions within the cell in order to balance osmotic pressure. This balance consists of an internal concentration of K^+ at around 5M and an outside concentration of Na^+ at around 4M.

The salt-in-cytoplasm mechanism is considered the typical archaeal strategy of osmoadaptation in halobacteria (archaea), the halophilic, anaerobic haloanaerobiales and *Salinibacter ruber* (Oren *et al.* 2002). Organisms following the salt-in-cytoplasm mechanism adapt the interior protein chemistry of the cell to high salt concentration. Organisms following this strategy adjust the water potential of the cell by raising the KCl salt concentration in the cytoplasm and must adapt the interior protein chemistry to a high salt concentration (Lanyi 1974, Dennis and Shimmin 1997). They accumulate and adjust the internal concentration of inorganic ions such as K^+ and Cl^- to values that counteract the external osmolarity. As a consequence, organisms using the salt-in-cytoplasm strategy display a relatively narrow adaptation and their growth is restricted to high saline environments. To adjust to increase in external NaCl, cells in all three kingdoms accumulate a variety of small molecules in the cytoplasm to counteract the external osmotic pressure. Inorganic cations K^+, and in some cells Na^+, are often key players in osmotic balance and the osmotic response.

The halophilic membrane has been shown to have a low Na^+ and H^+ permeability, at high salt concentrations (Vossenberg *et al.* 1999). Halobacteria seem to have an inwardly directed, light driven chloride pump that functions similarly to bacteriorhodopsin. Potassium ions also enter the cell via a chlorine gradient. Halobacteria have been shown to grow in the dark, indicating the presence of alternate transport systems (Grant *et al.* 1998). Bacteriorhodopsin (BR) creates a proton gradient and allows Na^+ to be pumped out of the cell. The pump is created by pushing one proton across the membrane for every photon absorbed. This proton gradient leads to the formation of ATP. The Na^+ gradient then allows K^+ to be taken into the cell and balance osmotic pressure. Halorhodopsin (HR) uses light in

order to pump Cl^- into the cells and balance the K^+ ions within. Fig. 1.1 shows the mechanism of salt in cytoplasm.

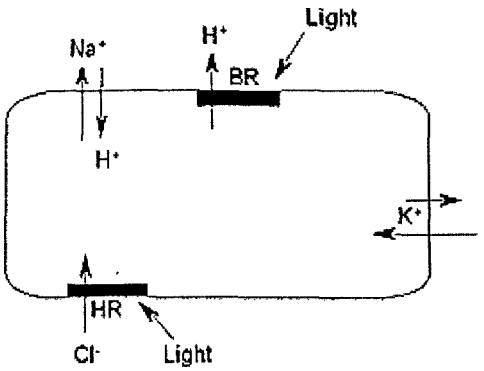


Fig. 1.1 Mechanism of salt in cytoplasm

Archaea are well adapted to saturating NaCl concentrations and have a number of novel molecular characteristics, such as enzymes that function in saturated salts, purple membrane that allows prototrophic growth, sensory rhodopsins that mediate the phototactic response and gas vesicles that promote cell flotation. Extremely halophilic organisms require a high internal potassium ion level, with some species requiring > 3M, to maintain osmotic pressure. Specific transport systems are needed to accumulate potassium ions within the cell and expel ions (Vossrnberg *et al.* 1999). Moderately halophilic bacterium *Halobacillus halophilus* requires chloride for growth (Roeßler and Müller 1998). *Halobacillus halophilus* copes with the salinity in its environment by the production of compatible solutes. At intermediate salinities of around 1M NaCl, these cells produce glutamate and glutamine in a chloride-dependent manner (Saum *et al.* 2006).

The bulk of proteins from halophiles are acidic in nature, with an excess of up to 20% aspartate and glutamate residues. These charged residues occur in clusters on the surface of the protein, and act as a screen attracting water and hydrated salt ions to the surface of the molecule (Jaenicke and Bohm 1998). At high solute levels, proteins tend to precipitate. High

concentration of salt in the cytoplasm is usually very unfavourable for proteins and other macromolecules. Salt causes protein aggregation due to enhanced hydrophobic interactions owing to hydration of ions. Salt decreases the availability of free water and also prevents intra and intermolecular electrostatic interactions (Dennis and Shimmin 1997). Halophilic proteins show a decrease in surface hydrophobicity, reducing their potential to precipitate (Danson and Hough 1997). In order to maintain the activity of the cellular metabolic systems under high concentrations of salt in the cytoplasm, specific adaptation of all proteins is required.

Salt resistance of the proteins of halophilic bacteria is achieved due to significant changes in the amino acid composition. Increased content of acidic amino acid residues glutamate and aspartate as compared to the proteins of the neutrophiles is the general direction of evolution of these proteins (Dennis and Shimmin 1997). The acidic residues, located mainly on the surface of the protein globule are highly hydrated. Their increased amount makes a protein more hydrophilic and its structural collapse or conglomeration is thus prevented. A number of enzymatic reactions depend on high K^+ concentrations they are also tolerant to NaCl (Oren and Gurevich 1993). Halophilic and halotolerant enzymes function optimally in high salt conditions and hence under conditions of low water activity (Grant *et al.* 1998).

1.2b Organic osmolyte mechanism

To prevent the loss of cellular water to the environment, halophiles accumulate osmolytes within the cytoplasm (Galinski 1993). This strategy is widespread and evolutionarily well conserved in all three domains of life (Bohnert 1995, Kempf and Bremer 1995, Kemph and Bremer 1998, Oren 2002). Organic osmolytes are used by cells of numerous water-stressed

organisms to maintain cell volume. They can be accumulated in molar concentration in the cytoplasm and allow for the adaptation of bacterial cells to varying salt concentrations. Microorganisms use not just one, but rather two or more osmolytes to meet this challenge (Galinski and Truper 1994, Ventosa 1998, Wood *et al.* 2001, Roberts 2005).

These solutes are small organic molecules that do not interfere, even at molar concentrations, with cellular metabolism and enzyme activity, although they may modulate individual enzyme activities. This behavior led to labeling them as 'compatible solutes' (Brown 1976). The selective advantages of the organic osmolyte systems are, first, a compatibility with macromolecular structure and function at high or variable (or both) osmolyte concentrations, and, second, greatly reduced needs for modifying proteins to function in concentrated intracellular solutions. Their accumulation helps to maintain turgor pressure, cell volume, and concentration of electrolytes, all important elements for cell proliferation. These substances also help to protect cells against stresses like high temperature, desiccation and freezing. Consequently, in surroundings with lower salt concentrations, the solubility of halophilic proteins is often very low (Danson and Hough 1997, Madern *et al.* 2000).

Most osmolytes are neutral - either zwitterionic or lacking charges at physiological pH, although some bacterial and archaeal osmolytes are anionic and are paired with K^+ to achieve neutrality (Martin *et al.* 1999). Organic osmolytes have been categorized into: (i) zwitterionic solutes, (ii) noncharged solutes, and (iii) anionic solutes, as detailed below, their structures shown in Fig. 1.2 and 1. 3 (Roberts 2005).

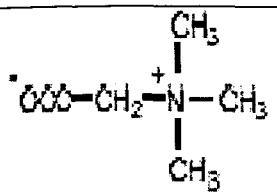
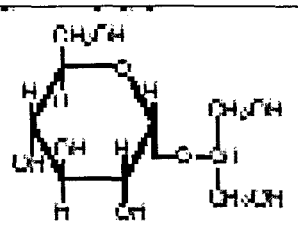
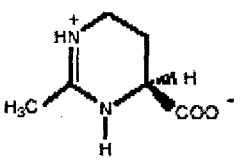
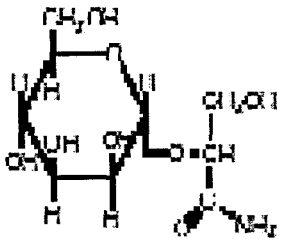
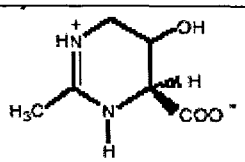
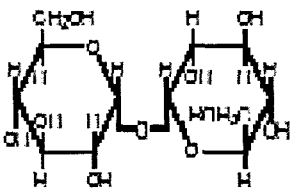
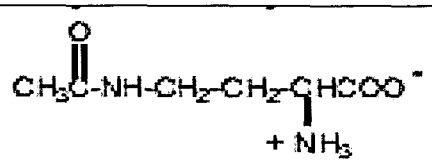
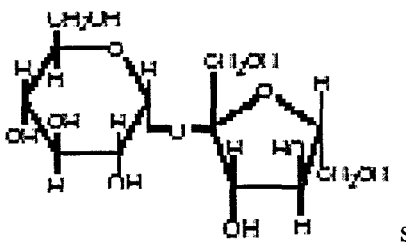
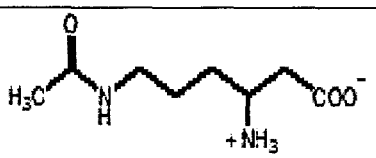
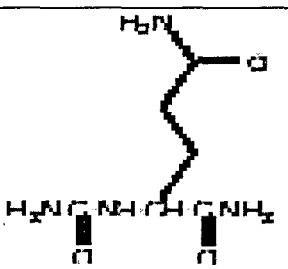
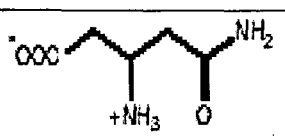
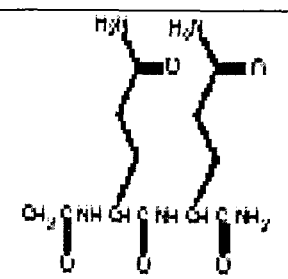
Zwitterionic solutes	Uncharged solutes
 betaine	 α -glucosylglycerol
 ectoine	 α- mannosylglyceramide
 hydroxyectoine	 trehalose
 N γ-acetyldiaminobutyrate	 sucrose
 N-€ acetyl-β-lysine	 N- α -carbamoyl–L- glutamine 1-amide
 β-glutamine	 N-acetylglutaminylglutamine amide

Fig. 1.2 Structure of zwitterionic and uncharged solutes

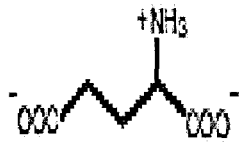
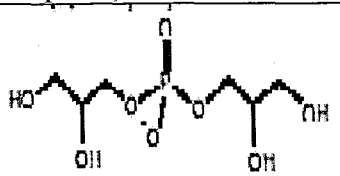
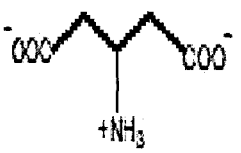
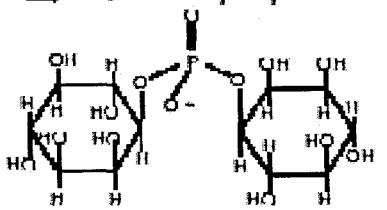
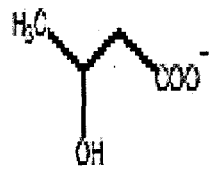
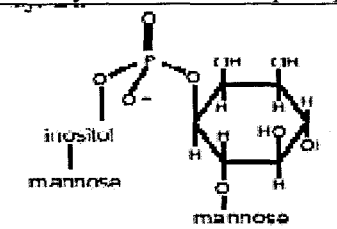
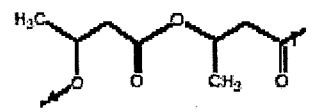
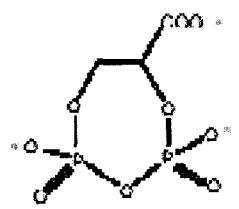
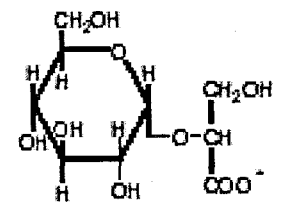
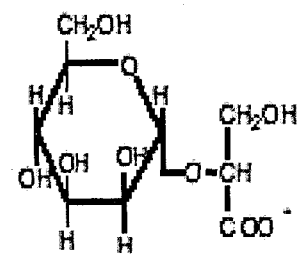
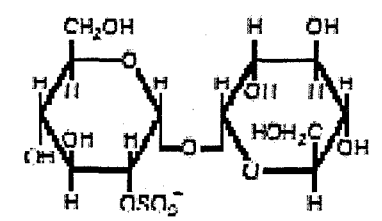
Anionic solutes (carboxylates)	Anionic solutes (phosphate, sulphate)
 L- α -glutamate	 α -diglycerol phosphate
 β- glutamate	 di-myo-inositol-1 1-phosphate
 hydroxbutyrate	 mannosyl-DII
 poly- β- hydroxyl butyrate	 cyclic 2,3 diphosphoglycerate
 α -glucosylglycerate	
 α - mannosylglycerate	 sulfotrehalose

Fig. 1.3 Structure of anionic solutes

A. Zwitterionic Solutes

Neutral amino acids are not accumulated to high concentrations, presumably because they are intermediates in protein biosynthesis. High and varying concentrations of these compounds could affect diverse cell pathways. Instead, many bacterial and archaeal cells synthesize and accumulate a few zwitterionic molecules derived from amino acids as compatible solutes.

1. Betaine

This ubiquitous solute, glycine with the primary amine methylated to form a quaternary amine, is found in halophilic bacteria (Imhoff 1984) and phototrophic bacteria (Galinski and Trüper 1982, Mackey *et al.* 1984). In most cells where it is accumulated as an osmolyte, the betaine is actively transported from the complex medium. A number of methanogens have also been observed to accumulate glycine-betaine when it is provided in the medium (Roberts 2005). Large number of bacteria transport betaine into the cell for use as an osmolyte; however *Actinopolyspora halophila*, *Halomonas elongata* and methanogen (*Methanohalophilus portucalensis*) are able to synthesize betaine either by oxidation of choline or methylate of glycine (Roberts *et al.* 1992, Nyyssola 2000)

2. Ectoine and hydroxyectoine

Ectoine, a cyclic tetrahydropyrimidine which is predominantly synthesized by chemoheterotrophic halophiles (Galinski 1993) can be considered a marker for halophilic bacteria. *H. elongata* displays a broad salt tolerance and synthesizes ectoine and hydroxyectoine and aspartate derivative of ectoine as compatible solutes (Galinski and

Herzog 1990). This solute was first detected in the halophilic, phototrophic *Halorhodospora halochloris* (Galinski *et al.* 1985). The intracellular ectoine concentration was shown to increase with increased extracellular NaCl. Hydroxyectoine, a variant of this solute, has been detected in halotolerant *Sporosarcina pasteurii* grown in high osmolarity medium (Kuhlmann and Bremer 2002).

B. Noncharged solutes

Molecules that are polar but lack any formal charges have been identified as osmolytes in halophilic bacteria, although they are well represented in eukaryotes. Glycerol is prevalent as an osmolyte in marine and halophilic *Dunaliella* (Borowitzka *et al.* 1974, Petrovic *et al.* 2002). Glycerol accumulation is also a characteristic of halotolerant yeast *Debaryomyces hansenii* as well as the black yeast *Hortea werneckii*, and adaptation of this eukaryotic organism to high NaCl requires glycerol accumulation (Plemenitas *et al.* 2002). Myo-inositol, another polyol, is used as an osmolyte in several eukaryotes. Negatively charged derivatives of both glycerol and inositol are accumulated by archaea. The few uncharged solutes that are used by halotolerant bacteria and archaea include several carbohydrates and an amino acid/dipeptide modified to neutralize all charged groups.

1. Carbohydrates

Carbohydrate osmolytes occur in archaea, fungi, algae, plants and possibly deep-sea invertebrates (Kültz and Chakravarty 2001). *Stenotrophomonas* accumulates α-glucosylglycerol, which has found biotechnological uses, and α-Mannosylglyceramide is accumulated in *Rhodothermus marinus* (Silva *et al.* 1999). The non-reducing glucose

disaccharide trehalose is used by organisms to counteract drying, but it also serves as an osmolyte.

2. Uncharged Amino Acids and Peptides

Carboxamine, and an acetylated neutral glutamine dipeptide have been identified as osmolytes. In both solutes, modifications mask the charged α-amino and α-carboxyl groups. N-α-Carbamoyl-L-glutamine 1-amide, an unusual amino acid derivative, is accumulated by halophilic phototrophic bacterium *Ectothiorhodospira marismortui* (Galinski and Oren 1991). The dipeptide N-acetylglutaminylglutamineamide is synthesized by several halophilic purple sulfur bacteria (Smith *et al.* 1989, D'Souza *et al.*1993).

C. Organic anions

Cells have a negative potential inside and often quite high intracellular K^+. Negatively charged solutes could serve balance high intracellular K^+ as well as counteract osmotic pressure. At lower external NaCl, many bacteria including *H. elongata* which also synthesizes ectoine and archaea use L-α-glutamate as an osmolyte. In the methanogen *Methanohalophilus portucalensis*, high NaCl often causes the cells to accumulate zwitterionic solute N ε-acetyl-β-lysine for osmotic balance (Robertson *et al.* 1992). Anionic solutes used by bacteria and archaea for osmotic balance can have a carboxylate negative charge or contain phosphate or sufate groups.

1. β-Glutamate

Methanogens such as *Methanothermococcus thermolithotrophicus* (Robertson *et al.* 1990) tend to accumulate β-glutamate as well as α-glutamate for osmotic balance. *Methanohalophilus portucalensis* synthesize de novo and accumulate β-glutamate. The negatively charged glutamates are accumulated when the external NaCl is less than 1M.

2 Hydroxybutyrate and derivatives

Soluble poly-β-hydroxybutyrates, normally used as carbon reservoirs in cells, have been detected in moderate concentrations in a number of organisms, including *Methylarcula marina* and *Methylarcula terricola* and in the deep sea organism *Photobacterium profundum* (Roberts 2005).

3. Anionic polyols and carbohydrates

In bacteria, high intracellular concentrations of negatively charged carbohydrates are not very common. However, α-glucosylglycerate and α-mannosylglycerate are two solutes that have been detected; these tie up the reactive end of the sugar in a glycosidic bond with a hydroxyl group of glyceric acid. α-Mannosylglycerate, accumulated by several Rhodothermus species, is higher in exponential phase cells and decreases abruptly as cells enter stationary phase (Martins *et al.* 1999). These cells accumulate both the anion mannosylglycerate and the neutral α-mannosylglyceramide. Stress conditions determine which of these two solutes dominates. Under temperature stress of the cells, *R. marinus* tends to accumulate mannosylglycerate; increased NaCl favored accumulation of the neutral

α-mannosylglyceramide rather than the organic anion (Martins *et al.* 1999). Glucosylglycerate has also been observed in *Methanohalophilus portucalensis* when those cells are grown with methanol rather than methylamine as the substrate for methanogenesis (Robertson 1992).

Moderate halophiles are expected to produce polar uncharged and zwitterionic solutes and halo-thermophilic microbes produce anionic compatible solutes (Shivanand and Mugeraya 2011).

1.3 Osmolyte Strategies

Most halotolerant and halophilic organisms use an array of solutes, not a single one, for osmotic balance. When a single solute is detected it is often supplied by the medium and efficiently transported into the cell. Bacterial or archaeal cells synthesize several molecules that together contribute to osmotic balance. Sometimes this is a combination of anions and zwitterions, but often several solutes with the same net charge.

Glycerol, the archetypical compatible solute (Brown and Simpson 1972), accumulates in certain water-stressed yeasts and algae to high levels (Wegmann 1986, Litchfield 1998). Expression of one of the genes putatively coding for glycerol-3-phosphate-dehydrogenase is salt dependent. Glycerol has been shown to be largely compatible with protein function, but its synthesis also requires the use of NADH. This may be essential for maintaining cellular redox balance, by regeneration of NAD^+ during anaerobic metabolism; indeed, mutant yeasts unable to make glycerol, are not only highly sensitive to osmotic stress but also accumulate excessive NADH and thus cannot grow (Ansell *et al.* 1997). The basic theme of

cellular response is known to be exclusion of the extracellular stress agent salt and intracellular accumulation of the compatible solute glycerol.

As a general rule of thumb, bacteria and eukaryotes usually accumulate neutral compatible solutes whereas archaea prefer negatively charged solutes (Martin *et al.* 1999, Roberts 2004). Interestingly, archaea tend to modify many of the same neutral or zwitterionic solutes accumulated by eukaryotes or bacteria to make them negatively charged.

Halotolerant and halophilic bacteria display certain plasticity in switching from N-containing compatible solute to sugar-polyol solute. A salinity-dependent switch in osmolyte strategy is observed in archaea and bacteria (Madkour *et al.* 1990, Regev *et al.* 1990, Pfluger *et al.* 2003).

At intermediate salinities *H. halophilus* uses glutamate and glutamine as osmolytes and chloride is involved in signaling the external NaCl concentration. *H. halophilus* switches its osmolyte strategy and produces proline as the dominant solute at higher salinities (2 to 3M NaCl). The solute glutamate is involved in the switch of osmolyte strategy from glutamate to proline as the dominant compatible solute during the transition from moderate to high salinity.

The accumulation of trehalose was strain-dependent and endogenous trehalose correlated with acquisition of tolerance to hyperosmotic stress. During osmotic stress, the permeability of the yeast membrane to glycerol and other solutes may be reduced by changes in the phospholipid composition (Watanabe and Takakuwa 1984, Tunblad-Johansson and Adler

1987) thus allowing solute accumulation. Some of these compatible solutes may have a role in the protection of cell components against thermal denaturation.

Salt-tolerant organisms synthesize disaccharides, whereas the more tolerant and halophilic species accumulate sugars-polyols and nitrogen containing compatible solute. Halotolerant microorganisms, which are often found in the marine environment, display a rather narrow salt tolerance compared with proteins which are slightly more acidic than the cytoplasmic proteins of *E. coli* (Falkenberg 1986).

Life at high salt concentrations is energetically expensive as it involves the build-up and maintenance of steep ion concentration gradients across the cell membrane, whether or not this is accompanied by the biosynthesis or accumulation of organic osmotic compounds. Salt-in option is energetically more favourable than the maintenance of a low salt cytoplasm with organic osmotic solutes to provide osmotic balance. Maintenance of suitable cell buoyancy and limits to the solubility of the compounds may be an additional reason why lighter solutes are made by microorganisms thriving at the highest salt concentrations.

Osmolytes increase thermodynamic stability of folded proteins and provide protection against denaturing stress. Osmolytes are typically accumulated in the intracellular environment at relatively high concentrations. Examination of microbial diversity in environments of increasing salt concentrations indicates that certain types of dissimilatory metabolism do not occur at the highest salinities. Examples are methanogenesis for $H_2 + CO_2$ or from acetate, dissimilatory sulfate reduction with oxidation of acetate, and autotrophic nitrification. Occurrence of the different metabolic types is correlated with the free-energy change associated with the dissimilatory reactions. Life at high salt

concentrations is energetically expensive (Oren 1999b). Not all metabolic types function in the presence of high salt concentrations. While processes such as oxygenic and anoxygenic photosynthesis, aerobic respiration, and denitrification can occur at or close to NaCl saturation, other physiological groups have never been shown to thrive at high salinities.

All halophilic microorganisms expend large amounts of energy to maintain steep gradients of Na^+ and K^+ concentrations across their cytoplasmic membrane. Microorganisms that balance the osmotic pressure of the medium with high concentrations of organic osmotic solutes also have to expend energy in ion pumps to keep intracellular ionic concentrations low and to counteract the diffusion of inorganic salts through their membranes (Oren 1999b).

When microbial cells are exposed to high salt concentrations, a hyper-osmotic shock is followed by a rapid osmotic adjustment, resulting in loss of turgor and volume in non adapted cells, while adapted fungal cells selectively accumulate compatible solute (Blomberg and Adler 1992, Plemenitas 2005).

Organic solutes are able to change in concentration in the cell in response to a change in external water potential, thus maintaining turgidity while having no significant effect on enzyme activity. They first are accumulated within the mycelium and then translocated to conidia during colonization. Stress conditions imposed on yeast can be diverse, such as entry into stationary phase, nutrition starvation, high and low temperature, high and low osmolarity, high ethanol concentration [illegible] and presence of heavy metals or oxidative compounds. It is well known that glycogen and trehalose accumulate in yeast under nutrient

starvation or entering into the stationary phase of growth. The salt-adapted strain showed a prolonged lag of about 16h, as compared to the unadapted culture.

It is observed that the total solute pool decreases during the stationary phase. Some compatible solutes replace others that accumulate during the exponential phase because they are not as easily lost to the external environment. Some compatible solute that accumulate during the exponential phase of growth may also be preferentially consumed when the concentrations of nutrients in the medium decrease below critical levels (Lamosa *et al.* 1998). A means of evaluating the efficacy of an osmolyte is provided by its m-value, an experimental quantity that measures the ability of the osmolyte to force a protein to unfold or fold. Common environmental stresses encountered by organisms tend to cause cellular water loss or gain. Vast numbers of organisms have evolved to handle water loss or gain by increasing or decreasing the intracellular levels of small organic molecules called osmolytes (Yancey *et al.* 1982). The environmental stress is accompanied by additional stresses that threaten the stability of intracellular macromolecules. In addition to their ability to control cell water loss or gain, many osmolytes stabilize cell components such as proteins (Hochachka and Somero 2002).

1.4 Transport and release of osmolytes

Osmolytes can either be synthesized by the cell or transported into the cell from the medium. However, halophilic microorganisms do not rely entirely upon de novo synthesis of solutes; they are also able to take up compatible solutes from the surrounding medium, which is a far more economical way to accumulate osmoprotectants (Oren 1999b).

Bacteria that produce compatible solutes often possess transport proteins in the membrane that enable them to take these solutes up from the medium, which is energetically more favorable than de novo synthesis. Such transport systems may be intended primarily to retrieve osmotic solutes that would otherwise be lost to the environment from leaky membranes. Thus, glycine-betaine producing cyanobacteria have an efficient glycine-betaine transporter in their membrane (Moore *et al.* 1987). The presence of transport systems for osmotic compounds reduces the energy to be spent on salt adaptation when suitable compounds are available in the medium. This relieves the organisms much of the cost of de novo solute synthesis. Halophilic bacteria that take up compatible solutes from the surrounding medium must be equipped with osmoregulated transporters to facilitate this process (Peter *et al.* 1998, MacMillan *et al.* 1999, Kunte *et al.* 1999, Rubenhagen *et al.* 2000). Osmolyte transporters also play important roles in the osmotic response. Some of these transporters are very specific and serve to recover any solute released by cells. Others scavenge the solute or osmolyte precursors so that the more wasteful biosynthetic resources of the cell are not used. Mechanosensitive channels are the membrane proteins involved in osmolyte movement.

Osmolytes are accumulated at relatively high concentration in the intracellular environment. Osmolytes increase thermodynamic stability of folded proteins without perturbing other cellular processes or biomolecular interactions. The release of osmolytes was rapid and specific and not affected by reduced temperatures. Osmolytes were not metabolized but released on exposure to hypotonic condition (Kayingo *et al.* 2001). The amount of osmolyte released was proportional to the shock intensity. Osmolyte release occurs with little cell lysis and thus do not affect survival and growth of yeast cells after hypo-osmotic shock.

Conservation and release of osmolytes by yeasts takes place during hypo-osmotic stress (Kayingo *et al.* 2001).

1.5 Halophilic Yeasts and fungi

To overcome water stress, most fungi produce compatible solutes. They are first accumulated within the mycelium and then translocated to the conidia during conidiation. *D. hansenii* accumulates ions and polyols (Ramos 2005). A shift in polyol pools was observed in halotolerant *D. hansenii,* which accumulates glycerol in the exponential phase (Andre *et al.*1988) and arabitol in stationary phase cells (Adler and Gustafsson 1980), whereas *A. niger* replaces glycerol with mannitol and erythritol in older mycelia (Witteveen and Visser, 1995). Some investigated fungi use high affinity glycerol transporters to maintain high levels of glycerol, despite continuous efflux (Blomberg and Adler 1992).

In general, when fungi grow under water stress, the amounts of sugar alcohols accumulated by mycelia change quantitatively but not qualitatively (Pfyffer and Rast 1988). In the higher fungi, low molecular weight sugar alcohols, glycerol and erythritol are accumulated at the expense of high molecular weight mannitol under water stress (Jennings 1995). This selective accumulation probably decreases the internal osmotic potential in conidia because glycerol and erythritol molecules are smaller and more polar than mannitol (Hallsworth and Magan 1994, Jennings 1995). Fungal germination and growth generally have been demonstrated to be more sensitive to matrix than osmotic potential stress (Brownell and Schneider 1985, Magan 1988, Magan *et al.* 1995).

The accumulation of osmoprotective compounds such as polyols; glycerol, arabitol (Brown1978), sugars (trehalose and manitol), amino acids derivatives (peptides, N acetylated amino acid) and some organic acids in fungi are a common response to salt stress (Csonka 1989, Casonka and Hanson 1991, Fougere and Streeter 1991, Luxo *et al.*1993). Osmotic adjustment of fungal cells may take place by exclusion of Na+ or by decreasing the cell volume, leading to an increase in the concentration of osmotic solutes (Han and Prade 2002, Kogej *et al.* 2005).

Mechanisms of salt tolerance have been studied in salt-sensitive *Saccharomyces cerevisiae* (Blomberg and Adler 1992, Blomberg 2000, Hohmann 2002) and in some halotolerant fungi, such as filamentous *Aspergillus nidulans*, and yeasts such as *Debaryomyces hansenii* (Adler *et al.* 1985, Andre *et al.* 1988, Larsson *et al.* 1990), *Candida versatilis* (Silva-Graca *et al.* 2003) and *Rhodotorula mucilaginosa* and *Pichia guillermondii* (Lahav *et al.* 2002). Increased production and accumulation of glycerol, trehalose, and other organic compatible solutes helps to maintain positive turgor pressure at high salinity. *Debaryomyces hansenii*, *Hortaea werneckii*, and *Wallemia ichthyophaga* have been isolated from natural hypersaline environments and are model organisms to study halotolerance in eukaryotes (Gunde-Cimerman 2009, Kuncic *et al.* 2010).

The black yeast *Hortaea werneckii* is the dominant fungal species in hypersaline waters (Gunde-Cimerman *et al.* 2000, Gunde-Cimerman *et al.* 2005, Petrovic *et al.* 2002). Different species of black yeasts have been isolated from hypersaline waters of solar salterns (Gunde-Cimmerman 2000) and at salinities greater than 20% (Zalar *et al.* 2005). Under both saline and non-saline growth conditions *H. werneckii* synthesizes dihydroxynaphthalene (DHN) melanin. Melanin granules in the cell walls are organized in a salt-dependent way, implying

the potential osmoprotectant role of melanin. Two mycosporins and three UV-absorbing compounds were detected. The structures of fungal mycosporin are shown in Fig. 1.4.

H. werneckii grown in high NaCl concentrations maintains very low intracellular amounts of potassium and sodium. *A. pullulans*, halotolerant black yeast isolated from salt marshes and from the water of solar salterns at lower salinities (Zalar *et al.* 1999, Gunde-Cimmerman *et al.* 2000) grows best without NaCl (Zalar *et al.* 1999) but can tolerate up to 17% NaCl in the growth medium. In black yeasts, ions do not contribute significantly to osmoadaptation and compatible solutes accumulate in these organisms to counterbalance the osmotic imbalance.

Both *H. werneckii* and *A. pullulans* keep very low intracellular amounts of potassium and sodium when the cells are grown in the presence of high NaCl concentrations and accumulate glycerol as the main compatible solute to counterbalance the increase of external salinity (Kogej *et al.* 2005). Low NaCl concentrations stimulate growth of *H. werneckii*, but not in *A. pullulans*, which grew best without NaCl. This confirms the halophilic character of *H. werneckii* and the halotolerance of *A. pullulans*. In halophilic *H. werneckii* and halotolerant *A. pullulans*, the ratio between potassium and sodium was highest in the cells grown without added NaCl in the medium and it decreased with the increasing concentration of NaCl, being lowest at 20 and 10% NaCl, respectively. 20% NaCl slowed down the growth rate of *H. werneckii*, whereas addition of 10% NaCl inhibited growth of *A. pullulans*. Both species have efficient transport systems for sodium exclusion. The HOG pathway in *Hortaea wernekii*, is important for sensing the changes in environment osmolarity. The expression of HwHog1 in salt adapted cells depends on the environmental salinity. Osmotic stress is caused by high concentration of either salts or non-ionic solutes in the surrounding medium (Da Costa 1989, Blomberg and Alder 1992).

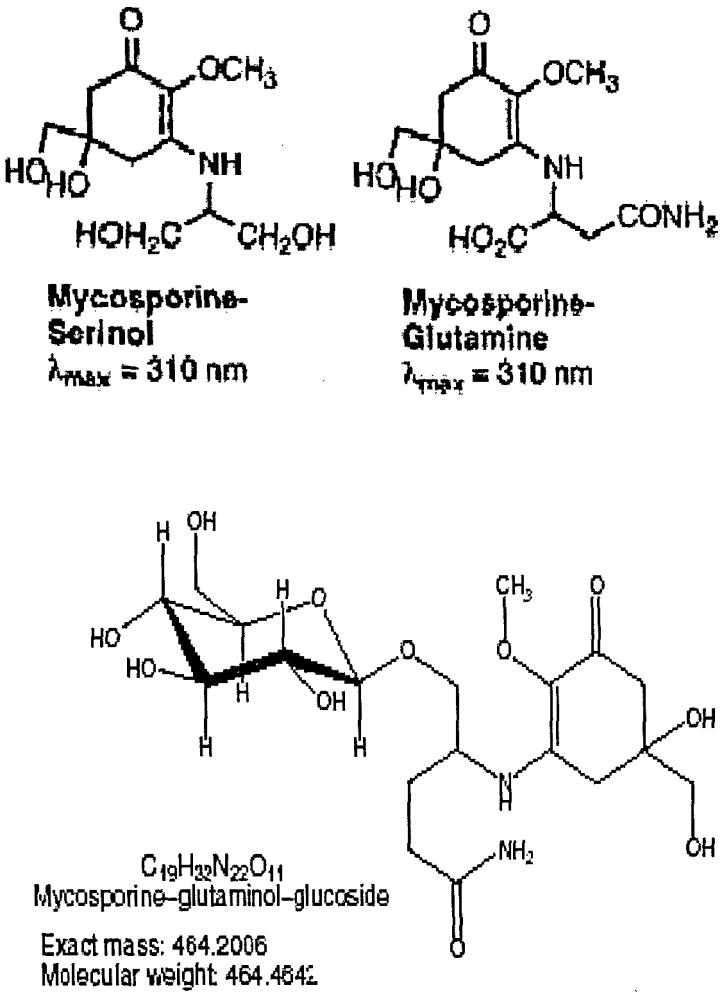


Fig. 1.4 Fungal mycosporins

Although mycosporines may act as supplementary compatible solutes, glycerol is the main compatible solute which accumulates in the cytoplasm, complemented by erythritol and partially by mycosporine-glutaminol-glucoside in the stationary-phase. The amount of erythritol increases gradually during the exponential growth phase and reaches its highest level during the stationary phase (Plemenitas *et al.* 2008).

The production and accumulation of glycerol rose, while the amounts of other polyols remained low as salinity increased over the optimal range for *H. werneckii.* Glycerol is the main compatible solute in actively growing fungal cells in most cases. Glycerol, mannitol, trehalose, arabitol and erythritol are observed in various growth stages and under different environmental conditions. In fungi, glycerol and erythritol is linked to osmotic stress and growth at low water activity. Mannitol and trehalose provide protection against heat, cold and drought, and trehalose is linked to prolonged existence. Spores of the fungus *Talaromyces macrosporus* are packed with trehalose and can survive many years of storage (Dijksterhuis *et al.* 2002).

At higher salinities, the cost of maintaining osmotic balance in the cells is much higher. The higher energetic demands of *H. werneckii* are revealed by reduced growth rates and biomass yield at salinities above 17% NaCl (Kogej *et al.* 2007). In response to fluctuations in environmental osmolarity, yeast cells adjust their intracellular solute concentrations in order to maintain a constant turgor pressure and ensure continuation of cellular activity.

The composition of this mixture depends not only on the external salinity, but also on the age of the cells. Similar observations have been made for some halotolerant/ halophilic

eubacteria (Galinski and Truper 1994), which can change the composition of the solute pool according to growth phase and growth medium.

H. werneckii accumulated a mixture of polyols with different lengths of carbon backbone from three to six atoms in various ratios at optimal growth salinity. Such mixtures may reduce the toxicity associated with high concentrations of a single osmolyte, and prevent feedback mechanisms that downregulate metabolic pathways in the presence of a high concentration of the product (Davis *et al.* 2000). *Candida sake* accumulates equimolar amounts of glycerol and arabitol (Abadias *et al.* 2000). Erythritol is produced by osmophilic yeasts such as *Pichia, Candida, Torulopsis, Trigonopsis, Moniliella and Aureobasidium sp.* (Kim *et al.* 1997). All four polyols have been detected in some *Aspergillus* species (Beever and Laracy 1986, Nesci *et al.* 2004) and in *C. sake* (Abadias *et al.* 2000). In addition to their use as compatible solutes, polyols can also serve as carbon storage compounds, they may help in balancing the cellular redox potential (Diano *et al.* 2006), and they can act as scavengers of reactive oxygen species (Voegele *et al.* 2005).

Amounts of ions remain low at all salinities (Kogej *et al.* 2005), while the amount of glycerol correlates with increasing salinity in the medium (Petrovic *et al.* 2002). Glycerol is known to be the primary osmolyte in many eukaryotic cells in fungi and yeasts, such as *Aspergillus niger* (Witteveen and Visser 1995), *S. cerevisiae* (Blomberg and Adler 1992), *Debaryomyces hansenii* (Adler *et al.*1985), *Zygosaccharomyces rouxii* (Kayingo *et al.* 2001) and *Yarrowia lipolytica* (Andreishcheva *et al.*1999). Besides glycerol, other polyols such as erythritol, arabitol and mannitol are also recognized to be compatible solutes in fungi.

The response of yeasts to environmental changes involves a dramatic increase in the metabolism of the non-reducing disaccharide trehalose, which functions as a carbohydrate reserve and stress metabolite (Hottiger *et al.* 1994). Synthesis of trehalose in the fission yeast *Schizosaccharomyces pombe* is a two-step process that includes the intermediate synthesis of trehalose 6-phosphate (T6P) by T6P synthase, encoded by the *tps1*$^+$ gene (Blázquez *et al.* 1994) and its subsequent dephosphorylation to trehalose by T6P phosphatase, encoded by the *tpp1*$^+$ gene (Franco *et al.* 2000). Hydrolysis of trehalose to glucose is catalysed by the enzyme neutral trehalase, encoded by the *ntp1*$^+$ gene (Soto *et al.* 1999).

Two major cellular responses in the budding yeast *S. cerevisiae* have been shown to counteract the lethal physiological effects of heat: the induction of heat shock proteins and an increase in intracellular production of the dissacharide, trehalose (Atttfield 1987). In saline environments, cells encounter stress due to increased electrolyte concentrations that tend to inhibit metabolic functions (Andre *et al.* 1988). In *S. cerevisiae*, hyperosmotic stress induces the synthesis of stress proteins and an increase in the intracellular production of glycerol (Lewis *et al.* 1995, Nass and Rao 1998). Stress tolerance also involves solute accumulation under conditions of heat and salt stress known to induce osmotic and thermotolerance in this hypersaline isolates. Trehalose has been proposed to play an important role in the ability of organisms to with stand environmental stress (Hounsa *et al.* 1998, Van Laere 1989, D'Amore *et al.* 1991). A correlation between intracellular trehalose concentration and thermotolerance has been reported in *S. cerevisiae* (Hottiger *et al.* 1987, Lewis *et al.* 1995). In some halotolerant yeast species, the amount and type of polyol accumulation are also related to the growth phase (Nobre and da Costa 1985) and the carbon sources used for growth (Van Eck *et al.* 1989). Besides heat, other stress agents such as

ethanol, freezing-thawing and dehydration can induce trehalose accumulation (Attfield 1987and Hottiger *et al.* 1987).

Glycerol is the primary osmoticum produced by many fungal cells subjected to NaCl stress. Osmolytes were detected in *A. flavus* and *A. parasiticus* (Nesci *et al.* 2004) and *F. graminaerum* (Ramirez *et al.* 2004).

A lower water potential is generated by synthesis of a mixture of polyols and amino acids derived from stored sugars, accumulation of negatively charged amino acids would facilitate uptake of counter ions without any perturbation to the metabolism. A reduction in the unsaturation of fatty acids and an increase in sterol content would rigidify the membrane so as to retain the compatible solutes synthesized as a response to osmotic stress. Deposition of melanin would further stabilize the wall and possibly prevent the leakage of osmolytes, apart from rendering the hyphae less susceptible to lysis due to sudden and drastic changes in the external salinity. Cell wall melanization is an important mechanism that helps to maintain a high intracellular concentration of glycerol in *H. werneckii* despite its highly fluid membrane. A melanized cell wall might decrease the energetic needs of the cell, as glycerol accumulation from the medium as well as de novo synthesis can be reduced due to more effective glycerol retention. *Cirrenalia pygmea* synthesizes different osmolytes in order to regulate its intracellular solute potential (Ravishankar 2006).

When thermal stress is applied, the most prominent physiological reactions are the production of a set of proteins or an increase in the quantity of certain types of proteins (Kuei-Yu Chen and Zuei-Cing Chen 2004). Salar and Aneja (2006) studied and isolated thermophilic and thermotolerant fungi from temperate soils of northern India.

Thermotolerant eubacteria are less temperature tolerant as compared to other groups. The maximum temperature limit for Eukaryota has been found to be 62°C. (Tansey and Brock 1978). Fungi are considered to be thermophilic if they grow at or above 50°C and fail to grow at or below 20°C (Cooney and Emerson, 1964).

1.6 Halophiles and its uses

Uses of halophiles are summarized in Table 1.1. Halotolerant microorganisms play an essential role in food biotechnology for the production of fermented food and food supplements. The degradation or transformation of a range of organic pollutants and the production of alternative energy are other fields of applications of these groups of extremophiles. Halotolerant or halophilic microorganisms, able to live in saline environments, offer a large amount of potential applications in various fields of biotechnology (Robert 2005).

Other possible uses of halophilic microorganisms include treatment of saline and hypersaline wastewaters and production of exopolysaccharides, poly-β –hydroxyalkanoate bioplastics and biofuel. Halophilic bacteria are a potential source of extracellular hydrolases like proteases with a wide array of industrial applications. These enzymes exhibit stability over a range of saline conditions (Shivanand and Jayaraman 2009). Due to their stabilizing effects, they can be used for various research and industrial applications. Biodegradation of organic pollutants by halophilic bacteria and archaea has been reviewed (Borgne *et al.* 2008). These microorganisms are good candidates for the bioremediation of hypersaline environments and treatment of saline effluents.

Many osmolytes have been shown to increase the stability of proteins and are useful as stabilizers of biomolecules and whole cells, salt antagonists, or stress-protective agents (Margesin and Schinner 2001). Welch and colleagues (1996) suggested that stabilizing osmolytes, which they call 'chemical chaperones', might rescue misfolded proteins in human diseases (Welch and Brown 1996). Compatible solutes exert their effect through changes in solvent structure and/or subtle changes in the dynamic properties of the protein rather than by changing the structure of the protein itself (Lamosa *et al.* 2003). Compatible solutes also interact with nucleic acids and can influence protein–DNA interactions (Pul *et al.* 2007). The thermostabilizing role of osmolytes has also been exploited for various biotechnology purposes. Folded structures of most proteins are sensitive to changes in environmental conditions such as temperature, pressure, moisture content and the presence of salts and other solutes. Significant perturbations in thermodynamic conditions can cause changes in secondary and tertiary structure, leading to a partial or complete loss of their activity. Organisms are known to adapt to such perturbations in different ways, including evolutionary adaptations that endow stability under extreme conditions or through accumulation of small organic solutes called osmolytes.

Properties of osmolytes are becoming increasingly useful in molecular biology, agriculture and biotechnology (Cushman 2001, Yancey 2001). Carbohydrate osmolytes such as glucose, sorbitol and trehalose can serve as immediate sources of energy after an organism emerges from a stress-induced dormancy. Defense against predators is another possible function of some osmolytes. DMSP widespread in marine microalgae, can be broken down into a gas, DMS (dimethylsulfide), and acrylate, which may serve to repel grazers such as copepods (Wolfe 2000, Van Alstyne and Houser 2003).

Ectoines also find applications in the treatment of the mucous membranes of the eye. Ophthalmologic preparations containing these molecules are useful for eye treatment to decrease the dryness syndrome. Introduction of ectoine and its derivatives into preparations for oral care has also been suggested (Detkovaa and Boltyanskaya 2007, Shivanand and Mugeraya 2011). Ectoines have gained much attention in biotechnology as protective agents for enzymes, DNA and whole cells against stresses such as freezing, drying and heating. Betaines are the compatible solutes occurring in halophilic phototrophic bacteria, chemotrophic bacteria and archaebacteria. They have therapeutic potential for the treatment and prophylaxis of adipose infiltration of the liver, which is the initial stage of cirrhosis (Detkovaa and Boltyanskaya 2007). Betaines decrease side effects of anti-inflammatory preparations.

The potential use of bacteriorhodopsin, the retinal protein proton pump of *Halobacterium* is being explored in photochemical processes (Oren 2010). The technical applications of bacteriorhodopsin comprise holography, spatial light modulators, optical computing, and optical memories. Biopolymers such as biosurfactants and exopolysaccharides, are of interest for microbially enhanced oil recovery. Other useful biosubstances are enzymes such as new isomerases and hydrolases that are active and stable at high salt contents (Robert 2005).

Table 1.1 Applications of halophiles

Halophile / Biomolecules	Applications
Microorganisms	a. Fermenting fish sauces and modifying food textures and flavours b. Waste transformation and degradation (e.g. hypersaline waste brines contaminated with a wide range or organics). Whole organism is used as ion exchange resin regenerant disposal
Membranes	Surfactants, pharmaceuticals
Osmolytes	a. Use in cosmeceuticals and pharmaceuticals b. Cryo-protection of microorganisms c. Enhancing PCR d. Chemical chaperones for Protein Folding e. Generation of stress-resistant transgenic organisms f. Protein and cell protectants in a variety of industrial uses (e.g freezing, heating)
Bacteriorhodopsin	Optical switches and photocurrent generators in bioelectronics
Polyhydroxyalkanoates	Medical plastics
Rheological polymers	Oil recovery
Eukaryotic homologues	Cancer detection, screening antitumour drugs
Lipids	Liposomes for drug delivery and cosmetic packaging
Lipids	Heating oil
Enzymes (nucleases, amylases, proteases)	Various industrial uses (e.g. flavouring agents)
□-Linoleic acid, β-carotene and cell extracts	Health foods, dietary supplements, food colouring, and feedstock (*Spirulina* and *Dunaliella*)

Aims of the thesis

Biodiversity in extreme habitats attracts great attention among researchers because the study of these systems can increase our understanding of the relationship between organisms and their environment, and the mechanisms of their adaptation to extreme conditions. Fungi are generally easily dispersed and are able to colonize a very wide variety of different substrata and withstand many different environmental conditions.

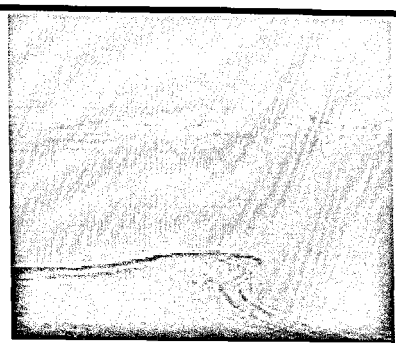
Many fungi are known to function well at very low water activities. In addition, fungi generally prefer a slightly acidic pH. Therefore, the acidic pH, low a_w of the Dead Sea, the organic material being available in the mangroves and Mandovi estuary would appear suitable for fungal life. Studying the mechanisms of physiological cell responses enables us to understand how organisms may thrive in a particular environment.

With this scenario in the background, this topic was chosen with the objective of extraction of osmolytes from the fungi and to understand the mechanism of survival and their salt tolerance.

The main aim of the thesis was to isolate halophilic fungi, study their cultural and morphological changes and study their osmolyte production in relation to growth phase and at increased temperature.

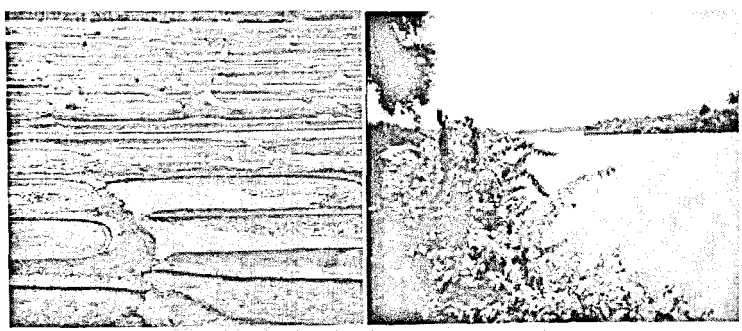
The present study deals with isolation of halophilic fungi from Dead Sea, Mandovi estuary, mangroves and salterns, its adaptation to varying salt and increased temperature, the levels

of salt tolerance, mechanism of osmolyte action, presence of osmolytes at different growth phases and at increased temperature.



CHAPTER 2

ISOLATION OF HALOPHILIC FUNGI FROM ESTUARINE AND HYPERSALINE ENVIRONMENTS



CONTENTS

2.1 Introduction

This Chapter focuses on the isolation of halophilic and halotolerant fungi from different econiches: Dead Sea, estuary, mangroves and salterns.

The Dead Sea located in the Syrian-African Rift Valley, between Israel and Jordan, a unique and extremely hypersaline environment, is a natural habitat for a variety of microorganisms and has evolved much interest and importance (Buchalo *et al.* 1998, Grishkan *et al.* 2003). It is one of the most hypersaline waters in the world, almost six times as salty as the ocean. Compared with the world's oceans, the Dead Sea is more abundant in calcium, magnesium, potassium, and bromide, and lower in sodium, sulfate, and carbonate (Buchalo *et al.* 1998). The Dead Sea is completely landlocked and it gets saltier with increasing depth. Salt precipitates out and piles up on the bottom of the sea. When water evaporates, it leaves behind all the dissolved minerals in the sea, thus increasing its salinity. The dual action of continuous evaporation as well as minerals salts that are carried into the sea from the local river makes the sea salty.

The Mandovi estuary Goa, flows into the Arabian Sea, during the pre-monsoon dry season, the water in the estuarine system remains well mixed and the intrusion of salt water is felt as far as 65 km upstream (Qasim and Gupta 1981). The flow of the estuarine system is regulated by the entry of seawater with the incoming tide which reaches Mandovi through the mouth of the river.

The mangroves are situated at the intertidal zone, also known as the littoral zone. Intertidal zone in marine aquatic environments is the area of the shore and seabed that is exposed to

the air at low tide and submerged at high tide, the area between tide marks. Mangroves are one of the richest and most productive of the habitats, and litter from mangrove trees form the base of the food chain in tropical estuarine environments. Marine fungi play an important role in nutrient regeneration cycles as decomposers of dead and decaying organic matter (Fell and Master 1980). Microorganisms are exposed to varying conditions: an intermittent supply of water and wave action which can wash away or dislodge poorly suited or adapted organisms.

The mangrove water flows into the adjacent man-made salterns which are subject to high intensity of solar radiation and heat, with consequent evaporation in the summer season, giving rise to increasing salt concentrations and eventual salt precipitation. Microorganisms can proliferate in a diverse range of saline concentrations from low saline environments such as freshwater lakes to saturated brines found in solar salterns. Halophiles could be expected to be in larger numbers in such an environment.

Fungi have been isolated from Dead Sea (Grishkan *et al.* 2003, Kis-Papo *et al.* 2003c, Wasser *et al.* 2003, Das *et al.* 2006), mangrove regions (Borse *et al.* 1998, Kathiresan 2000, Sarma *et al.* 2001, Vittal and Sarma 2006) and salterns (Diaz-Munoz and Montalvo-Rodriguez 2005, Cantrell *et al.* 2006).

The present study describes the isolation of high salt-tolerant fungi from the mangroves and salterns in Goa.

2.2 Materials and methods

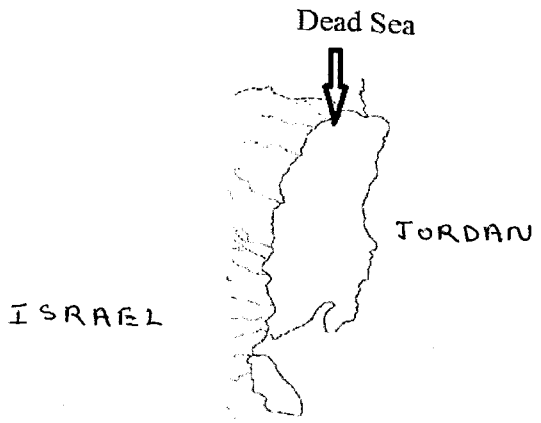
2.2.1 Isolation of fungi from Dead Sea, Mandovi estuary, mangroves and salterns, Sample collection and processing

2.2.1.1 Dead Sea

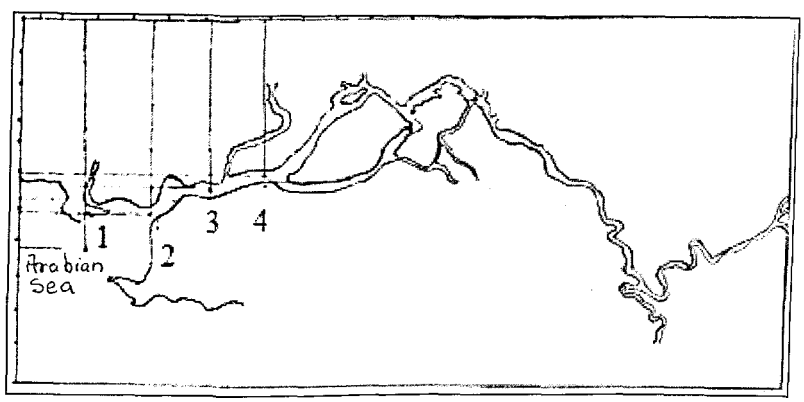
Sampling was done at Kalya beach (31°45'50.62"N and 35°30'13.10"E) during the month of May. Dead Sea water sample was collected in sterile glass bottle. The sediment samples were collected in sterile petri plate. Fig 2.1a gives the map showing sampling sites. The sampling sites are shown in Fig 2.2a

Water sample, 100ml, was filtered through 0.45µm millipore filter. The pellet was re-suspended in 1ml of 2% saline and immediately spread- plated, 100µl was then plated on 10 plates of Czapek-Dox Agar+20% solar salt (20%S-CzA) and on CzA+10% solar salt (10%S-CzA) plate, then incubated at 30°C for 30d.

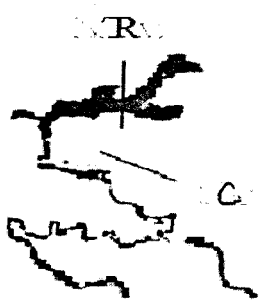
Sediment sample (5g) was suspended in Dead Sea filtrate (50ml) and kept on a shaker at 70rpm for 10min. Flasks were kept standing for a brief while to allow the particulate matter to settle. Supernatant was decanted and then filtered through 0.45µm filter. The pellet was then processed in similar manner as the water sample and plated on 10 plates of CzA+20% solar salt (20%S-CzA) and on CzA+10% solar salt (10%S-CzA) plate, then incubated at 30°C for 30d.



a



b



c

Fig. 2.1 Map showing the location of the sites in a) Dead Sea b) Mandovi estuary c) R. Ribander, and Santa Cruz Mangroves and Salterns

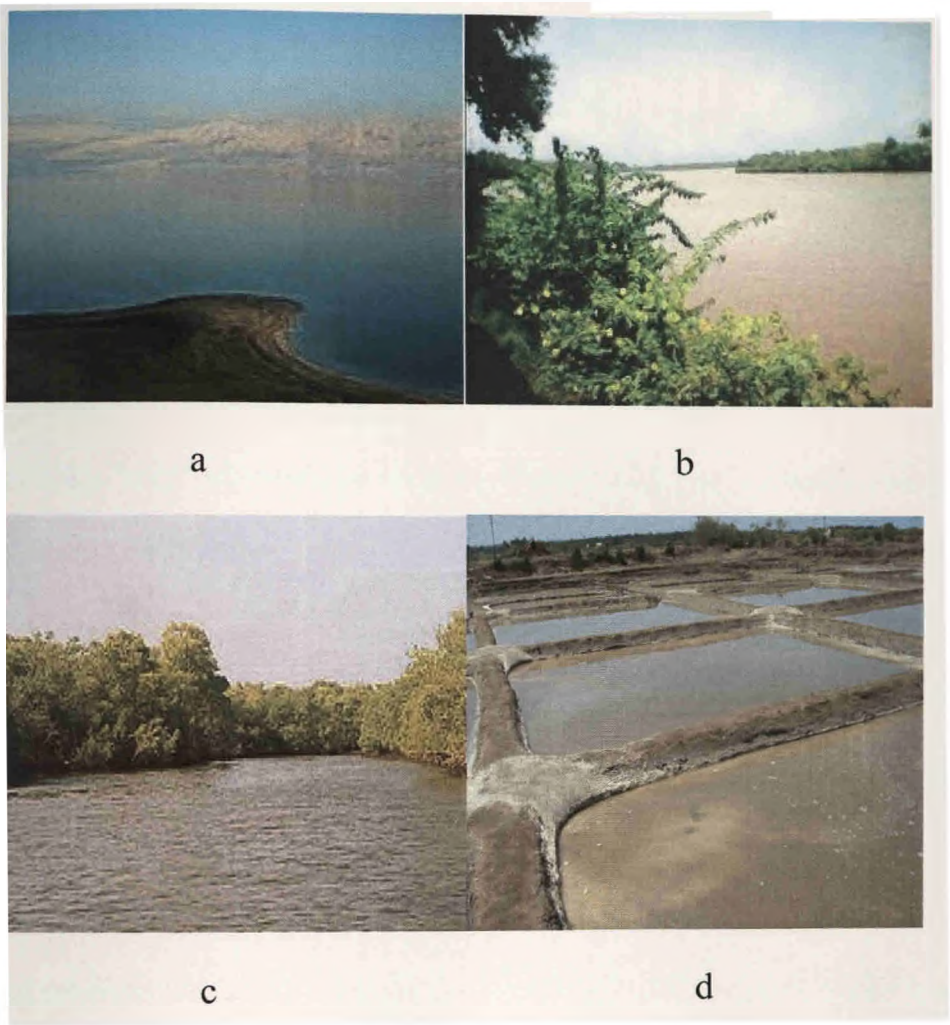


Fig. 2.2 Sampling sites a) Dead Sea b) Mandovi estuary c) Mangroves d) Salterns

2.2.1.2 Mandovi estuary

The sampling sites in the Mandovi estuary were located between 15°28.75'N-15°30.7'N and 73°46.65'E-73°51.9'E. Fig. 2.1b gives the map showing sampling sites of Mandovi estuary. The sampling sites are shown in Fig. 2.2b

Top water, bottom water and sediment samples were collected from four different stations. Water samples were collected in sterile plastic bottles by a Niskin sampler while the sediment samples were collected by Grab sampler and put into sterile zip lock bags.

Surface water and bottom water samples, 20ml each, were filtered under vacuum using 0.45µm filter. The pellet was scraped and suspended in 100µl of 2% saline and spread plated on CzA+20% solar salt. The plates were then incubated at 30°C.

Sediment samples, 1gm each were added in 10ml 2% saline and was kept on the shaker at 175rpm for 15min. It was allowed to settle for 5min and filtered through 0.45µm filter. The pellet was processed in the similar way as the water sample and was spread plated on plate, containing CzA+20% solar salt. The plates were then incubated at 30°C.

2.2.1.3 Mangroves

Samples of water (w) and sediment (s) were collected from Mangroves (M) and Salterns (S) in Goa, at Ribander (R): 15°30'0N and 73°50'60E, and at Santa Cruz (C): 15°28'0N and 73°49'60E. Fig 2.1c gives the map showing the location of sampling sites of mangroves. The sampling sites of mangroves are shown in Fig. 2.2c.

Samples from mangroves were obtained at the intertidal zone, during low tide, at a distance of 1m from the shore and a depth of 0.5m; sampling was done from five different sites at an approximate distance of 15cm apart and pooled together. The water samples were collected in sterile Mc Cartney's bottle. The sediment samples were collected in sterile petri plates.

Water sample, 100ml, was centrifuged aseptically at 7000rpm for 5min (Remi centrifuge). The pellet was suspended in 500µl saline. 100µl each was spread-plated on five plates containing CzA with 20% NaCl and streptomycin (0.5g/l). The plates were incubated at 30°C for 15 days.

Sediment sample 4gm was suspended in 4ml saline and kept on a shaker at 150rpm for 5min. This was filtered aseptically through muslin cloth. The pellet was processed in the similar way as the water sample and spread-plated on five plates containing CzA+20% solar salt and streptomycin (0.5g/l).The plates were incubated at 30°C for 15 days.

2.2.1.4 Salterns

Similarly samples from salterns were obtained from five different saltpans and pooled. Processing of the water and sediment samples from saltern was done in the similar way as that of mangrove samples. Three isolates obtained on 10% S-CzA in a previous work, were also examined Fig 2.1d gives the map showing the location of sampling sites of salterns. The sampling sites of salterns are shown in Fig. 2.2d.

2.2.2 Physicochemical analysis

Physicochemical conditions of temperature, pH and salinity of the water samples were determined while pH and salinity was determined for the sediment samples.

2.2.2.1 Temperature:

The temperatures of the water samples of the mangroves, as well as salterns were measured in situ at 7.00 am and 12.00 noon with the help of a thermometer. The temperature of the water samples of the Mandovi estuary was measured immediately on collection.

2.2.2.2 pH:

The pH of the water samples was determined using a Cyberscan pH meter. Sediment samples, 1g was suspended and shaken in 5ml distilled water, centrifuged at 3000rpm for 10min to remove particulate matter and pH measured.

2.2.2.3 Salinity:

The salinity was measured using Atago Handheld refractometer. The salinity of the sediment samples was measured as follows

Sediment sample 1gm was suspended in 5ml distilled water and kept on a shaker for 15min. It was then centrifuged at 3000rpm for 10min (Remi centrifuge), and the supernatant was collected to check salinity.

2.2.3 Cultures and maintainence

Isolates were picked based on the apparent dissimilarity of cultural characteristics. They were purified and were maintained on CzA +10% salt. They were regularly sub-cultured every six months.

2.2.3.1 Identification

The isolates picked, were identified based on their colonial and morphological characteristics (Raper and Fennell 1965, Domsch *et al.* 1993).

Cultural characteristics:

The cultures were observed for nature of growth, colour of the spores and the mycelia, presence of pigmentation.

Morphological characteristics:

The cultures were observed for septation of the hyphae, the structure of the fruiting body and the arrangement of spores.

Statistical analysis

The correlation of viable counts with salinity of the sample was performed by the Pearson correlation test. Fungal diversity of water or of sediment, each on 10%S-CzA and on 20%S-CzA, was analyzed by Shannon-Weaver index (H).

2.3 Results

2.3.1 Physicochemical characteristics

Temperature, pH and salinity of Dead Sea, Mandovi estuary, mangrove and saltern samples are shown in Fig. 2.3a, 2.3b, 2.3c, 2.3d respectively.

2.3.1.1 Dead Sea

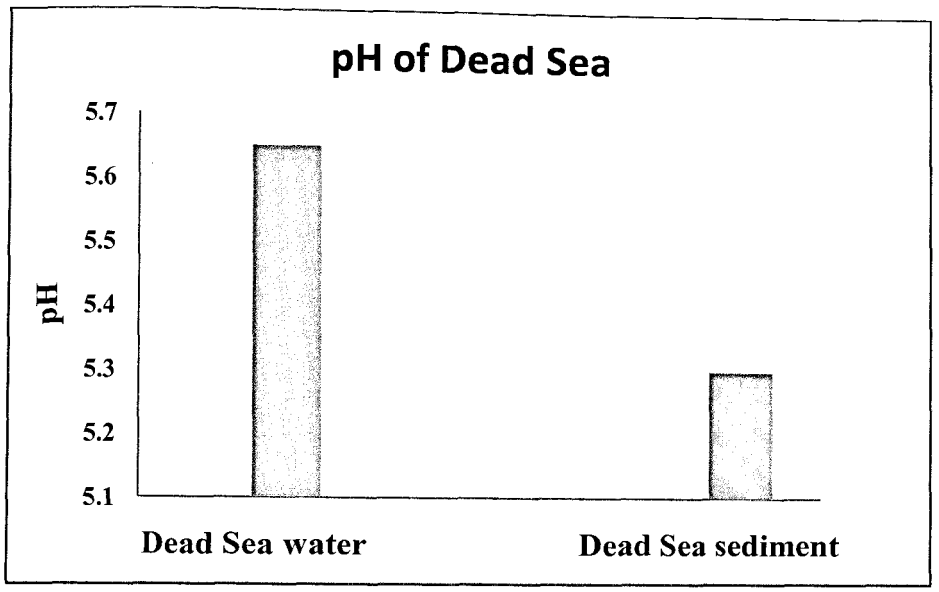
The water sample of the Dead Sea had a salinity of 370‰ and pH of 5.65 while the sediment showed 450‰ salinity and pH 5.3.

2.3.1.2 Mandovi estuary

The temperature of the surface water was 32°C±1, and that of bottom water was 32°C±1, salinity of the surface water was 34±1‰, bottom water was 34±2‰ and that of sediment was 10‰, except for station 1 which had 25‰ in water and pH of the Mandovi estuary sample was 7.5±0.25.

2.3.1.3 Mangroves

The temperatures recorded at the mangroves, Ribander (MR) varied from 23°C in the morning to 32°C at noon, and from 23°C to 33°C at Santa Cruz (MC).



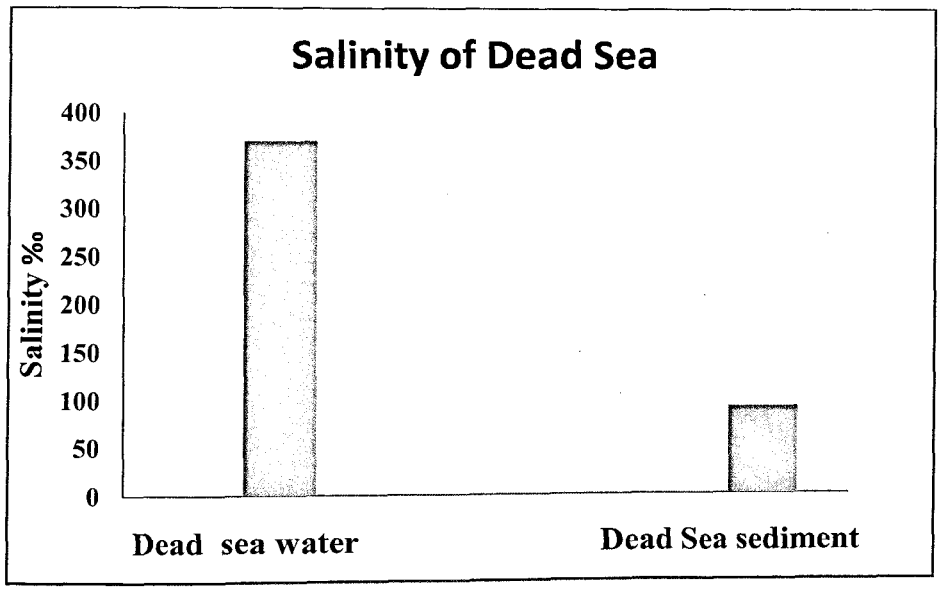
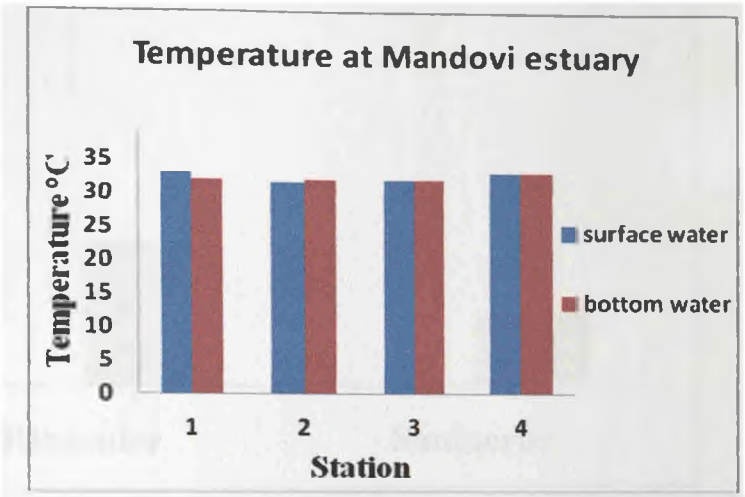
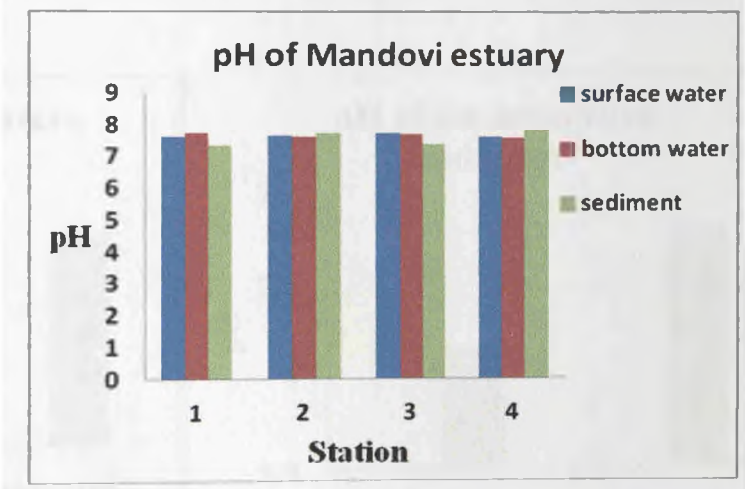


Fig. 2.3a. Physicochemical properties: pH and salinity of the Dead Sea samples





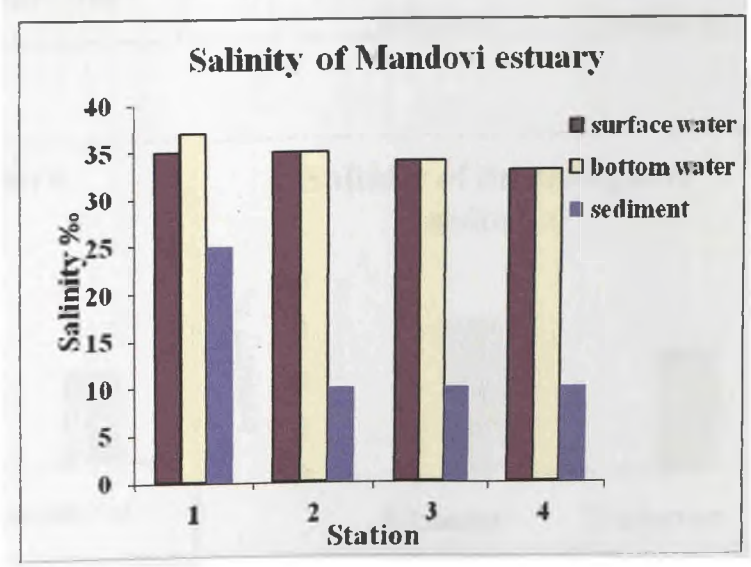
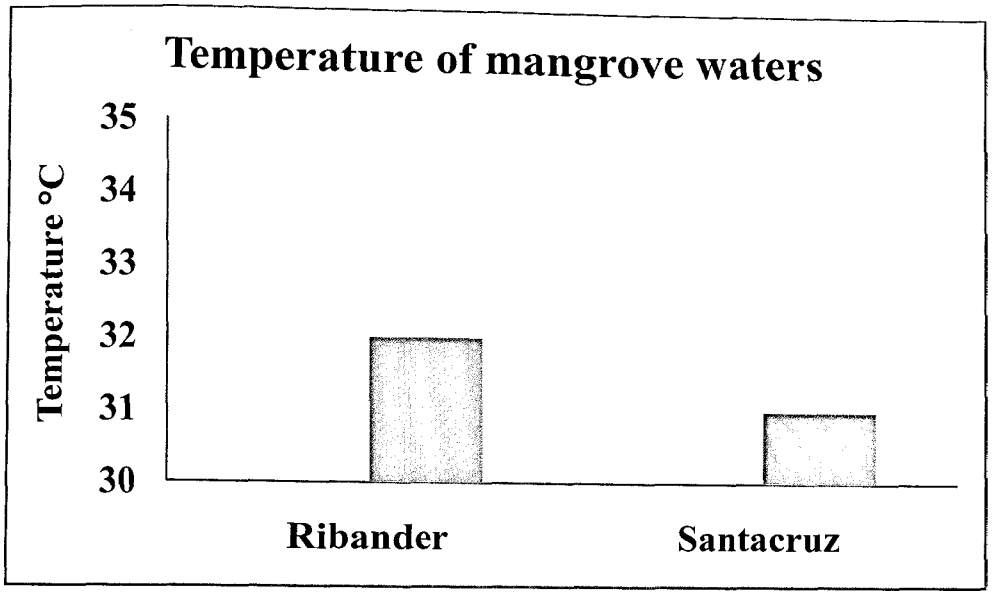
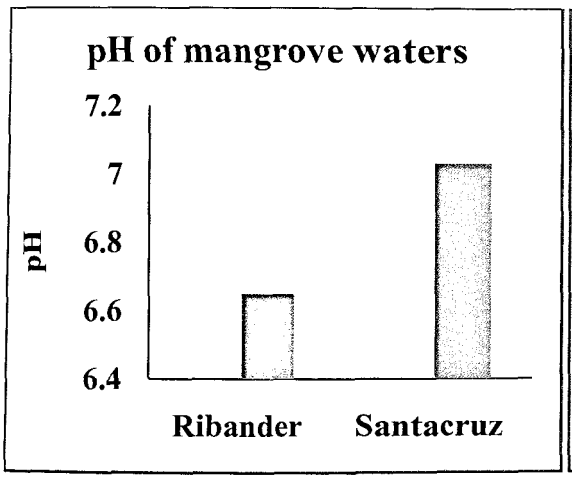
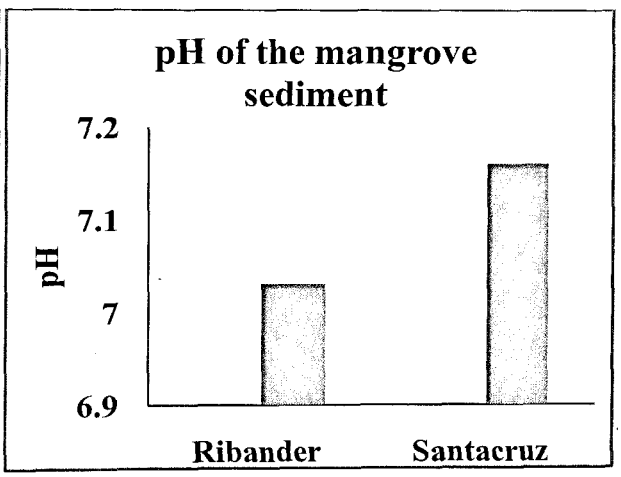
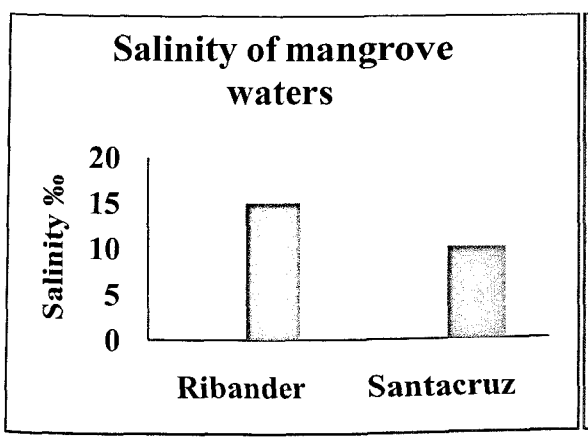


Fig. 2.3b. Physicochemical properties : temperature, pH and salinity of Mandovi estuary.









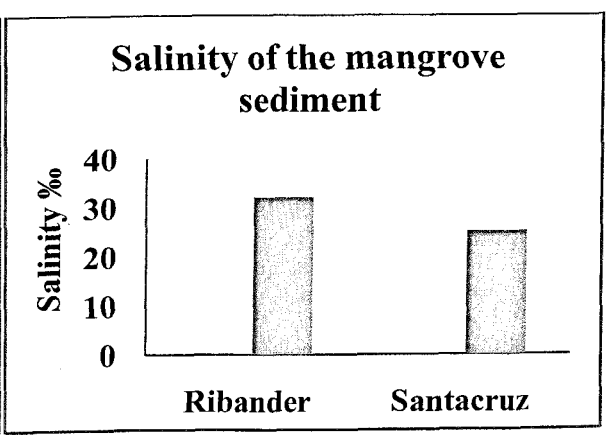
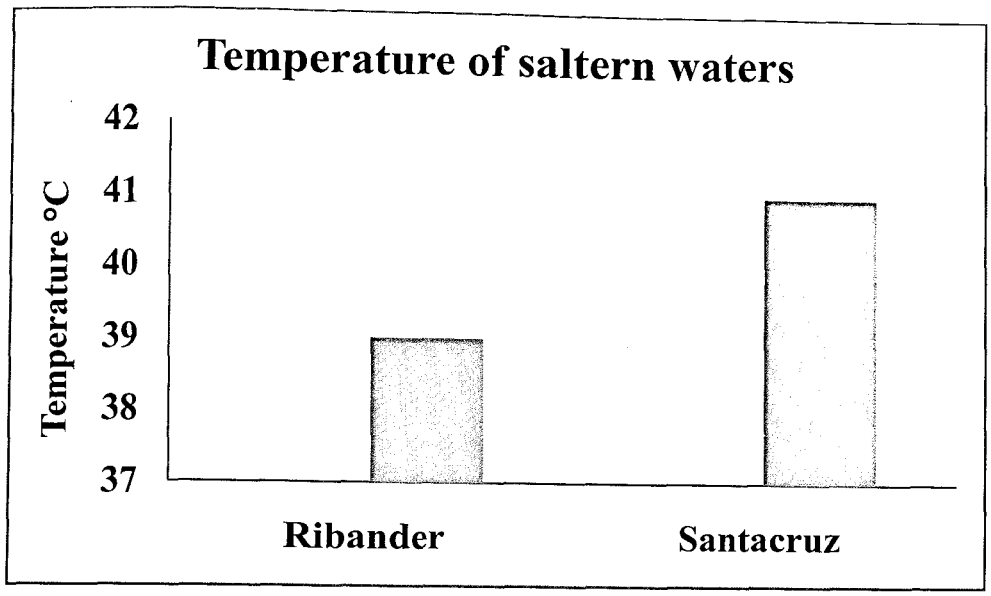
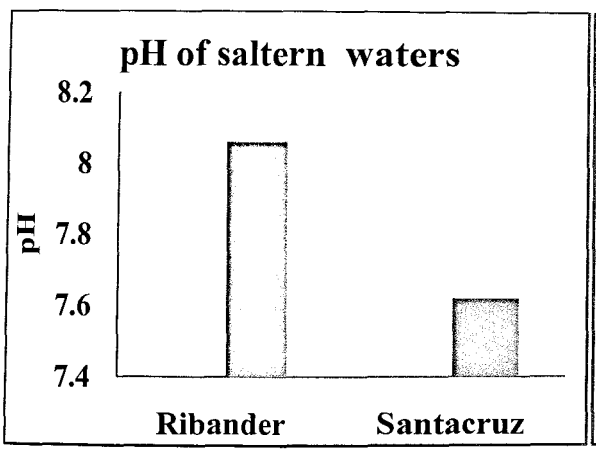
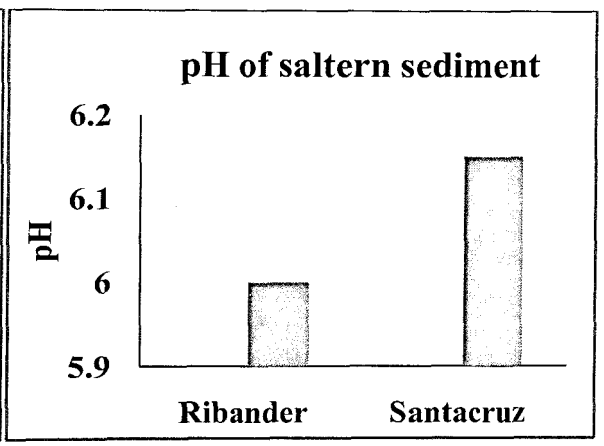
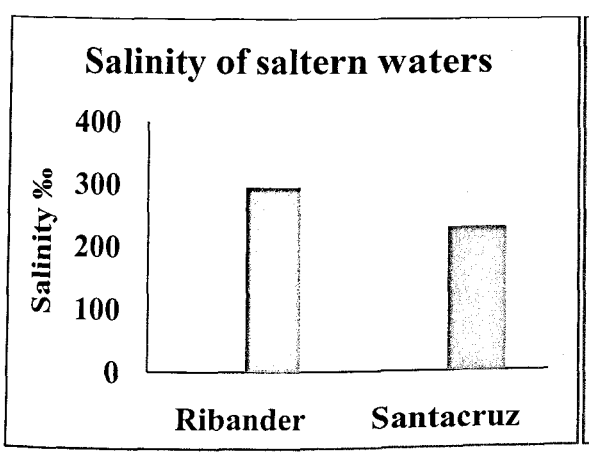


Fig. 2.3c. Physicochemical properties: temperature, pH, salinity of the mangroves.









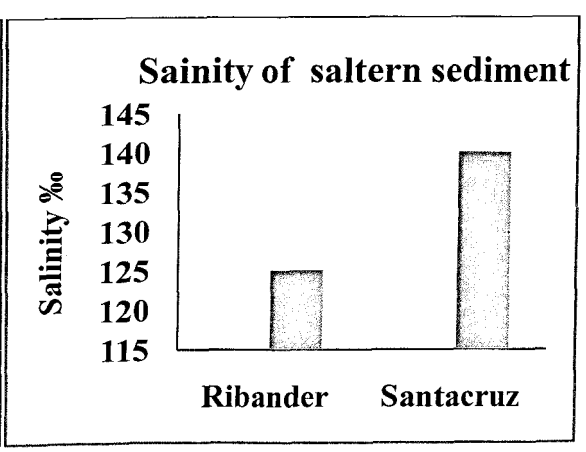


Fig. 2.3d. Physicochemical properties: temperature, pH, salinity of the salterns.

The pH of the mangrove samples was close to neutral MR water 6.65, MC water 7.03, that of the sediment samples Ribander 7.03, Santa Cruz 7.16, being marginally higher than that of the water samples.

The salinity of the mangrove waters and sediment at Ribander was 32‰ and 15‰ respectively and that at Santa Cruz was 25‰ and 10‰ respectively.

2.3.1.4 Saltern

The temperatures recorded at the salterns Ribander (SR) was 39°C and at Santa Cruz (SC) was 41°C.

The pH of the saltern waters was basic:Ribander water 8.06, Santa Cruz water 7.62, with that of the sediment being a little acidic Ribander 6.00 , Santa Cruz 6.15.

The salinity of the saltern water and sediment was 295‰ and 125‰ respectively at Ribander, and 230‰ and 140‰ respectively at Santa Cruz.

2.3.2 Fungal isolates

Viable count of the Dead Sea, Mandovi estuary, mangroves and salterns are given in Table 2.1a, 2.1b, 2.1c, 2.1d respectively. The density of fungal isolates showed a positive correlation with salinity of the sample (r=1) and with salt concentration used in the isolation medium (r=1).

2.3.2.1 Dead Sea

The total number of isolates obtained from the water sample was 0.2 cfu ml^{-1} on 20% S-CzA and 0.1 cfu ml^{-1} on 10% S-CzA; from the sediment sample, 91.1 cfu g^{-1} on 20% S-CzA and 8.9 cfu g^{-1} on 10% S-CzA were obtained.

2.3.2.2 Mandovi estuary

The total number of isolates obtained from the water sample-: From top water was 3.25 cfu ml-^{1}, 3 cfu ml-^{1}, 1.05 cfu ml-^{1} respectively for stations 2, 3 and 4 respectively. No isolates were obtained from station 1. From bottom water was 0.5 cfu ml-^{1}, 0.65 cfu ml-^{1}, 9 cfu ml-^{1}, 0.9 cfu ml-^{1} for stations 1, 2, 3 and 4 respectively and from sediment sample was 6 cfu g-1, 9 cfu g-^{1}, 120 cfu g-^{1}, 22 cfu g^{-1} for stations 1, 2, 3 and 4 respectively.

2.3.2.3 Mangroves

Total number of highly salt-tolerant isolates obtained in 100ml water samples or 4g of sediment samples were 0.17 cfu ml-^{1} from MRw, 0.04 cfu ml-^{1} from MCw and 1.75 cfu g^{-1} from MCs. No isolates were obtained from MRs.

2.3.2.4 Salterns

Total numbers of highly salt-tolerant isolates obtained in the 100ml water samples or of 4g of sediment samples, were 1.12 cfu ml-^{1} from SRw, 7.5 cfu g^{-1} from SRs, and 0.09 cfu ml-^{1} from SCw. No isolates were obtained from SCs.

Table 2.1a. Viable count of Dead Sea samples

Dead sea	Viable count	
	CzA+20% salt	CzA+10% salt
Water	0.22 cfu ml^{-1}	0.04 cfu ml^{-1}
Sediment	82.8 cfu g^{-1}	23 cfu g^{-1}

Table 2.1b. Viable count of Mandovi estuary samples

Mandovi estuary	Viable count on CzA+20% salt		
Station	Surface water	Bottom water	Sediment
1	No growth	0.5 cfu ml^{-1}	6 cfu g^{-1}
2	3.25 cfu ml^{-1}	0.65 cfu ml^{-1}	9 cfu g^{-1}
3	3 cfu ml^{-1}	9 cfu ml^{-1}	120 cfu g^{-1}
4	1.05 cfu ml^{-1}	0.9 cfu ml^{-1}	22 cfu g^{-1}

Table 2.1c. Viable count of mangrove samples

Mangrove	Viable count on CzA+20% salt
Ribander water	0.17 cfu ml^{-1}
Ribander sediment	No growth
Santa Cruz water	0.04 cfu ml^{-1}
Santa Cruz sediment	1.75 cfu g^{-1}

Table 2.1d. Viable count of saltern samples

Saltern	Viable count on CzA+20% salt
Ribander water	1.12 cfu ml^{-1}
Ribander sediment	7.5 cfu g^{-1}
Santa Cruz water	0.09 cfu ml^{-1}
Santa Cruz sediment	No growth

2.3.3 Fungal diversity

30 species were identified from the Dead Sea, 28 from Mangroves and saltern and 28 from Mandovi estuary.

The identification of the isolates obtained from the Dead Sea, Mandovi estuary, mangroves and salterns are shown in Table 2.2a, 2.2b, 2.2c, 2.2d respectively, Table 2.3 gives the biochemical tests for *H. werneckii.*

2.3.3.1 Dead Sea

Those isolated from water samples on 20% S-CzA were identified as *Aspergillus* species: *A. penicillioides*, *A. versicolor*; *Penicillium* species: *P. corylophilum*, *P. steckii*, On 10% S-CzA, only one genus, namely *Cladosporium*, was obtained and identified as *C. cladosporioides.*

The sediment was dominated exclusively by *Aspergillus* species, the predominant species being *A. penicillioides* isolated mainly on 20% S-CzA, but a few also on 10% S-CzA. In addition, *A. versicolor* was obtained on 10% S-CzA and a few on 20% S-CzA. On 20% S-CzA, DSw showed seven species (H= 0.72), DSs yielded two species (H=0.15); on 10% S-CzA, DSw and DSs each showed only one species (H=0).

2.3.3.2 Mandovi estuary

The isolates obtained from Mandovi estuary station 1 yielded *P. canescens* (top water), *P. steckii* (top water), *P. chrysogenum* (sediment). Station 2 yielded *A. flavus* (sediment) *A. versicolor,* (top water, bottom water, sediment), *A. penicillioides* (bottom water, sediment) *P. corylophilum* (sediment).

E. repens, (sediment) *P. steckii,* (top water and sediment), *P. corylophilum*, (bottom water) were isolated from station 3. *P. steckii,* (top water), *A. penicillioides*, (top water, bottom water, sediment), *C. cladosporioides* (top water and bottom water), *E. amstelodami* (bottom water), *E. repens* (sediment) were isolated from station 4.

2.3.3.3 Mangroves

The isolates obtained from MRw were identified as *A. penicillioides, E. amstelodami, E. repens, P. asymmetrica sec fasciculata* and *P. corylophilum. A. tamarii and H. wernekii* were isolated from MCw and *A. flavipes, A. terreus var terreus, A. versicolor, E. amstelodami, E. repens* from MCs.

2.3.3.4 Salterns

The isolates obtained from Ribander brine were identified as *A. versicolor, A. wentii, P. asymmetrica sec fasciculata, P. chrysogenum, P. corylophilum, P. griseofulvum* and those from SRs were *A. sydowii, A. versicolor, A. wentii, H. werneckii, E. amstelodami.* Santa

Cruz brine samples yielded isolates of *A. penicillioides, A. versicolor, H. werneckii, P. asymmetrica sec fasciculata,* no isolates were obtained from Santa Cruz saltern sediment.

The numbers of species picked from Dead Sea, Mandovi estuary, mangrove and saltern samples are given in Fig. 2.4a, 2.4b, 2.4c, 2.4d respectively.

Isolates from previous work, obtained from the Ribander saltern water SRw182, Siridao saltern water SSw135 and Santa Cruz saltern water SCw167 isolated on 10%-S CzA were identified as *A. niger, A. terreus, A.glaucus.*

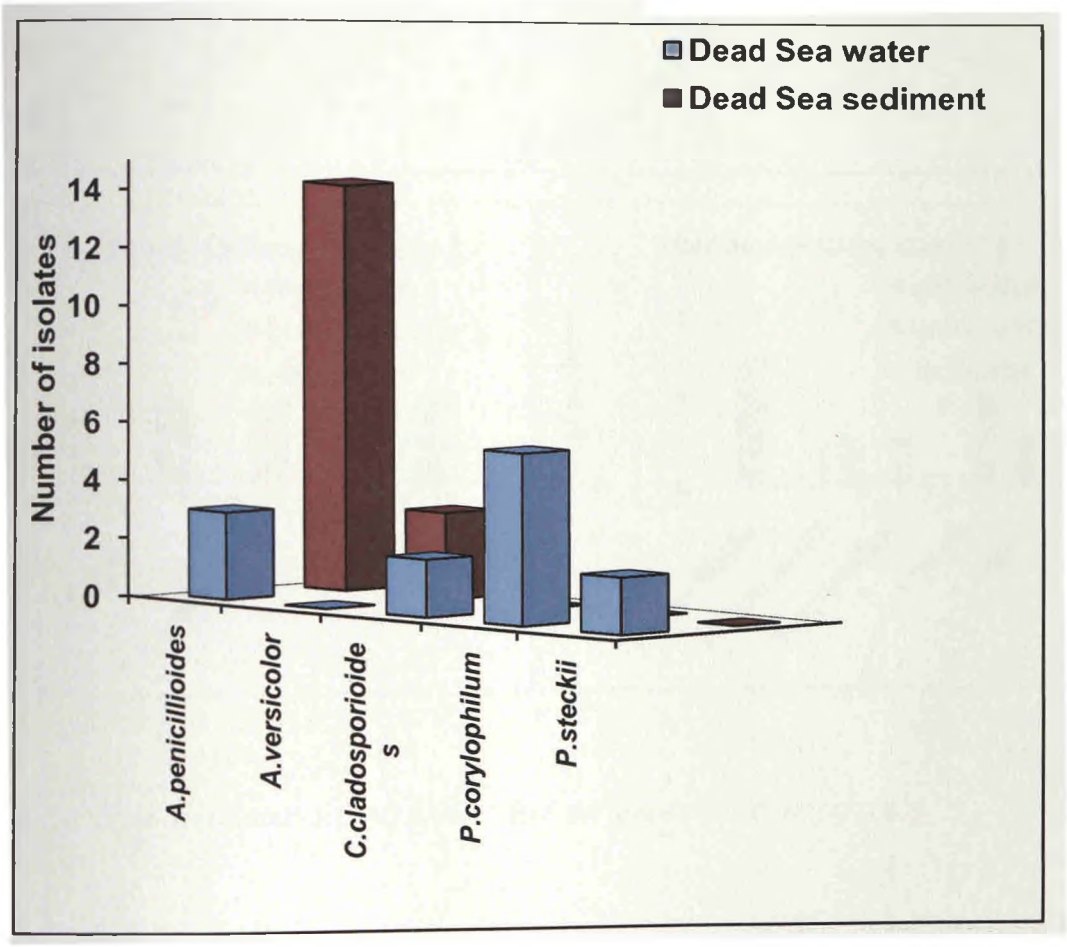


Fig. 2.4a. Number of isolates picked from the Dead Sea sample.

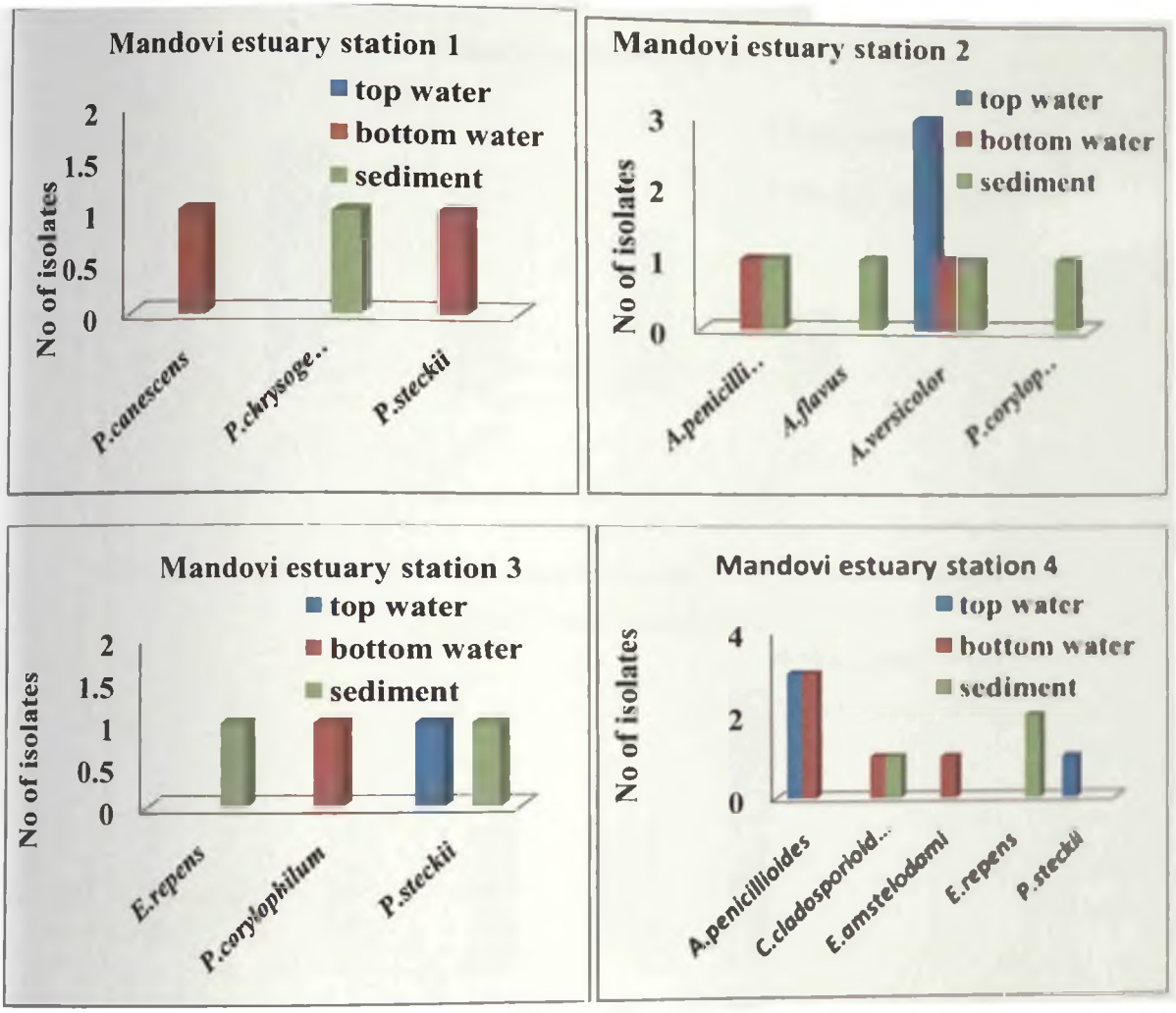


Fig. 2.4b. Number of isolates picked from different Mandovi estuary sample.

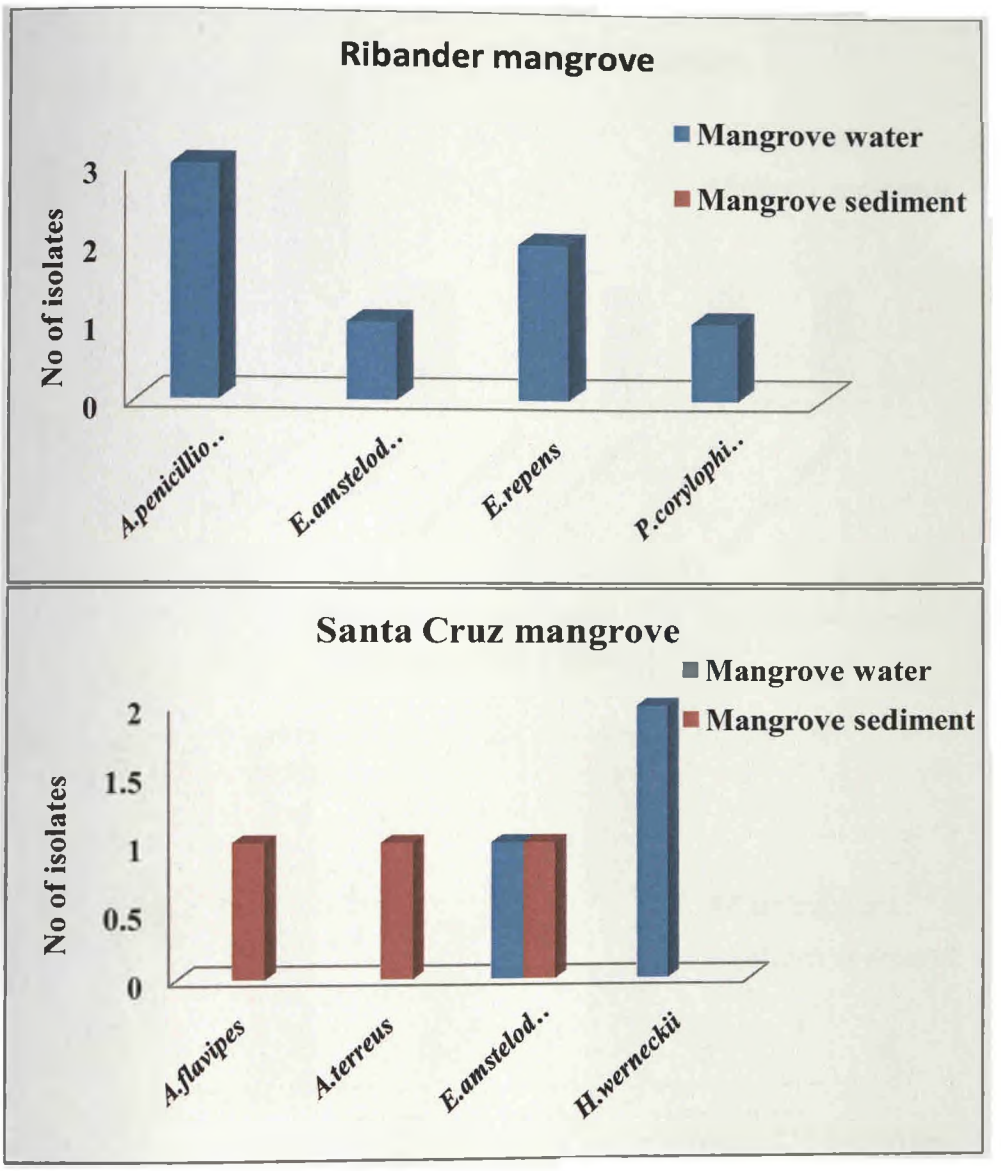


Fig. 2.4c. Number of isolates picked from the mangrove sample

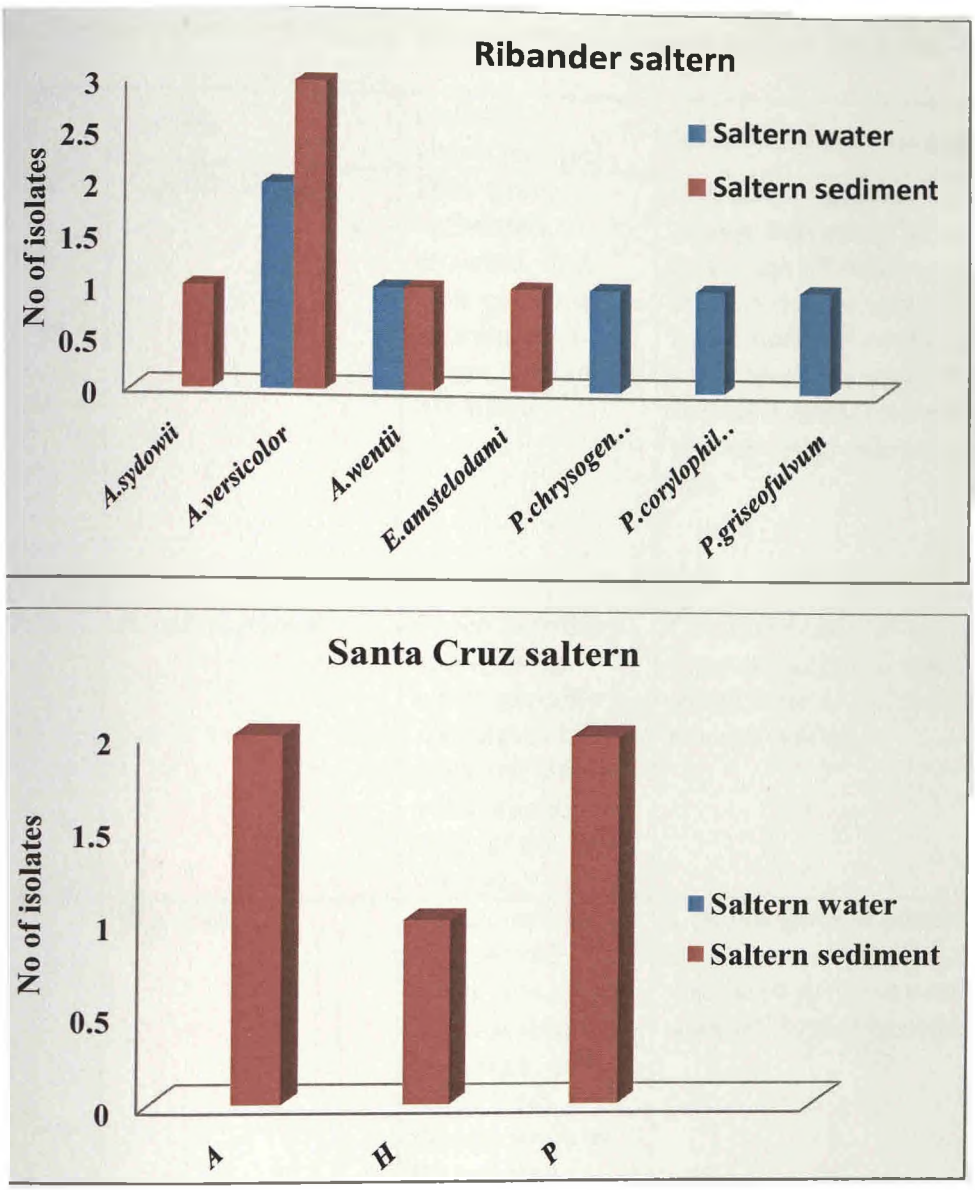


Fig.2.4d. Number of isolates picked from the saltern sample

Table 2.2a. Cultural and morphological characteristics of isolates from Dead Sea

Culture no	Isolates	Cultural characteristics	Morphological characteristics
DSw1, DSw5 DSw17 DSs 29, DSs 31, DSs 33, DSs 35, DSs 37,DSs 39, DSs 41, DSs 43, DSs 45, DSs 47, DSs 49, DSs 51, DSs 53, DSs 55	*A. penicillioides*	Dark green, undulated, elevated, dry, slow growing, sporulated in 7days, reverse off white.	Conidial heads radiate when young, becoming loosely columnar. Phialides borne directly on the vesicle covering more than half of the vesicle. Conidia ellipsoidal often with flattened ends, becoming ellipsoidal to subglobose with age.
DSw 4, DSw 8 DSw11 DSw13, DSw18, DSw19	*P. corylophilum*	Green powdery spores with white mycelium, sporulates in 5 days, off white with orange tinge at the reverse.	Conidiophores with metulae of unequal length. Conidia subglobose to ellipsoidal, smooth walled
DSw7 DSw 21	*P. steckii*	Green powdery spores with white mycelium, dry, sporulates in 5 days, off white with orange tinge at the reverse.	Conidia globose smooth walled, conidiophores bearing divergent whorls of metulae usually without further branching.
DSw23 DSw25	*C. cladosporioides*	Brown spores, undulated velvety, elevated, sporulates in 5 days, reverse black.	Conidia ellipsoidal to lemom shaped, mostly smooth-walled. Conidiophores long, bearing numerous conidial chains arising below septa.
DSs57 DSs 59 DSs 61	*A. versicolor*	Green spores, undulated, dry, sporulated in 5 days, reverse off white.	Conidiophores colourless, vesicles elongate with metulae and phialides covering most of the surface, conidial heads radiating, conidia globose.

Table 2.2b. Cultural and morphological characteristics of isolates from Mandovi estuary station1

Station 1	Culture no	Isolates	Cultural characteristics	Morphological characteristics
Surface water	Nil			
Bottom water	EM1wb101	*P. canescens*	Dark green powdery spores, sporulated in 5 days, reverse off white.	Conidiophores rough, metulae strongly divergent. Conidia ovate, later becoming globose, smooth-walled or slightly roughened.
	EM1wb102	*P. steckii*	Dark green powdery spores, sporulates in 5 days, reverse off white with orange tinge.	Conidia globose smooth walled, conidiophores bearing divergent whorls of metulae usually without further branching.
Sediment	EM1s103	*P. chrysogenum*	Green powdery spores, sporulates in 5 days, reverse off white with orange tinge.	Conidia smooth walled, ellipsoidal, globose to subglobose.

Contd.....

Table 2.2b. Cultural and morphological characteristics of isolates from Mandovi estuary station 2

Station 2	Culture no	Isolates	Cultural Characteristics	Morphological characteristics
Surface water	EM2wt104 EM2wt105 EM2wt106	*A. versicolor*	Colony aerial, green spores, white mycelium, sporulated in 5 days, reverse off white.	Conidiophores colourless, vesicles elongate with metulae and phialides covering most of the surface, conidial heads radiating, conidia globose.
Bottom water	EM2wb107	*A. penicillioides*	Light green spores,dry, sporulated in 6 days, reverse off white.	Conidial heads radiate, becoming loosely columnar. Phialides borne directly on the vesicle covering more than half of the vesicle. Conidia ellipsoidal often with flattened ends, becoming ellipsoidal to subglobose.
	EM2wb108	*A. versicolor*	Light green spores white at the sides, sporulated in 5 days, reverse off white.	Same as above
Sediment	EM2s109	*P. corylophilum*	Light green powdery spores, sporulated in 5 days, reverse off white.	Conidiophores with metulae of unequal length. Conidia smooth walled, subglobose to ellipsoidal.
	EM2s110	*A. versicolor*	Colony powdery, green spores, sporulated in 5 days, reverse off white.	Same as above
	EM2s111	*A. flavus*	Colony aerial, green spores yellow mycelium, sporulated in 5 days, reverse off white.	Conidiophores hyaline, long and rough walled. Conidial heads radiating, conidiophores having a layer of metulae supports the phialides.
	EM2s112	*A. penicillioides*	Colony dry spores, green spores yellow pigment, sporulated in 6 days, reverse off white.	Same as above

Contd.....

Table 2.2b. Cultural and morphological characteristics of isolates from Mandovi estuary station 3

Station 3	Culture no	Isolates	Cultural characteristics	Morphological characteristics
Surface	EM3wt113	*P. steckii*	Colony powdery green spores, sporulates in 5 days, reverse off white with orange tinge.	Conidia globose smooth walled, conidiophores bearing divergent whorls of metulae usually without further branching.
Bottom	EM3wb114	*P. corylophilum*	Colony powdery, green spores, sporulated in 5 days, reverse off white.	Conidiophores with metulae of unequal length. Conidia smooth walled subglobose to ellipsoidal.
Sediment	EM3s115	*P. steckii*	Colony powdery, green spores sporulated in 5 days, reverse off white.	Conidia globose smooth walled, conidiophores bearing divergent whorls of metulae usually without further branching.
	EM3s116	*E. repens*	Colony aerial, yellowish orange spores sporulated in 5 days, reverse yellow to orange pigment with age.	Cleistothecia yellow, globose, ascospores smooth walled, conidia globose often roughened.

Contd.....

Table 2.2b. Cultural and morphological characteristics of isolates from Mandovi estuary station 4

Station 4	Culture no	Isolates	Cultural Characteristics	Morphological characteristics
Surface water	EM4wt117	*P. steckii*	Colony powdery, green spores, sporulated in 5 days, reverse off.	Conidia globose smooth walled, conidiophores bearing divergent whorls of metulae usually without further branching.
	EM4wt118 EM4wt119 EM4wt120	*A. penicillioides*	Colony undulated, green spores sporulated in 6 days, reverse off.	Conidial heads radiate when young, becoming loosely columnar. Phialides borne directly on the vesicle, covering more than half of the vesicle. Conidia ellipsoidal often with flattened ends, becoming ellipsoidal to subglobose
Bottom water	EM4wb121 EM4wb123 EM4wb125	*A. penicillioides*	Same as above	Same as above
	EM4wb122	*C. cladosporioides*	Colony velvety, brown spores, undulated, elevated, sporulates in 3 days, reverse black.	Conidia ellipsoidal to lemom shaped, mostly smooth-walled. Conidiophores long, bearing numerous conidial chains arising below septa.
	EM4wb124	*E. amstelodami*	Colony undulated green spores, black mycelium, sporulated in 4 days, reverse yellow to orange pigment with age.	Cleistothecia yellow, globose, ascospores rough walled with an equatorial line or ferrow, Conidia globose often roughened.
Sediment	EM4s126 EM4s127	*E. repens*	Green powdery spores sporulated in 4 days, reverse yellow to orange pigment with age.	Cleistothecia yellow, globose, ascospores smooth walled, Conidia globose often roughened.
	EM4s128	*C. cladosporioides*	Same as above	Same as above

Table 2.2c. Cultural and morphological characteristics of isolates from mangroves.

Culture no	Ribander Isolates	Cultural characteristics	Morphological characteristics
MRw202 MRw203 MRw208	*A. penicillioides*	Dark green spores, undulated, elevated, dry, slow growing, sporulated in 7days plate reverse off white.	Conidial heads radiate, becoming loosely columnar. Phialides borne directly on the vesicle, covering more than half of the vesicle. Conidia ellipsoidal often with flattened ends, becoming ellipsoidal to subglobose.
MRw205	*P. corylophilum*	Colony dry, powdery green spores, plate reverse pale sporulated in 4 days.	Conidiophores with metulae of unequal length. Conidia subglobose to ellipsoidal, smooth walled
MRw210 MRw212	*E. repens*	Fast growing, sporulated in 3 days, aerial green spores, dry, reverse colourless.	Cleistothecia yellow, globose, ascospores smooth walled, Conidia globose often rough.
MRw211	*E. amstelodami*	Fast growing, sporulated in 3 days, aerial, dry greenish spores, reverse colourless turning yellow, brown to black with age	Cleistothecia yellow, globose, ascospores rough walled with an equatorial line or ferrow, Conidia globose often rough.
MCw214 MCw215	*H. werneckii*	Slow growing, spoulated in 6 days, moist slimy black colony with a thin layer of mycelium, small oval, reverse black with no pigment.	Septate hyphae, bicellular, yeast like conidia.
MCs219	*A. flavipes*	Slow growth,brown aerial spores, dry, sporulated in 6 days, reverse colourless.	Conidial head pale buff commonly with sulphur yellow sclerotia
MCs221	*E. amstelodami*	Fast growing, sporulated in 3 days, aerial greenish yellow spores, dry, yellow pigment turning brown to black with age.	Cleistothecia yellow, globose, ascospores rough walled with an equatorial line or ferrow, Conidia rough and globose.
MCs223	*A. terreus var terreus*	Fast growing, brown spores, powdery, dry, sporulated in 2 days, reverse colourless.	Conidiophores long, smooth walled, hyaline, with hemispherical vesicles, metulae present, conidial heads columnar, conidia globose to ellipsoidal

Table 2.2d. Cultural and morphological characteristics of isolates from saltern water.

Culture no	Ribander Isolates	Cultural characteristics	Morphological characteristics
SRw225	*P. chrysogenum*	Green spores powdery, sporulated in 4 days, reverse pale.	Conidia smooth walled, ellipsoidal, globose to subglobose
SRw228	*P. griseofulvum*	Dark green spores undulated, sporulated in 4 days, reverse pale.	Conidiophores three to four stage branched, phialides. Conidia ellipsoidal, smooth walled.
SRw230, SRw235	*A. versicolor*	Dark green spores with white mycelium at sides.	Conidiophores, colourless, vesicles elongate with metulae and phialides covering most of the surface, conidial heads radiating,conidia globose.
SRw233	*P. corylophilum*	Light green spores, sporulated in 4 days, reverse is pale.	Conidiophores with metulae of unequal length. Conidia subglobose to ellipsoidal, smooth walled.
SRw234	*A. wentii*	Yellow spores, powdery, sporulated within 5 days, dry, reverse is colourless with no pigment.	Conidia ellipsoidal at first but later becoming subglobose smooth to broadly verrucose. Conidiophores long and radiating conidial head.

Contd.....

Table 2.2d. Cultural and morphological characteristics of isolates from saltern sediment

Culture no	Ribander Isolates	Cultural characteristics	Morphological characteristics
SRs237 SRs239 SRs241	*A. versicolor*	Green spores, undulated, sporulated within 5 days, dry, reverse colourless with no pigment.	Conidiophores, colourless, vesicles elongate with metulae and phialides covering most of the surface, conidia globose conidial heads radiating.
SRs243	*A. sydowii*	Light green spores, rough powdery, dry, sporulated within 5 days, dry, reverse colourless with no pigment.	Conidiophores, colourless, vesicles elongate with metulae and phialides covering most of the surface, conidia are echinulate conidial heads radiating.
SRs245	*E. amstelodami*	Yellow aerial spores, dry, sporulated within 5 days, dry, reverse colourles, no pigment.	Cleistothecia yellow, globose, ascospores rough walled with an equatorial line or ferrow, Conidia globose often rough.
SRs247	*A. wentii*	Brown aerial spores, dry, sporulated within 5 days, dry, reverse colourles, no pigment.	Conidia ellipsoidal but later becoming subglobose smooth to broadly verrucose. Conidiophores long with radiating conidial head.
SCs250	*H. werneckii*	Slow growing, spoulated in 6 days, moist slimy black colony with a thin layer of mycelium with age, small oval, reverse black, no pigment.	Septate hyphae, bicellular, yeast like conidia.
SCs252, SCs254	*A.versicolor*	Green spores, dry, sporulated within 5 days, dry, reverse colourles, no pigment.	Conidiophores, colourless, vesicles elongate with metulae and phialides covering most of the surface, conidia globose conidial heads radiating.
SCs256, SCs257	*P.asymmetrica sec fasciculata*	Green powdery spores, dry, sporulates in 5 days, off white orange tinge	Penicilli branched below level of metulae, assymetrical.

Contd.....

Table 2.2d Cultural and morphological characteristics of isolates from salterns*

Culture no	Ribander Isolates	Cultural characteristics	Morphological characteristics
SRw135	*A. terreus*	Brown spores sporulated within 3 days. No pigment	Conidial heads compactly columnar, conidiophores smooth colourless, vescicles hemispherical,metulae present, conidia globose.
SSw167	*A. glaucus*	Arial mycelium, green spores with yellow colour in patches.	Hyphae are septate and hyaline, conidial heads are radiate to loosely columnar.Conidiophores are smooth walled, vescicles are globose cliestothecia are yellow ,globose
SCw182	*A. niger*	Arial mycelium, sporulated within 3 black spores.	Conidial head carbon black, globose, radiate, conidiophores hyaline to brown, vescicles globose, conidia globose to subglobose.

*Isolates previously obtained on 10% S-CzA

Table 2.3. Biochemical tests for *H.werneckii*

	Casein	Tyrosine	Growth at 37°C (PDA)	Growth at 42°C (PDA)	
MCw214	+	+	+	-	
MCw215	+	+	+	-	
SCs250	+	+	+	-	

2.4 Discussion

In this study obligate halophiles and facultative halophiles as well as halotolerant fungi were identified from Dead Sea, Mandovi estuary, mangroves and salterns. The fungal isolation was carried on media containing high concentration of salt to inhibit the growth of the faster-growing terrestrial fungi and thus enhance selection for halophiles.

Due to its unique ecology, the study of the microflora from the Dead Sea has attracted much interest in recent decades. There has been an increased interest towards the presence of filamentous fungi in these waters. The acidic pH of the Dead Sea (Momani *et al.* 2009) would be favourable to fungal life, unlike other hypersaline lakes with neutral or alkaline pH (Oren 2003). Greater diversity amongst the isolates was found in the sediment as compared to water, possibly as a result of different nutrients and salts present in the sediment.

The mycobiota obtained in the Dead Sea could be indigenous as well as of terrestrial source, or could also be by the movement of water at the sea shore, as well as by airborne contamination and/or by human activity (Buchalo 2003). Marine ascomycetes have been shown to have their origin in terrestrial sources (Vijaykrishna *et al.* 2006).

The salinity of the upper water layers of the Dead Sea is reduced as a result of inflow of freshwater. This becomes an important feature for development of mycelium and hence its survival in these waters (Kis-Papo *et al.* 2003b). Thus, fungi are adapted in the respective hypersaline environment. Microbes in the dormant state may return to the physiologically active state under favorable conditions (Kunte *et al.* 2002).

The principal genera obtained from Dead Sea, mangroves, salterns and mandovi estuary were *Aspergillus* and *Penicillium.* At low water potential the active mycota is dominated by species of *Aspergillus* and *Penicillium* and is numerically the most common taxa (Buchalo 2003, Nazareth *et al.* 2011). *Aspergillus* and *Penicillium* were also dominant amongst the isolates obtained from Mandovi estuary, mangroves and salterns of Goa.

Amongst the genera obtained in the Dead Sea, the genera *Penicillium* and *Cladosporium* were found exclusively in the water and *Aspergillus* species was only obtained from sediment. Among the *Aspergillus* species were *A. penicillioides* and *A. versicolor. Cladosporium* species were isolated only on medium containing 10% salt but not in presence of 20% salt, indicating that the genus as a whole is not as extremely halotolerant as the genera of *Aspergillus*, and *Penicillium*; *A. versicolor* was found exclusively in the sediment.

A. penicilloides was present in high density in the sediment in Dead Sea. This could be due to the high salinity and matrix potential and consequently a lower a_w. Only those organisms which could survive such conditions could grow; this accounted for lower diversity in the sediment (Kunte *et al.* 2002). Microorganisms can resist denaturation by salt and are capable of balancing the osmotic pressure in the sediment (Oren 1999a, Das Sarma and Arora 2002). This could be the reason why the sediment showed high population density. Amongst the species obtained, *A. versicolor* and *C. cladosporioides* were native flora of the Dead Sea water (Kis-Papo *et al.* 2003c). The presence of *C. cladosporioides*, *P. steckii* and *P. corylophilum* have also been reported (Buchalo 2003, Grishkan *et al.* 2003, Kis-Papo *et al.* 2003c, Mbata 2008, Wasser *et al.* 2003). *Gymnascella marismortui* from the Dead Sea have been reported as obligate halophiles (Buchalo 2003).

The study on the Mandovi estuary showed a predominance of *Aspergillus* species, with *Penicillium* being less predominant. *Eurotium* and *Cladosporium* were found in fewer numbers. *Penicillium* was isolated from (Auguada Bay) station 1, *A. versicolor* from (Miramar) station 2, *Cladosporium* from (Ribander) station 4. *Eurotium* was found in station 3 (Panaji) and 4 whereas *A. penicillioides* were from station 4 (Ribander) and 2 (Miramar). There were great variations between the species composition from station 1 to station 4 indicating a change in fungal communities from the freshwater to marine environments. Fluctuations in river discharge and mixing processes are the major factors that affect the dispersion of microfungi in the estuary (Cooke *et al.* 1980). Rivers eventually flow into the sea and freshwater fungi are likely to be transported with woody substrata into the sea. Fungi have been examined along a gradient from salinity range of 10‰-35‰.

The study of mangroves and salterns revealed a predominance of *Aspergillus* species, with *Penicillium* also in fairly high proportion and the telemorphic form *Eurotium* as well as the black yeast *Hortaea werneckii* were obtained in fewer numbers. At lower salinities, dematiaceous or melanized fungi occur occasionally and are replaced by moniliaceous fungi. Recent evidence indicates that some species are capable of growing and reproducing in hypersaline environment (Gunde-Cimerman *et al.* 2004).

The black yeast *H. werneckii* was found in both mangroves as well as salterns; most of the *Eurotium* species and *A. penicillioides* were found in the mangroves while most of the *Penicillium* species were from salterns and were isolated only from the water samples. *A. flavipes, A. terreus* were exclusive to mangrove sediment samples. *A. flavus, A. wentii* and *A. sydowii* were exclusive to salterns, all isolated from the sediment, and *A. wentii* found

also in the brine. The presence of *Eurotium* species corroborates the finding on the genus as part of the indigenous fungal community in hypersaline waters of salterns (Butinar *et al.* 2005b) the results here further reveal their presence also in the mangroves. The genus *Eurotium* is regarded as xerophilic, and its representatives have been reported to live in concentrated salt or sugar solutions at water activities as low as 0.70 (Lacey and Magan 1991). Some species of *Aspergillus* and *Penicillium* grow at above 25% NaCl (Tresner *et al.* 1971).

Most of the fungi that can be found in the extreme environments belong to the imperfect stage of the Ascomycota, which have been reported in mangroves, saline soils, marine sediments, sea water, salt marshes, and sand dunes (Guiraud *et al.* 1995, Moubasher *et al.* 1990, Newell 1996, Díaz-Muñoz and Montalvo-Rodríguez 2005). Marbaniang and Nazareth (2006) reported the isolation of slight halophilic *Penicillium species* with maximum salt tolerance of 17.5%, from mangroves and salterns in Panaji, Goa. *A. flavipes; A. flavus; A. sydowii;* was also isolated from sediments of Chapora and Mandovi rivers in Goa (D'Souza *et al.* 1979). *A. flavipes, A. flavus, A. ochraceus, A. penicillioides, A. sydowi, A. terreus, A. versicolor, A. wentii, E. amstelodami* and *P. chrysogenum* were the common isolates identified. However, these have not been identified as halophiles.

Mangicolous fungi have been isolated along the South-West Coast; from Honnavar (Karnataka) and Panaji (Goa) mangroves (Maria and Shridhar 2002) and West Coast of India (Sarma *et al.* 2001), the Sunderbans (Rai and Chowdhery 1978) and the Bay of Bengal region (Vittal and Sarma, 2006). However, no work has been recorded on the diversity of halophilic fungi from mangroves and salterns of Goa, India.

Wide range of fungi occurs in the mangrove ecosystem although these differ as to their location and some fungi occur more frequently than others (Hyde and Jones 1988). Many factors such as salinity, temperature, availibility and diversity of substrata, quantity of propagules in the water, the nutrient status of the water and host specificity appear to have an effect on species occurance (Jones and Alias 1997).

A. candidus, A. melleus, A. niger, A. ochraceous, A. restrictus, Chaetomium globosum, Cladosporiun spp, H. werneckii, Myrithecium roridum, P. citrinum and *P. chrysogenum* were isolated from Cabo Rojo saltern and have been reported in other hypersaline waters from temperate to tropical regions isolated (Kis papo *et al.* 2001, 2003a, Gunde-Cimmerman *et al.* 2004, Butinar *et al.* 2005a). Gunde-Cimerman *et al.* 2000 isolated *H. werneckii* along with some other Black yeasts and *Cladosporium* from Secorlje saltern situated at the southeast part of the Piran bay.

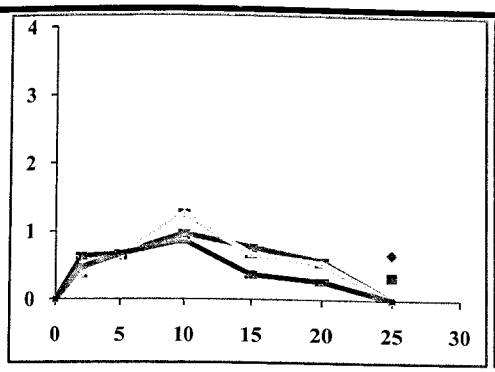
It appears therefore that a hypersaline condition is not necessarily an indication of the existence of a high number of obligate halophilic fungi more than that of low saline environments.

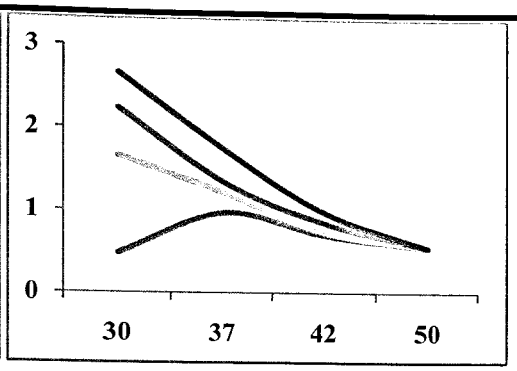
2.5 Conclusion

The study revealed a diversity of halophilic fungi in sediment and water. Dead Sea water yielded *A. penicillioides, P. corylophilum, P. steckii* on CzA+20% salt whereas *C. cladosporioides* was isolated on CzA+10% salt. Among the sediment sample, *A. penicillioides, A. versicolor, P. corylophilum, P. steckii* were isolated on CzA+20% salt whereas *A. penicillioides, A. versicolor* was isolated from CzA+10%.

Isolates obtained from salterns at Ribander, Sirdao and Santa Cruz isolated on CzA+10% salt grew faster than those isolates obtained on CzA+20% salt from mangroves and saltern. *Eurotium* was not isolated from the Dead Sea sample. *Aspergillus, Penicillium, H. werneckii* and *Eurotium* were isolated from mangroves and salterns. *H. werneckii* was neither isolated from the Dead Sea nor from the Mandovi estuary.

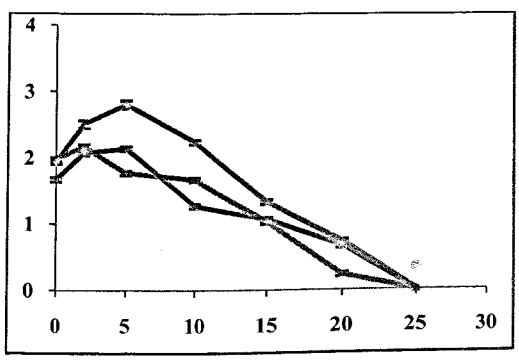
Although having low salinity many obligate halophiles were isolated from the Mandovi estuary. *H. werneckii, E. repens, E. amstelodami, P. corylophilum, P. chrysogenum, P. steckii* were facultative halophiles.

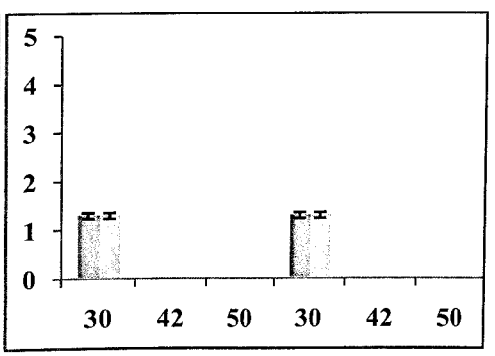




CHAPTER 3

TOLERANCE OF HALOPHILIC FUNGI TO SALT AND HEAT





CONTENTS

CHAPTER 3: TOLERANCE OF HALOPHILIC FUNGI TO SALT AND HEAT

3.1 Introduction

This chapter focuses on the halotolerance and heat tolerance of the isolates from Dead Sea, Mandovi estuary, mangrove and salterns.

Fungi are heterotrophic eukaryotes which exhibit a great diversity in morphology and distribution. Some species can survive at an elevated temperature of hot springs, in the high salinity of sea water or in various other adverse environments.

Fungi have the ability to tolerate varying temperature. When thermal stress is applied, the most prominent physiological reactions are the production of a set of novel proteins or an increase in the quantity of certain proteins. This will change the structure or metabolism of the organism by acting as elicitors. As a result gene expression will be affected and result in the synthesis of stress-specific compounds to protect the organisms. Under conditions where the temperature exceeds the normal growth range, cells experience stress due to the damaging effect of heat on intracellular molecules, such as heat sensitive enzymes, and on cell membranes.

Halophiles can be classified as slight, moderate and borderline extreme halophiles, extreme halophiles, requiring salt concentrations of 0.2 to 0.5M, 0.5 to 2.5 M, 1.5 to 4.0M and 2.5 to 5.2M NaCl, respectively, non halophiles being those that grow at a concentration of less than 0.2M (Kushner 1978). Facultative halophiles are organisms that grow even in the absence of added salt; organisms which have an absolute requirement for salt added to the medium are termed as obligate halophiles. Microorganisms can proliferate in a diverse range of saline concentrations from low saline environments such as freshwater lakes to saturated brines

found in solar salterns. Cell-size and intracellular water activity must remain relatively constant to maintain physiological processes.

In the summer, sun-heated bodies of water, mud, margins of ponds, and springs in temperate latitudes are at temperatures above 40°C for fairly long periods of time (Deacon and Minckley 1974). Thermophilic fungi do occur in these places. Seasonality of occurrence and abundance of particular species of thermophilic fungi in soil is known (Tansey and Jack 1976). Mesophiles are the organisms that grow best in moderate temperature which are neither too hot nor too cold, typically between 25°C and 40°C.

The present study describes the salt tolerance and heat tolerance of the isolates from Dead Sea (water and sediment), Ribander (Salterns and Mangrove), Santa Cruz (Mangrove and Salterns), Mandovi (surface water, bottom water and sediment).

3.2 Materials and methods

3.2.1 Halotolerance test

Spore suspension containing 10^3 spores was made in 2% saline + 0.05% Tween 80. This was inoculated on CzA containing solar salt (0-30%). and incubated at 30°C. These were then sub-cultured by inoculating 10^3 spores in triplicate on plates containing corresponding salt concentrations. Growth was measured in terms of colony diameter, on 7^{th} day, or on 15^{th} day for slow-growing cultures. Statistically significant difference ($P<0.05$) in the effect of different salt concentrations on growth of all isolates, as well as in growth within the species, was analysed by two-way ANOVA.

Selected isolates as representatives of the obligate halophiles DSw35, MRw202 and of black yeast *H. werneckii* MCw215 were identified by ITS sequence analysis from Merck-GeNei Services, and the GenBank accession number of the obligate isolate and facultative halophile was obtained.

Alignment and phylogenetic tree were constructed in Clustal X version 2 and the NJ distance method.

To confirm its obligate halophilic nature the obligate halophiles *A. penicillioides* were grown on different media PDA (Potato Dextrose Agar), MEA (Malt extract agar), CzA, SA Sabourads agar with 10% salt and without salt.

3.2.2 Screening for Heat tolerance

Spore suspension was prepared in 2% saline + 0.05% Tween 80 and was spot-inoculated on 2 sets of plates of CzA containing salt concentrations required for optimal growth (CzA-So); one set was incubated at 42°C and one set at 50°C. Growth was measured in terms of colony diameter, on 7th days.

3.2.2.1 Heat tolerance

3.2.2.1A Spore germination and growth of cultures at elevated temperatures

Cultures which could grow at 42°C / 50°C were re-grown by spot-inoculating 10^3 spores in triplicates on 4 sets of CzA-So, each set incubated at 30°C, 37°C, 42°C, 50°C. The colony diameter was recorded on the 7th day. Statistically significant difference ($P<0.05$) of the isolates at increased temperature was analysed by two-way ANOVA.

3.2.2.1B Growth of germinated spores at elevated temperatures

Cultures which could not grow at 42°C / 50°C, were inoculated as above and incubated at 30°C to allow spore germination, and then at 42°C and 50°C . The period for germination was determined after every three hours by removing agar plugs, staining it with lactophenol cotton blue stain and then observing it under the microscope. The sets were then incubated at 42°C and 50°C and observed for growth; results were recorded on the 7th day. Statistically

significant difference ($P<0.05$) of the isolates on germinated and ungerminated spores was analysed by two-way ANOVA.

The isolates were selected for the further studies based on the salt and heat tolerance and variation in species.

3.2.3 Effect of salt on growth rate

Spore suspension was made as above; spot inoculated on plates of CzA+ 0%, 2%, 5%, 15% solar salt and were incubated at 30°C. The diameter of the culture was noted day-wise from 4^{th} day for the facultative halophiles and from 7^{th} day for the obligate halophiles, for 4 succeeding days. The linear regression of the increase in radius versus incubation time (in days) was used to obtain the growth rates.

3.2.4 Effect of heat on growth rate

Spore suspension was made as above and spot inoculated on plates of CzA-So and incubated at 30°C, 37°C, 42°C and 50°C. The growth of the culture was noted as above and the linear regression of increase in radius versus incubation time (in days) was used to obtain the growth rates.

Statistically significant difference ($P<0.05$) of growth rate at varying salt concentrations and temperature on growth of all isolates was analysed by two-way ANOVA.

3.3 Results

3.3.1 Halotolerance levels

Salt tolerance curves of the isolates from Dead Sea, Mandovi estuary, mangroves and salterns are shown in Fig. 3.1a, 3.1b, 3.1c, 3.1d respectively.

3.3.1.1 Dead Sea

Among the isolates from Dead Sea water sample, *A. penicillioides* DSw1, DSw5, DSw17 had a range of 2%-20% salt with optimum growth at 10% salt; DSw5 grew at 25% salt in 15days. *P. steckii* DSw7, DSw21 with the optimum growth at 5% and 2% salt,respectively had a range of 0%-20% salt. *P. corylophilium* DSw4, DSw8, DSw11, DSw18 and DSw19 grew optimally at 5% salt with a salt tolerance range of 0%-20%. *P. corylophilium* DSw13 grew optimally at 2% and had a salt tolerance range of 0%-20%. *C. cladosporioides* DSw23 had a salt tolerance range 0%-15% and grew on 20% salt in 15 days and DSw25 had a salt tolerance range of 0-20% with optimum growth at 5% salt. All *A. penicillioides* showed optimal growth at salt concentration of 10%.

Among the sediment isolates, *A. versicolor* DSs59, DSs61 grew optimally at 0% salt and had at a range of 0%-20% salt, *A. versicolor* DSs57 with a salt tolerance range of 0%-20% had an optimum growth at 10% salt. All the *A. penicillioides* DSw49, DSw35, DSw41, DSw43, DSw51 grew optimally at 10% salt with a range of 5%-20% salt; DSw29, DSw31, DSw33, DSw37, DSw43, DSw45, DSw47, DSw51, DSw55 had a salt tolerance range of 2%-20% and had an optimum growth at 10% salt. DSw49, DSw35, DSw39 grew at 2% salt

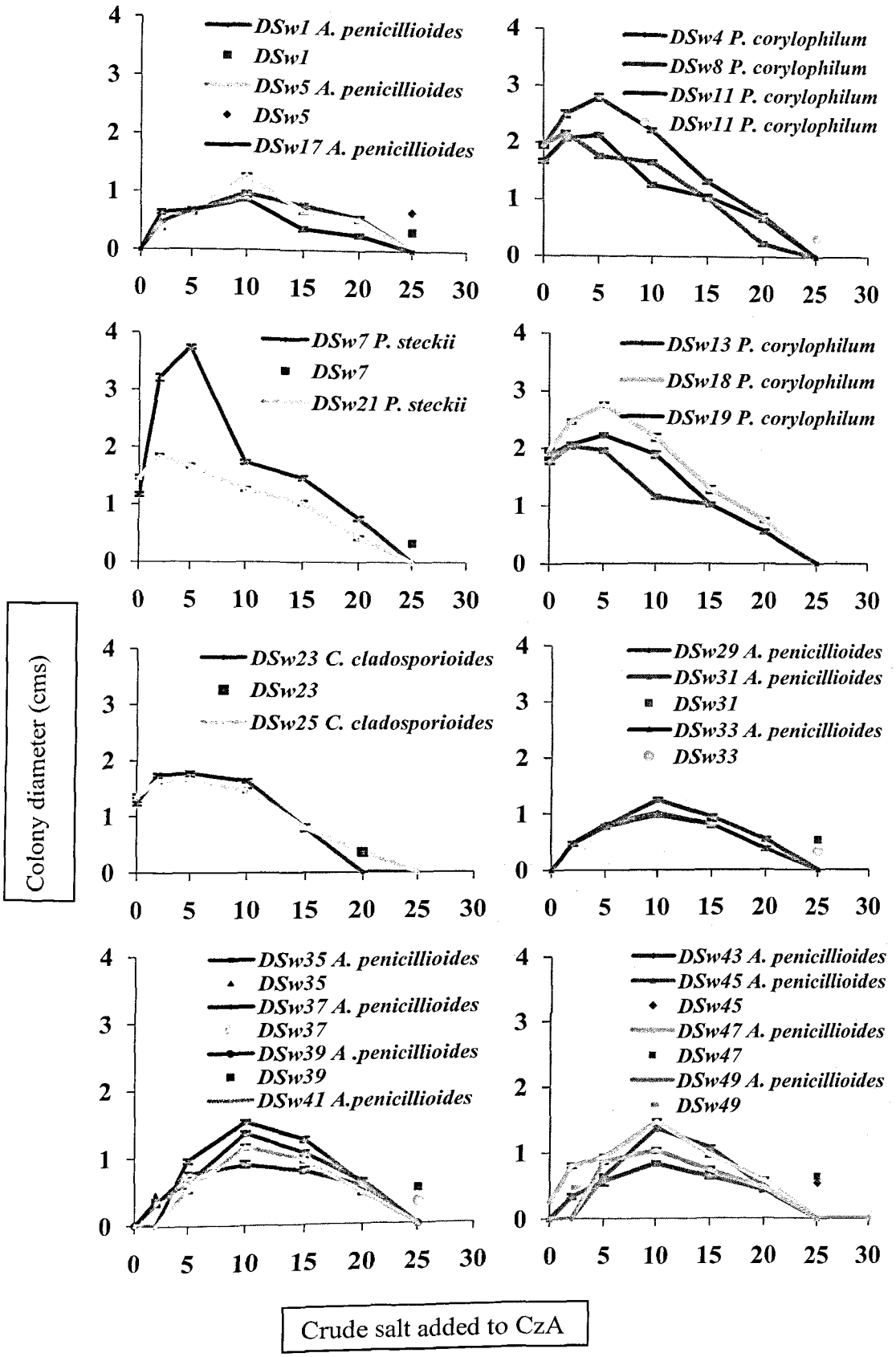


Fig. 3.1a. Salt tolerance curves of isolates from Dead Sea as recorded after 7d incubation; unconnected points indicate delayed growth at respective salt concentrations, after 15d incubation.

Contd.....

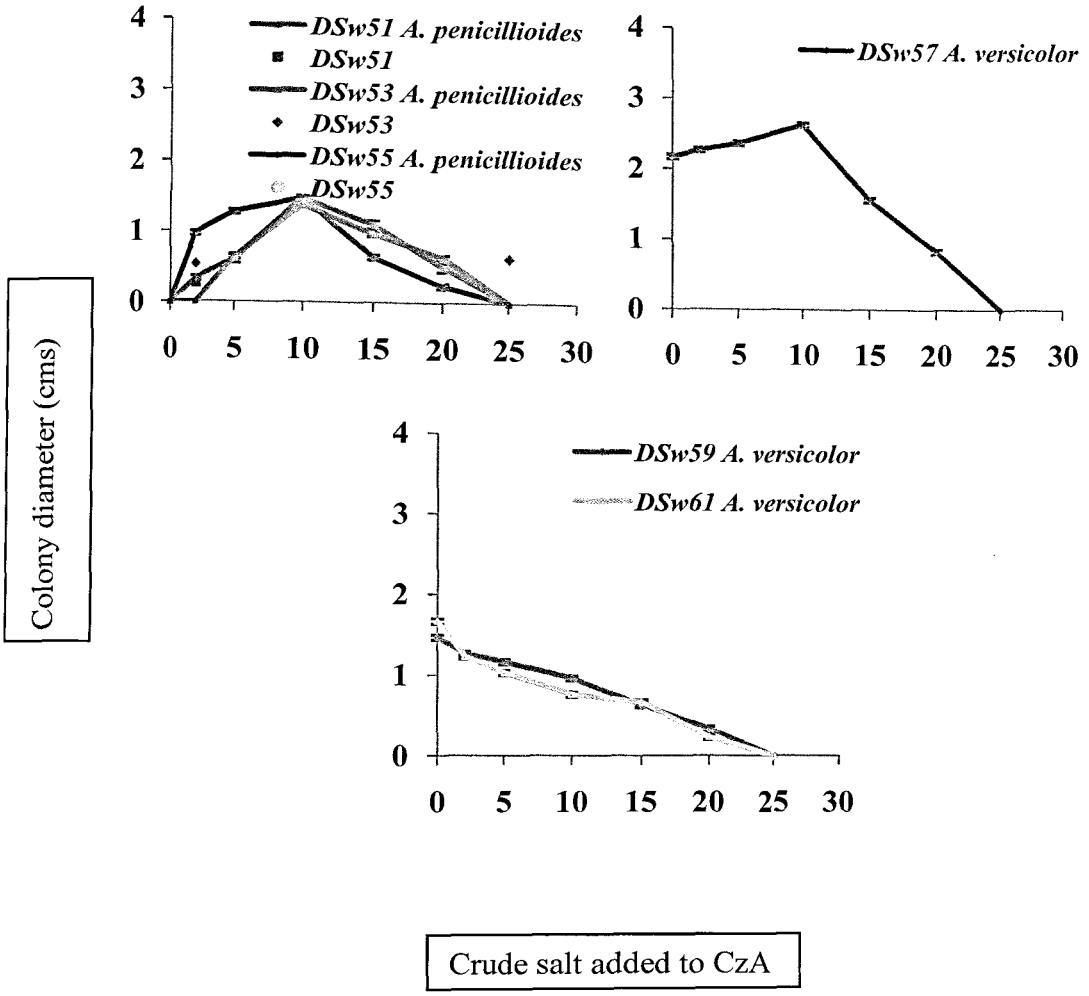


Fig. 3.1a. Salt tolerance curves of isolates from Dead Sea as recorded after 7d incubation; unconnected points indicate delayed growth at respective salt concentrations, after 15d incubation.

in 15 days, DSw31, DSw33, DSw39, DSw41, DSw53 grew at 25% salt in 15 days. DSw55 grew at 2% salt in 15 days

3.3.1.2 Mandovi

A. penicillioides EM4wt118, EM2wt107, EM4wt119, EM4wt120 with a range of 2%-20% salt, *A. penicillioides* EM4wb121 with range of 5%-25% salt, *A. penicillioides* EM2s112, EM4wb123 with range 2%-25% salt had optimum growth at 10% salt. *C. cladosporioides* EM4wb122 and EM4s128 and *A. versicolor* EM2wt105, EM2wt106 with optimum growth at 2% salt had a salt tolerance range of 0%-20% and 0%-25% respectively. *A. versicolor* EM2wt104 EM2s110 and EM2wb108, had a salt tolerance range of 0-20% with an optimum growth of 2% salt. EM2wb108 had an optimum growth at 5% salt. EM2wb108, EM2s110 grew at 25% salt in 15 days. *E. repens* EM4s127 and *E. amstelodami* EM4wb124 had a range of 0%-20% salt and grew optimally at 5% salt. They grew on 25% salt in 15days. *E. repens* EM4s126 and EM3s116 had an optimum growth at 10% and 5% salt respectively with a range of 0%-25% salt. *P. steckii* EM1wt102, EM3wt113 grew at 25% salt in 15 days having an optimum growth at 5% salt and had a salt tolerance range of 0%-20%. *P. steckii* EM3s115, EM4wt117 and *P. chrysogenum* EM1s103 had a salt tolerance range 0%-25% and 0%-20% respectively and grew optimally at 2% salt. *P. chrysogenum* EM3wb114 grew optimally at 5% salt and grew on 25% salt in 15 days. *P. canescens* EM1wb101 grew at a range of 0%-20% salt and at 25% salt in 15 days, it grew optimally at 2% salt. *P. corylophilum* EM2s109 grew optimally at 10% salt and had a range of 0%-25% salt.

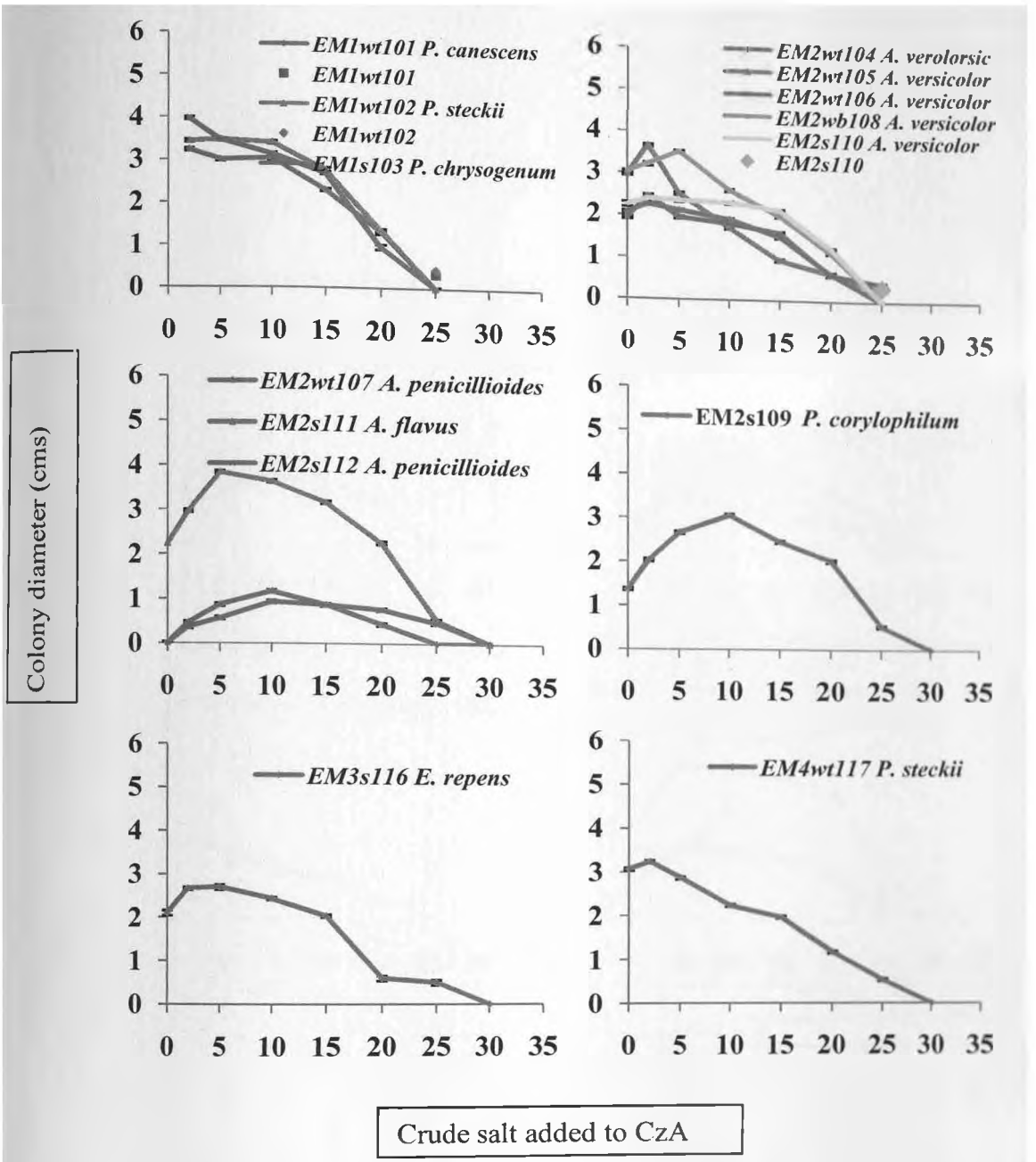


Fig. 3.1b. Salt tolerance curves of isolates from Mandovi estuary as recorded after 7d incubation; unconnected points indicate delayed growth at respective salt concentrations, after 15d incubation.

Contd.....

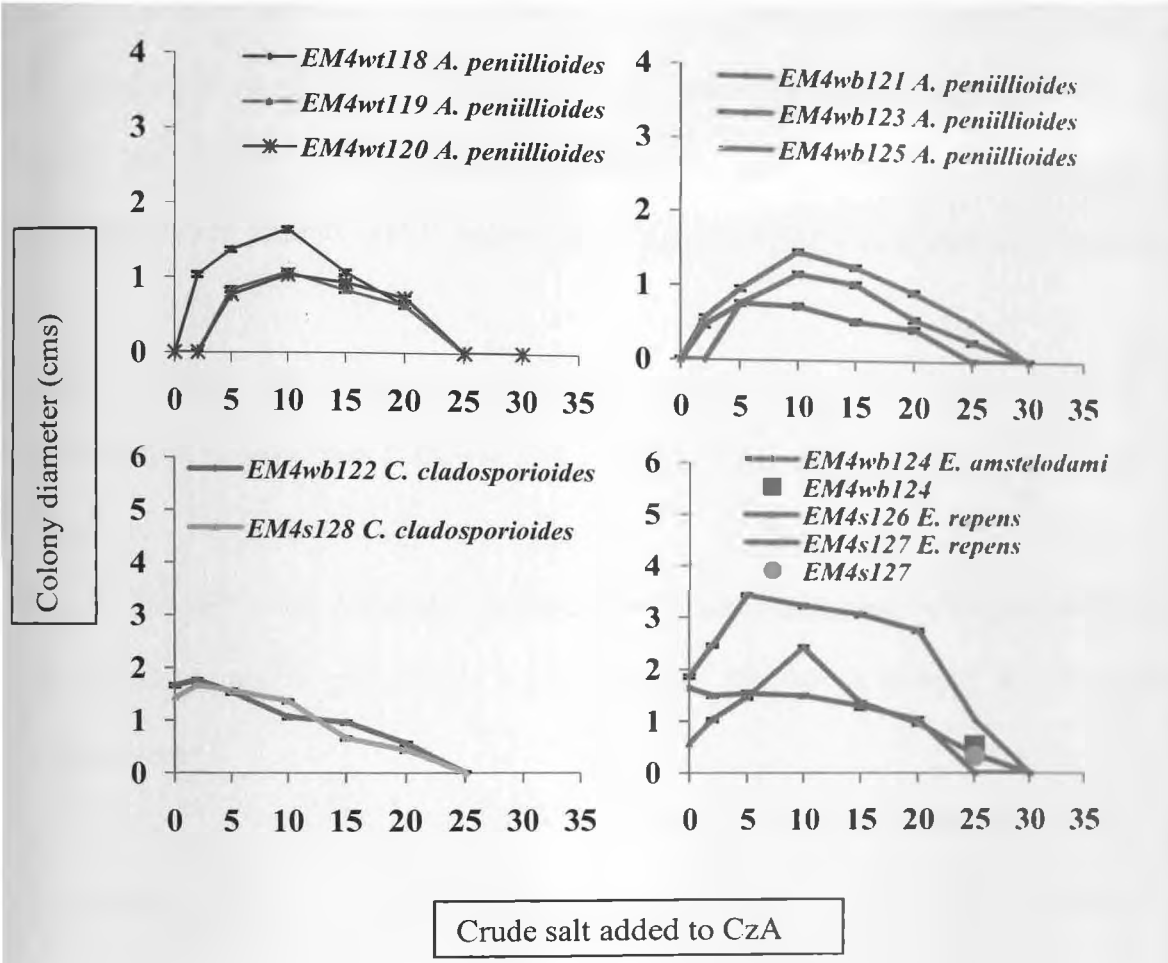


Fig. 3.1b. Salt tolerance curves of isolates from Mandovi estuary as recorded after 7d incubation; unconnected points indicate delayed growth at respective salt concentrations, after 15d incubation.

3.3.1.3 Mangroves

Most of the cultures grew in the presence of 0%-20% salt within 7 days, with optimal growth at salt concentrations of 2% / 5% / 10% salt as shown:, *A. terreus var terreus* MCs223, had optimum growth at 2% salt; *A. flavipes* MCs219, optimum growth at 2% - 5% salt and, *A. sydowii* SRs 243, *E. amstelodami* MRw211 and *P. corylophilum* MRw205 all growing optimally at 5% salt and *E. repens* MRw210, MRw212 with optimum at 10% salt.

Some isolates grew at the range of 0%-25% salt in 7 days, as in *H. werneckii* MCw215, MCw214, optimum growth at 10% salt and *E. amstelodami* MCs221, optimum growth at 10 % salt.

A. penicillioides MRw202, MRw203, MRw208 required a minimum of 2% added salt for growth and could grow in presence of up to 20% salt. All showed optimal growth at 10% salt concentration.

3.3.1.4 Salterns

Most of the cultures grew in the presence of 0%-20% salt within 7 days, with optimal growth at salt concentrations of 2% / 5% / 10% salt as shown:, *A. versicolor* SRw235 and SRs239, *A. wentii* SRs247, *P. asymmetrica sec fasciculata* SCw256 and *P. corylophilum* SRw233, had optimum growth at 2% salt; *A. sydowii* SRs243, *A. versicolor* SRw230, SRs237, SCw252 and SCw254, *A. wentii* SRw234, *P. asymmetrica sec fasciculata* SCw257, *P. chrysogenum* SRw225 and SRw227 and *P. griseofulvum* SRw228, all growing optimally at 5% salt.

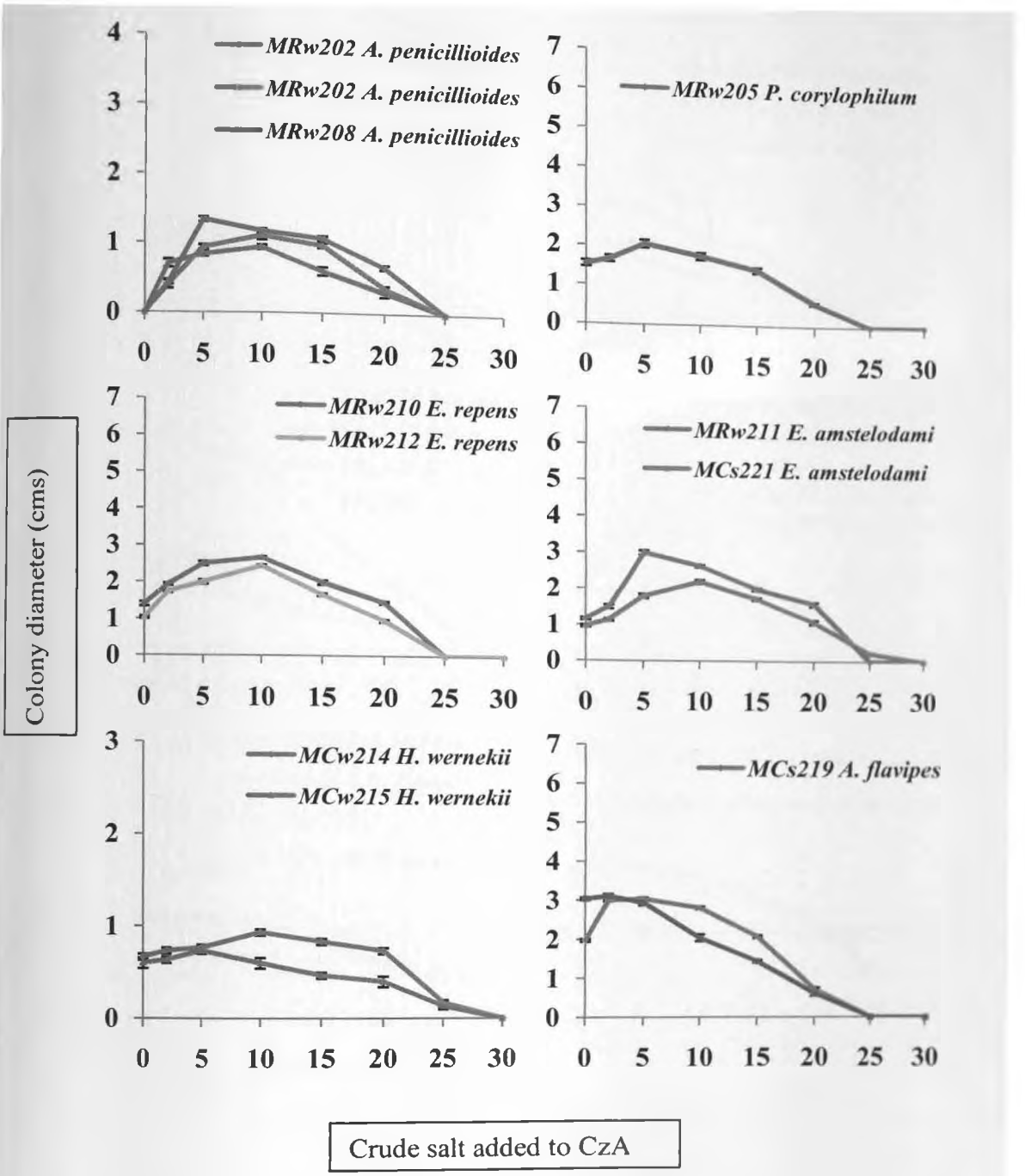


Fig. 3.1c Salt tolerance curves of isolates from mangroves as recorded after 7d incubation; unconnected points indicate delayed growth at respective salt concentrations, after 15d incubation.

Contd.....

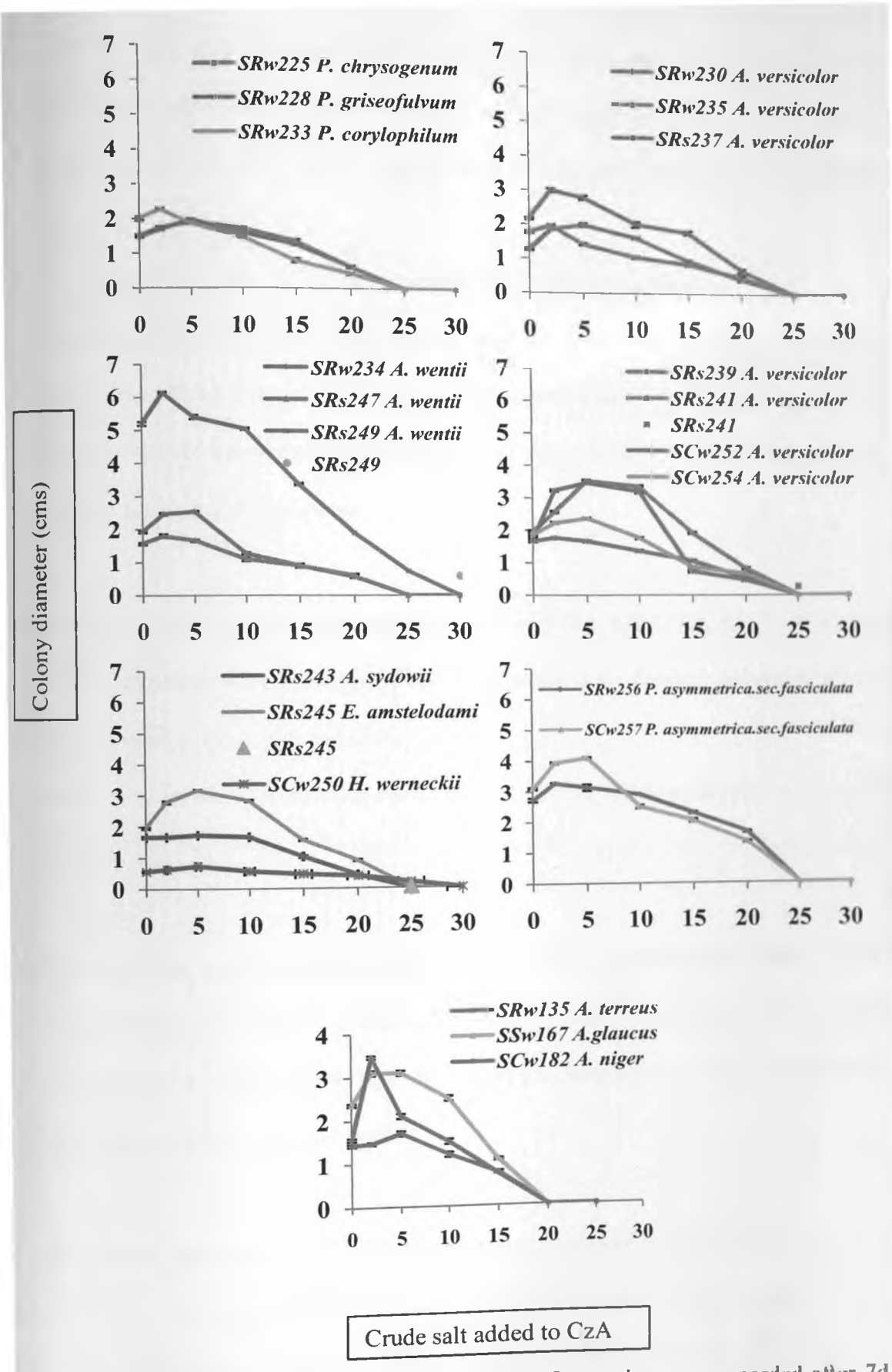


Fig. 3.1d Salt tolerance curves of isolates from salterns as recorded after 7d incubation; unconnected points indicate delayed growth at respective salt concentrations, after 15d incubation.

Some isolates were able to grow in the presence of 25% salt in 15 days, namely *A. versicolor* SRs241 and *E. amstelodami* SRs245, all with optimal growth at 5% salt, some grew at the range of 0% - 25% salt in 7 days, as in *H. werneckii*, SCw250 with optimum at 5% salt.

SCw182, SSw167 and SRw135 isolated on 10% CzA grew at a range of 0%-20% salt with an optimum growth at 5% salt, SRw135 had an optimum growth at 2% salt.

The isolates from Dead Sea, Mandovi estuary, mangrove and saltern were categorised as obligate or facultative halophiles, or as halotolerant respectively in Table 3.1a, 3.1b, 3.1c, 3.1d based on the above observations.

As shown in Fig 3.2 *A. penicillioides* did not grow on PDA, MEA, SA and CzA in absence of salt. With optimum concentration of salt *A. penicillioides* showed enhanced growth in MEA as compared to PDA, SA and CzA.

The ANOVA values were significantly different for varying salt concentrations. P value is less than 0.05.

Identification of the selected representative isolates of *A. penicillioides* DSw35, MRw202 and *Hortaea werneckii* MCw215 was confirmed by ITS sequence analysis, GenBank Accession No. HQ702384, HQ891824 and HQ71162 respectively. Fig. 3.3 indicates the phylogenetic relationship of these isolates.

All the obligate halophiles *A. penicillioides* from the Dead Sea, Mandovi estuary and mangroves were morphologically as well as culturally similar to DSw35 and MRw202. MCw214 from Mangrove and SCw250 from salterns also showed colony and morphological similarity with MCw215.

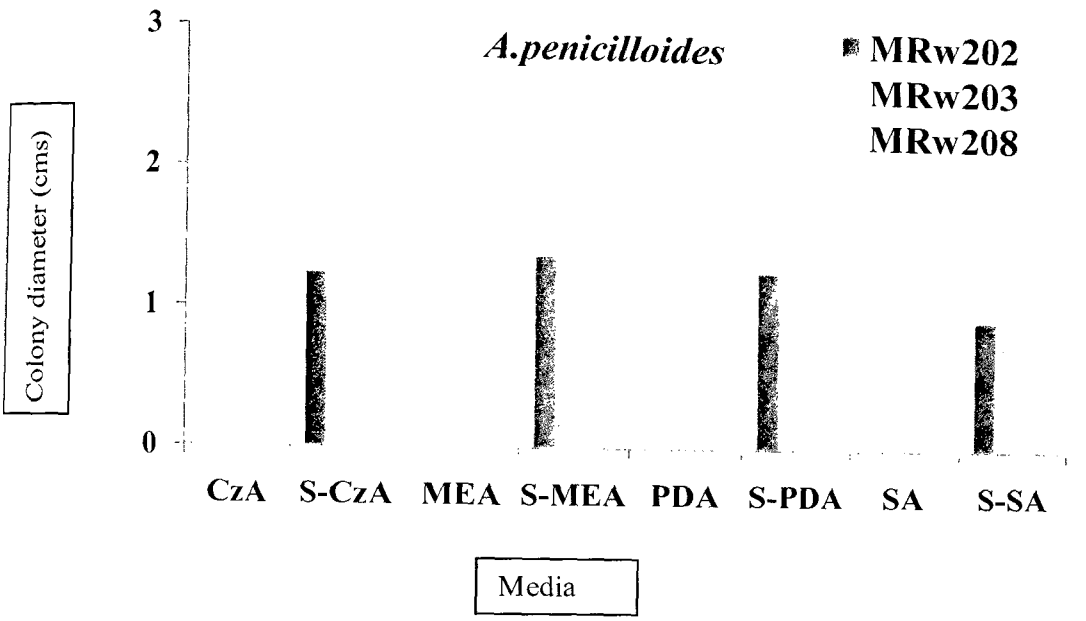


Fig 3.2 Growth of *A. penicillioides* on different media

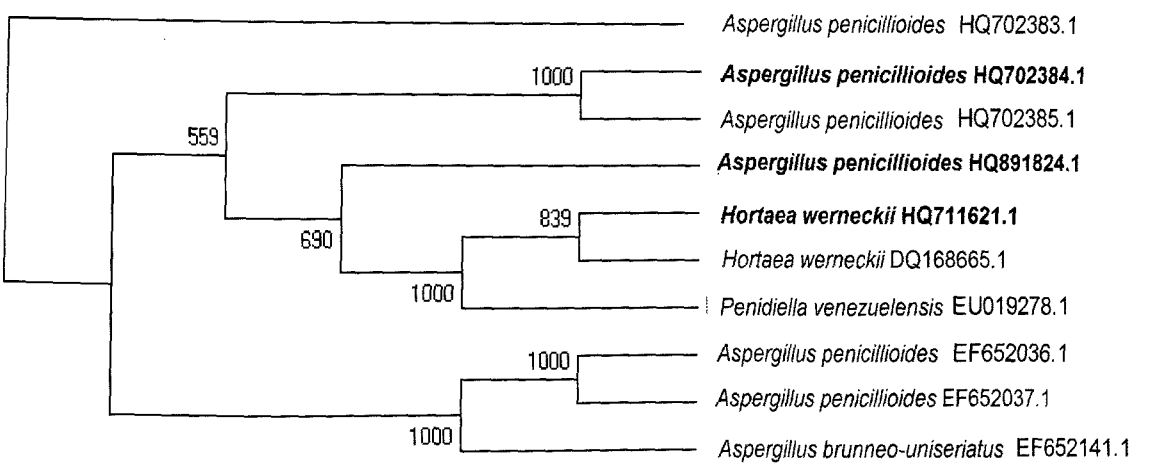


Fig. 3.3 Phylogenetic tree obtained from the alignment of ITS region of rDNA of *Aspergillus* species and *H.werneckii*. DSs35 *A. penicillioides* HQ702384.1, MRw202 *A. penicillioides* HQ891824.1 and MCw215 *H.werneckii* HQ711621.1 are the isolates obtained in the present study

Table 3.1a Obligate and facultative halophiles from Dead Sea water and sediment samples isolated on Czapek Dox Agar with 10% or 20% salt.

Isolate		Salt levels supporting growth		Halophilic nature
Species	Number	Range (%)	Optimal(%)	
A. penicillioides	DSw1, DSw17	2%-20%	10%	Obligate
	DSw5	2%-20%(25%)	10%	Obligate
C. cladosporioides	DSw25	0%-20%	5%	Facultative
	DSw23	0%-15%(20%)	5%	Facultative
P. corylophilum	DSw13	0% -20%	2%	Facultative
	DSw11, DSw4, DSw8 DSw18, DSw19	0%-20%	5%	Facultative
P. steckii	DSw21	0%-20%	2%	Facultative
	DSw7	0%-20%	5%	Facultative
A. penicillioides	DSs29, DSs35, DSs37, DSs55	2%-20%	10%	
	DSs31, DSs33, DSs39 DSs45	2%-20%(25%)	10%	Obligate
	DSs43	5%-20%	10%	Obligate
	DSs 41, DSs53	5%-20%(25%)	10%	Obligate
	DSs49	5%-20%(2%)	10%	Obligate
	DSs 51, DSs47	0%-20%	10%	Facultative
A. versicolor	DSs57	0%-20%	10%	Facultative
	DSs59	0%-20%	0%	Halotolerant
	DSs 61	0%-20%	0%	Halotolerant

*Salt tolerance of isolates recorded after 7d incubation; data in brackets indicate delayed growth at the respective salt concentration, recorded after 15d incubation

Table 3.1b Obligate and facultative halophiles from Mandovi estuary samples isolated on Czapek Dox Agar with 20% salt.

Isolate		Salt levels supporting growth		Halophilic nature
Species	Number	Range (%)	Optimal(%)	
A. flavus	EM2s111	0%-25%	5%	Facultative
A. penicillioides	EM2wb107 EM4wt118 EM4wt119 EM4wt120	2%-20%	10%	Obligate
	EM2s112, EM4wb123	2%-25%	10%	Obligate
	EM4wb125	2%-20%	5%	Obligate
	EM4wb121	5%-25%	10%	Obligate
A. versicolor	EM2wt105 EM2wt 106	0%-25%	2%	Facultative
	EM2wt104 EM2s110	0%-20%	0%/2%	Facultative
	EM2wb108	0%-20%(25%)	5%	Facultative
C. cladosporioides	EM4wb122 EM4s128	0%-20%	2%	Facultative
E. amestelodami	EM4wb124	0%-20%(25%)	5%	Facultative
E. repens	EM3s116	0%-25%	5%	Facultative
	EM4s126	0%-25%	10%	Facultative
	EM4s127	0%-20%(25%)	5%	Facultative
P. canescens	EM1wb101	0%-20%(25%)	2%	Facultative
P. chrysogenum	EM1s103	0%-20%	2%	Facultative
P. corylophilum	EM3wb114	0% - 20%(25%)	5%	Facultative
	EM2s109	0%-25%	10%	Facultative
P. steckii	EM1wb102 EM3wt113	0%-20%(25%)	5%	Facultative
	EM3s115 EM4wt117	0%-25%	2%	Facultative

*Salt tolerance of isolates recorded after 7d incubation; data in brackets indicate delayed growth at the respective salt concentration, recorded after 15d incubation

Table 3.1c Obligate and facultative halophiles from mangroves isolated on Czapek Dox Agar with 20% salt

Isolate		Salt levels supporting growth		Halophilic nature
Species	Number	Range (%)	Optimal(%)	
A. flavipes	MCs219	0%-20%	2%/5%	Facultative
A. penicillioides	MRw202, MRw203, MRw208	2%-20%	10%	Obligate
A. terreus var terreus	MCs223	0%-20%	2%	Facultative
H.werneckii	MCw214	0%-25%	10%	Facultative
	MCw215	0%-25%	5%	Facultative
E. amstelodami	MRw211	0%-20%	5%	Facultative
	MCs221	0%-25%	10%	Facultative
E. repens	MRw210, MRw212	0%-20%	10%	Facultative
P. corylophilum	MRw205	0%-20%	5%	Facultative

Table 3.1d Obligate and facultative halophiles fungi from salterns isolated on Czapek Dox Agar with 20% salt

Isolate		Salt levels supporting growth		Halophilic nature
Species	Number	Range (%)	Optimal(%)	
A. sydowii	SRs243	0%-20%	5%	Facultative
A. versicolor	SRw230, SCw254	0%-20%	5%	Facultative
	SRw235 ,SRs239	0%-20%	2%	Facultative
	SRs237	0%-20%	5%	Facultative
	SRs241	0%-20%(25%)	5%	Facultative
	SCs252	0%-20%	5%	Facultative
A. wentii	SRw234	0%-20%	5%	Facultative
	SRs247	0%-20%	2%	Facultative
H. werneckii	SCw250	0%-25%	5%	Facultative
E. amstelodami	SRs245	0%-20%(25%)	5%	Facultative
P. asymmetrica sec fasciculata	SCw256	0%-20%	2%	Facultative
	SCw257	0%-20%	5%	Facultative
P. chrysogenum	SRw225	0%-20%	5%	Facultative
P. corylophilum	SRw233	0%-20%	2%	Facultative
P. griseofulvum	SRw228	0%-20%	5%	Facultative

*Salt tolerance of isolates recorded after 7d incubation; data in brackets indicate delayed growth at the respective salt concentration, recorded after 15d incubation

3.3.2 Heat tolerance

Screening of the isolates from Dead Sea, Mandovi estuary, mangroves and salterns for growth at 42°C and 50°C is shown in Table 3.2a, 3.2b, 3.2c, 3.2d respectively. Most of the aspergilli and penicilli grew at selected temperature of 25°C, 30°C and 37°C but not at 42°C; *C. cladosporioides* and *H. werneckii* did not grow even at 37°C. However a few aspergilli and penicilli could grow even at 42°C.

The heat tolerance curves of the isolates from Dead Sea, Mandovi estuary, mangroves and saltern are shown in Fig. 3.4a, 3.4b, 3.4c, 3.4d respectively which grew at 42°C and some of the isolates which grew at 37°C.

3.3.2.1A Dead Sea

Isolates from water sample had the heat tolerance range as follows-: *A. penicillioides* DSw1, DSw5, DSw17 grew optimally at 30°C and had the heat tolerance range from 25°C-37°C. *P. corylophilum* DSw4, DSw8 DSw13, DSw11, and DSw19 had the heat tolerance range from 25°C-42°C all with optimum growth at 30°C. *P. corylophilum* DSw18, *P. steckii* DSw7 and DSw21 with an optimum growth at 30°C had a heat tolerance range of 25°C-37°C. *C. cladosporioides* DSw23 and DSw25 with an optimum growth at 25°C had a heat tolerance range at 25°C-30°C. Isolates from sediment sample had the heat tolerance range as follows-: *A. versicolor* DSs57 grew optimally at 30°C and had a heat tolerance of 25°C-42°C. *A. penicillioides* DSs29, DSs31, DSs33, DSs35, DSs37, DSs39, DSs41, DSs43, DSs45, DSs47, DSs49, DSs51, DSs53, Dss55 and *A. versicolor* DSs59 and DSs61 grew optimally at 30°C with heat tolerance range of 25°C-37 °C.

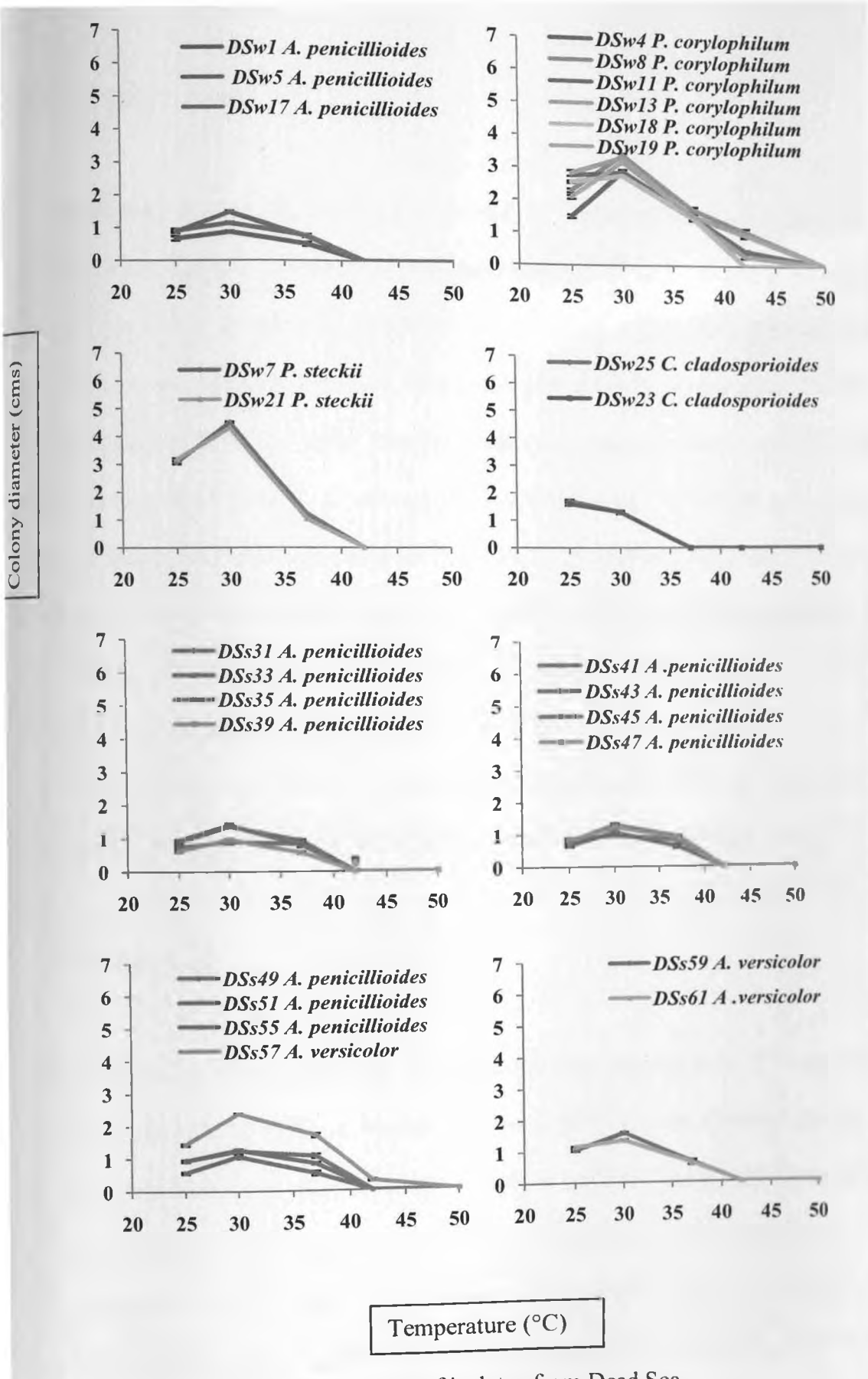


Fig. 3.4a. Heat tolerance curves of isolates from Dead Sea

3.3.2.2A Mandovi estuary

A. penicillioides EM2wt107, EM2s112, EM4wt118, EM4wt119, EM4wt120, EM4wt121 EM4wb123 and EM4wb125 had a heat tolerance range of 25°C-37°C and grew optimally at 30°C. *A. versicolor* EM2wt104, EM2wt105, EM2wt106 with a heat tolerance range of 25°C-37°C, *A. versicolor* EM2wb108, EM2s110 with a heat tolerance range of 25°C-42°C grew optimally at 30°C. *A. flavus* EM2s111 with an optimum growth at 30°C had a heat tolerance range of 25°C-42°C. *C. cladosporioides* EM4wb122, EM4s128 grew optimally at 25°C and had a heat tolerance range of 25°C-30°C. *E. repens* EM4s126, *E. amstelodami* EM3s116, EM4s127, EM4wb124 and *P. corylophilum* EM3wb114 grew optimally at 30°C with a heat tolerance range of 25°C-37°C. *P. canescens* EM1wb101, *P. chrysogenum* EM1s103 *P. corylophilum* EM2s109 and *P. steckii* EM1wb102, EM3wt113, EM3s115 had a heat tolerance range of 25°C-42°C with the optimum growth at 30°C. *P. steckii* EM4wt117 with an optimum growth at 30°C had a heat tolerance of range of 25°C- 37°C.

3.3.2.3A Mangrove

A. penicillioides MRw202, MRw203 and MRw208 grew optimally at 37°C and had a heat tolerance range of 25°C-30°C. *A. terreus var terreus* MCs223 with optimum growth at 37°C had a heat tolerance range of 25°C-50°C. *A. flavipes* MCs219 had a heat tolerance range of 25°C-37 °C and optimum growth at 30°C. *H. wernekii* MCw214 and MCw215 had the optimum growth at 30°C with a heat tolerance range of 25°C-30°C. *E. repens* MRw210, MRw212 with an optimum growth at 30°C had a heat tolerance range of 25°C-42°C. *E. amstelodami* MCs221 with optimal growth at 30°C and MRw211 with optimal growth at

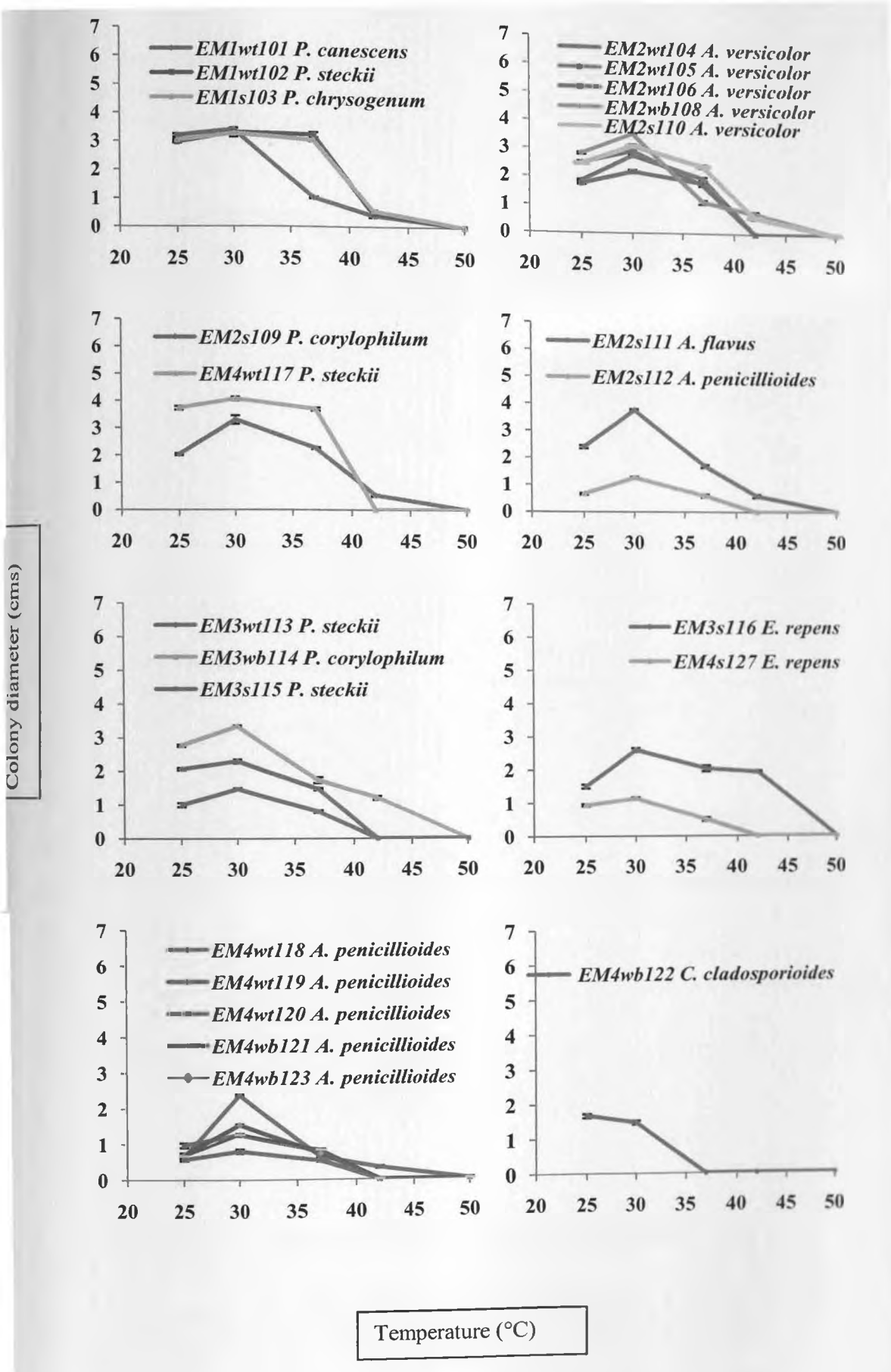


Fig. 3.4b. Heat tolerance curves of isolates from Mandovi estuary.

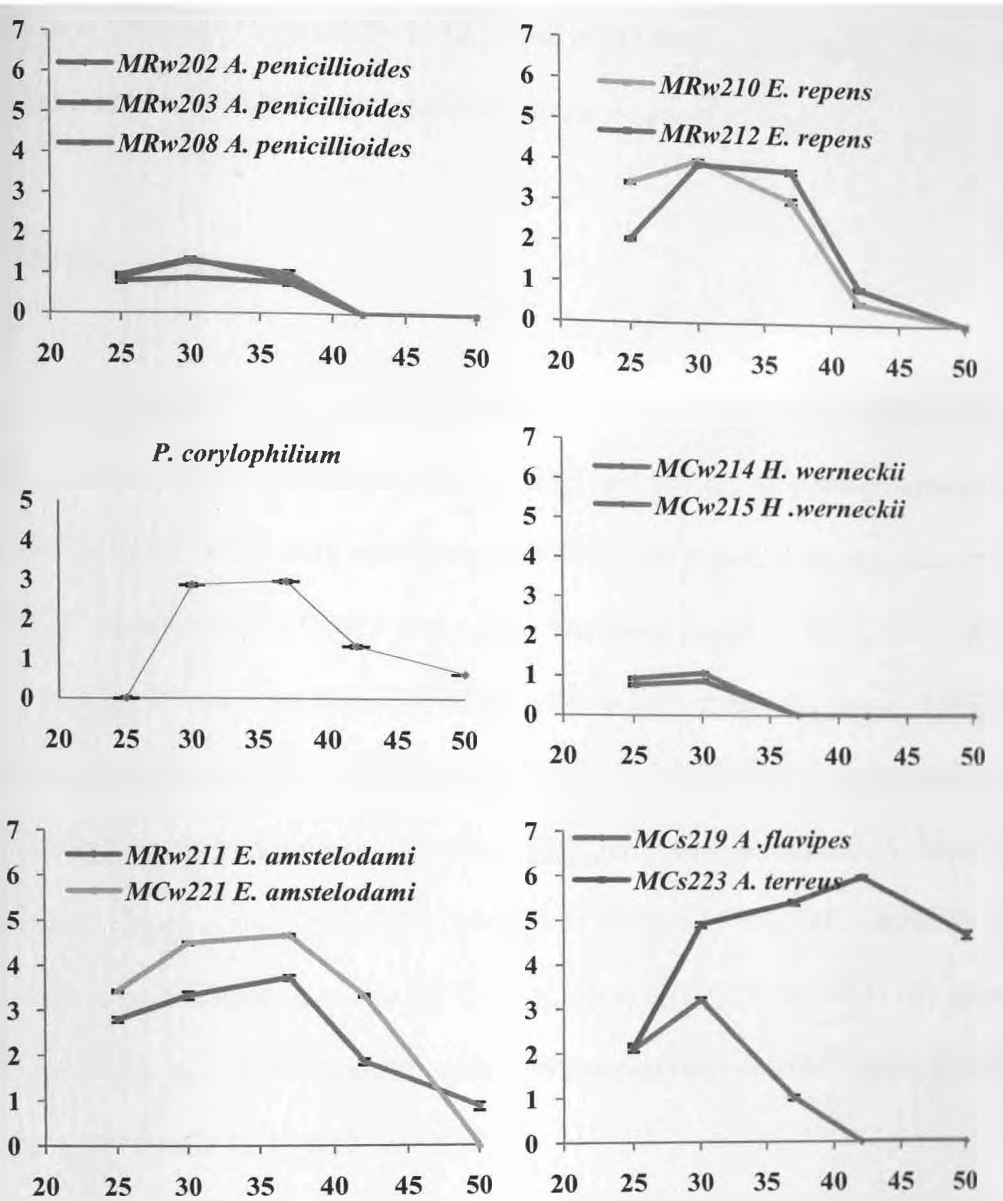


Fig. 3.4c. Heat tolerance curves of isolates from mangroves

37°C had a heat tolerance range of 25°C-42°C and 25°C-50°C respectively. *P. corylophilum* MRw205 grew optimally at 30°C with heat tolerance range of 25°C-42°C.

3.3.2.4A Saltern

A. wentii SRw234 and SRs247 had the heat tolerance range 25°C-42°C with optimal growth at 30°C. *A. sydowii* SRs243 grew optimally at 30°C and had a heat tolerance range of 25°C-42°C. *H. wernekii* SCw250 with optimum growth at 30°C had a heat tolerance range of 25°C-30°C. *E. amstelodami* SRs245 had a heat tolerance range of 25°C-42°C and optimal growth at 30°C. *P. corylophilium* SCw233 with a heat tolerance range 25°C-37°C, *P. asymmetrica sec fasciculata* SCw256, SCw257 with a heat tolerance range 25°C-37°C and 25°C-42°C respectively had optimal growth at 30°C. *A. versicolor* SRw235, SRw241 with a heat tolerance range 25°C-42°C, *A. versicolor* SCw230, SRs237, SRs239, SCw252, SCw254 with a heat tolerance range 25°C-42°C grew optimally at 30°C. *P. griseofulvum* SRw228 and *P. chrysogenum* SRw225 with optimum growth at 30°C had a heat tolerance range of 25°C-37°C and 25°C-42°C respectively.

Isolates SRw135 and SCw182 saltern had a heat tolerance range of 30°C-50°C and optimum growth at 37°C. SSw167 tolerated only 30°C. Table 3.3a, 3.3b, 3.3c, 3.3d gives the heat tolerance of the isolates from Dead Sea, Mandovi estuary, mangroves and salterns respectively germinated and grown at different temperature.

The ANOVA values were significantly different for varying temperature. P value is less than 0.05 with.

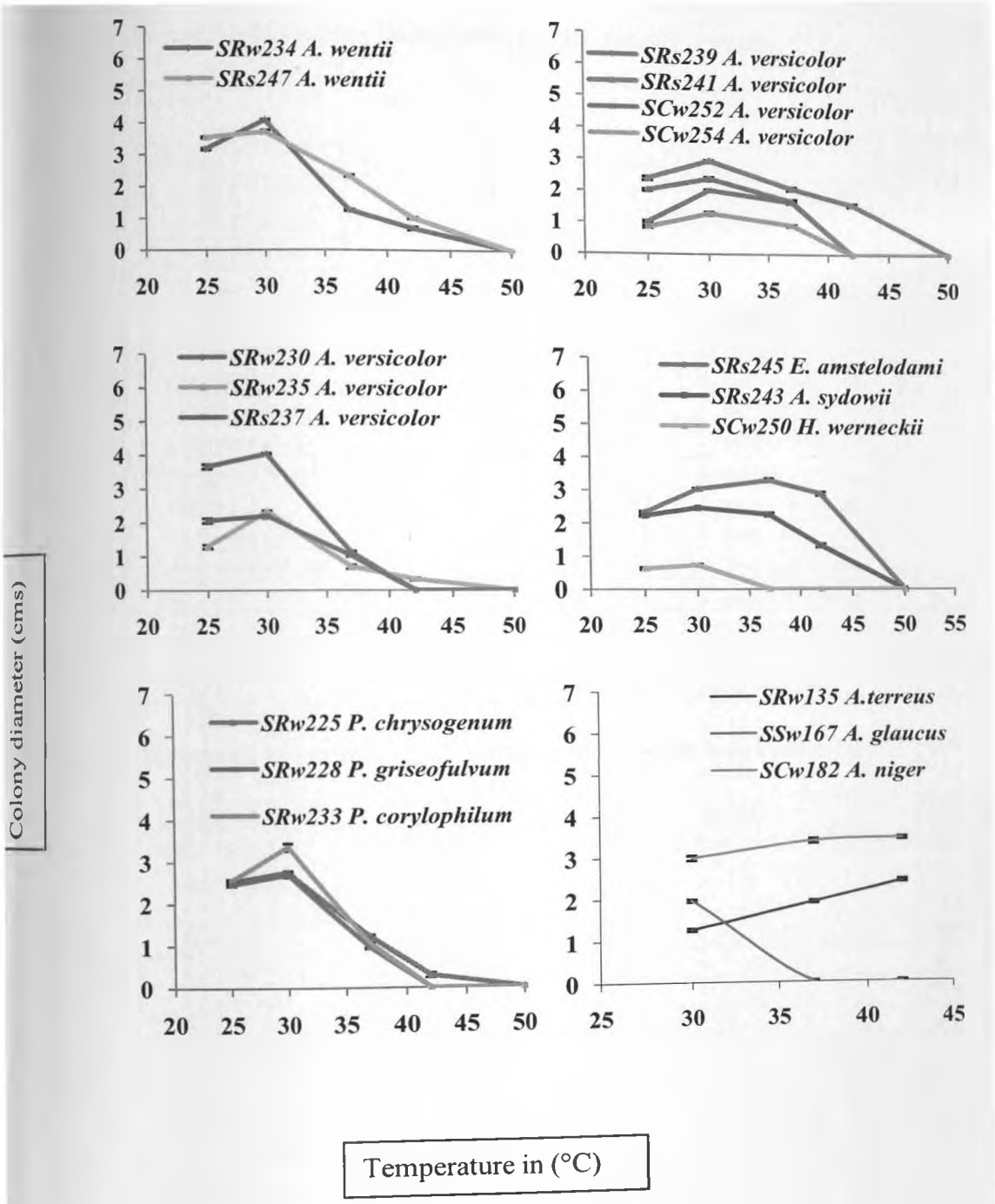


Fig. 3.4d. Heat tolerance curves of isolates from salterns.

Table 3.2a Screening of isolates from Dead Sea for heat tolerance

Isolate		Growth (colony diameter, cm) at		Isolate		Growth (colony diameter, cm) at	
Number	Species	42°C	50°C	Number	Species	42°C	50°C
DSw1	*A. penicillioides*	0	0	DSs29	*A. penicillioides*	0	0
DSw 4	*A. penicillioides*	0.467	0	DSs 31	*A. penicillioides*	0	0
DSw 5	*A. penicillioides*	0	0	DSs 33	*A. penicillioides*	0	0
DSw 7	*P. steckii*	0	0	DSs 35	*A. penicillioides*	0	0
DSw 8	*P. corylophilum*	1.067	0	DSs 37	*A. penicillioides*	0	0
DSw 11	*P. corylophilum*	0.467	0	DSs 39	*A. penicillioides*	0	0
DSw 13	*P. corylophilum*	0.67	0	DSs 41	*A. penicillioides*	0	0
DSw 17	*P. corylophilum*	0	0	DSs 43	*A. penicillioides*	0	0
DSw 18	*P. corylophilum*	0	0	DSs 45	*A. penicillioides*	0	0
DSw 19	*P. corylophilum*	0.367	0	DSs 47	*A.penicillioides*	0	0
DSw 21	*P. steckii*	0	0	DSs 49	*A. penicillioides*	0	0
DSw 23	*C. cladosporioides*	0	0	DSs 51	*A. penicillioides*	0	0
DSw 25	*C. cladosporioides*	0	0	DSs 53	*A. penicillioides*	0	0
				DSs 55	*A. penicillioides*	0	0
				DSs 57	*A. versicolor*	0.3	0
				DSs 59	*A. versicolor*	0	0
				DSs 61	*A. versicolor*	0	0

Table 3.2b Screening of isolates from Mandovi estuary for heat tolerance

Isolate		Growth (colony diameter, cm) at		Isolate		Growth (colony diameter, cm) at	
Number	Species	42°C	50°C	Number	Species	42°C	50°C
EM1wt101	*P. canescens*	0.4	0	EM3s115	*P. steckii*	0	0
EM1wt102	*P. steckii*	0.46	0	EM3s116	*E. repens*	1.93	0
EM1s103	*P. chrysogenum*	0.56	0	EM4wt117	*P. steckii*	0	0
EM2wt104	*A. versicolor*	0	0	EM4wt 118	*A. penicillioides*	0	0
EM2wt105	*A. versicolor*	0	0	EM4wt 119	*A. penicillioides*	0.8	0
EM2wt106	*A. versicolor*	0	0	EM4wt 120	*A. penicillioides*	0.33	0
EM2wb107	*A. penicillioides*	0	0	EM4wb121	*A. penicillioides*	0	0
EM2wb108	*A. versicolor*	0.73	0	EM4wb122	*C. cladosporioides*	0	0
EM2s109	*P. corylophilum*	0.53	0	EM4wb123	*A. penicillioides*	0	0
EM2s 110	*A. versicolor*	0.6	0	EM4wb124	*E. amstelodami*	0	0
EM2s111	*A. flavus*	0.6	0	EM4wb125	*A. penicillioides*	0	0
EM2s112	*A. penicillioides*	0	0	EM4wb126	*E. repens*	0	0
EM3wt113	*P. steckii*	0	0	EM4wb127	*E. repens*	0	0
EM3wb114	*P. corylophilum*	1.2	0	EM4wb128	*C. cladosporioides*	0	0

Table 3.2c Screening of isolates from mangroves for heat tolerance

Isolate		Growth (colony diameter, cm) at		Isolate		Growth (colony diameter, cm) at	
Number	Species	42°C	50°C	Number	Species	42°C	50°C
MRw202	*A. penicillioides*	0	0	MRw212	*E. repens*	0.9	0
MRw203	*A. penicillioides*	0	0	MCw214	*H. werneckii*	0	0
MRw205	*P. corylophilum*	0.63	0	MCw215	*H. werneckii*	0	0
MRw208	*A.penicillioides*	0	0	MCs219	*A. flavipes*	0.4	0
MRw210	*E. repens*	0.53	0	MCs221	*E. amstelodami*	3.3	0
MRw211	*E. amstelodami*	1.86	0.87	MCs223	*A.terreus*	5.97	4. 7

Table 3.2d Screening of isolates from salterns for heat tolerance

Isolate		Growth (colony diameter, cm) at		Isolate		Growth (colony diameter, cm) at	
Number	Species	42°C	50°C	Number	Species	42°C	50°C
SRw225	*P. chrysogenum*	0.9	0	SRs241	*A. versicolor*	1.6	0
SRw228	*P. griseofulvum*	0	0	SRs243	*A. sydowii*	1.3	0
SRw230	*A. versicolor*	0	0	SRs245	*E. amstelodami*	2.9	0
SRw233	*P. corylophilum*	1.07	0	SRs247	*A. wentii*	1.0	0
SRw234	*A. wentii*	0.7	0	SCs250	*H. werneckii*	0	0
SRw235	*A. versicolor*	0.3	0	SCs252	*A. versicolor*	0	0
SRs237	*A. versicolor*	0	0	SCs254	*A. versicolor*	0.6	0
SRs239	*A. versicolor*	0	0	SCs256	*P. asymmetrica*	0.5	0
				SCs257	*A. versicolor*	0.5	0

Table 3.3 a Heat tolerance range of isolates from Dead Sea.

Isolate		Growth Temperature[a]	
Species	Number	Maximum	Optimal
A. penicillioides	DSw1, Dsw5, DSw17	25°C-37°C	30°C
C. cladosporioides	DSw23 ,DSw25	25°C-30°C	25°C
P. corylophilum	DSw4, DSw8, DSw11, DSw13, DSw18	25°C-42°C	30°C
P. steckii	DSw7, DSw21	25°C-42°C	30°C
A. penicillioides	DSs29, DSs31, DSs33, DSs35, DSs37, DSs39 DSs41, DSs45, DSs47, DSs49 DSs 51, DSs 53, DSs55	25°C-37°C	30°C
A. versicolor	DSs57	25°C-42°C	30°C
	DSs59, DSs 61	25°C-37°C	30°C

[a] Temperatures used for growth: 25°C, 37°C, 42°C, 50°C

Table 3.3 b Heat tolerance range of isolates from Mandovi estuary.

Isolate		Growth Temperature[a]	
Species	Number	Maximum	Optimal
A. flavus	EM2s111	25°C-37°C	30°C
A. penicillioides	EM2wb107, EM2s112, EM4wt118, EM4wt119, EM4wt120, EM4wb121, EM4wb123 EM4wb125	25°C-37°C	30°C
A. versicolor	EM2wt104,EM2wt105, EM2wt 106	25°C-37°C	30°C
	EM2wb108, EM2s110	25°C-42°C	30°C
C. cladosporioides	EM4wb122, EM4s128	25°C-30°C	25°C
E. amestelodami	EM4wb124	25°C-37°C	30°C
E.repens	EM3s116, EM4s126, EM4s127	25°C-37°C	30°C
P. canescens	EM1wb101,	25°C-42°C	30°C
P. chrysogenum	EM1s103	25°C-42°C	30°C
P. corylophilum	EM3wb114	25°C-37°C	30°C
	EM2s109	25°C-42°C	30°C
P. steckii	EM1wb102, EM3wt113, EM3s115	25°C-42°C	30°C
	EM4wt117	25°C-37°C	30°C

[a] Temperatures used for growth: 25°C, 37°C, 42°C, 50°C

Table 3.3 c Heat tolerance range of isolates from mangroves

Isolate		Growth Temperature[a]	
Species	Number	Maximum	Optimal
A. flavipes	MCs219	25°C-37°C	30°C
A. penicillioides	MRw202, MRw203, MRw208	25°C-37°C	30°C
A. terreus var terreus	MCs223	25°C-50°C	37°C
H. werneckii	MCw214, MCw215	25°C-30°C	30°C
E. amstelodami	MRw211	25°C-50°C	37C
	MCs221	25°C-42°C	30°C
E. repens	MRw210, MRw212	25°C-42°C	30°C
P. corylophilum	MRw205	25°C-42°C	30°C

[a]Temperatures used for growth: 25°C, 37°C, 42°C, 50°C

Table 3.3 d Heat tolerance range of isolates from salterns.

Isolate		Growth Temperature[a]	
Species	Number	Maximum	Optimal
A. sydowii	SRs243	25°C-42°C	30°C
A. versicolor	SRw230, SRs237 SRs239, SCw254 SCs252	25°C-37°C	30°C
	SRw235 , SRs241,	25°C-42°C	30°C
A. wentii	SRw234, SRs247	25°C-42°C	30°C
H. werneckii	SCw250	25°C-30°C	30°C
E. amstelodami	SRs245	25°C-42°C	30°C
P. asymmetrica sec fasciculata	SCw256	25°C-37°C	30°C
	SCw257	25°C-42°C	30°C
P. chrysogenum	SRw225	25°C-42°C	30°C
P. corylophilum	SRw233	25°C-37°C	30°C
P. griseofulvum	SRw228	25°C-37°C	30°C

[a]Temperatures used for growth: 25°C, 37°C, 42°C, 50°C

The growth of pre-germinated spores at 42°C and 50°C is shown at Fig. 3.5a, 3.5b. As described below, some of the isolates which did not show growth initially at 42°C and 50°C, were able to tolerate better, these higher temperatures after pre-germination of the spores at 30°C; Fig. 3.5a gives the isolates which grew at 42°C, Fig. 3.5b gives the isolates which did not grow at 42°C.

3.3.2.1B Isolates which did not grow at 42°C

Dead Sea: *A. penicililoides* DSw1, *P. corylophilum* DSw11 *A. versicolor* DSs59, DSs61 did not grow at 42°C; however, after germination at 30°C and subsequent transfer to 42°C, they showed growth.

Mandovi estuary: *P. canescens* EM1wb101, *P. steckii* EM1wb102, *P. chrysogenum* EM1s103 *P. corylophilum* EM3wb114, *A. versicolor* EM2wt106 did not grow at 50°C; however, after germination at 30°C and subsequent transfer to 50°C, they showed growth.

Mangroves: *A. flavipes* MCs219 grew at 50°C after germination at 30°C.

Salterns: *A. versicolor* SRw230 did not show growth at 42°C; however, after germination at 30°C and subsequent transfer to 42°C, they showed growth. *A. versicolor* SRs239, SRs241, *P. chrysogenum* SRw225 grew at 50°C °C after germination at 30°C.

3.3.2.2B Isolates which grew at 42°C

Dead Sea: *P.corylophilum* DSw4, DSw8, .DSw13, DSS31 which could grow at 42°C, showed enhanced growth when grown at 42°C after germination at 30°C.

Mandovi estuary: *A. versicolor* EM2Wb108, *A. flavus* EM2s111, *E. repens* EM3s116, *P. steckii* EM3wt117 showed enhanced growth when grown at 42°C after germination at 30°C. EM2s112 grew at 42°C after germination at 30°C.

Mangroves: *P. corylophilum* MRw205, *A. penicillioides* MRw208, *E. repens* MRw210, *E. amstelodami* MCs221 all showed enhanced growth when grown at 42°C after germination at 30°C

Salterns: SRs245 *A. wentii* SRw234, SRs247, *A. sydowii* SRs243 all showed enhanced growth when grown at 42°C after germination at 30°C.

The ANOVA values were not significantly different for ungerminated and germinated spores where P value is less than 0.05. In *A. penicillioides* MRw202, MRw203, *P. corylophilum* MRw205, *E. repens* MRw212, *H. werneckii* MCw214, MCw215, *A. flavipes* MCs219, *P. chrysogenum* SRw225, *A. versicolor* SRw230, *A. wentii* SRw234, *A. versicolor* SRs239, SRs241, *A. sydowii* SRs243, *A. versicolor* SCs254, the ANOVA values were significantly different for ungerminated spores.

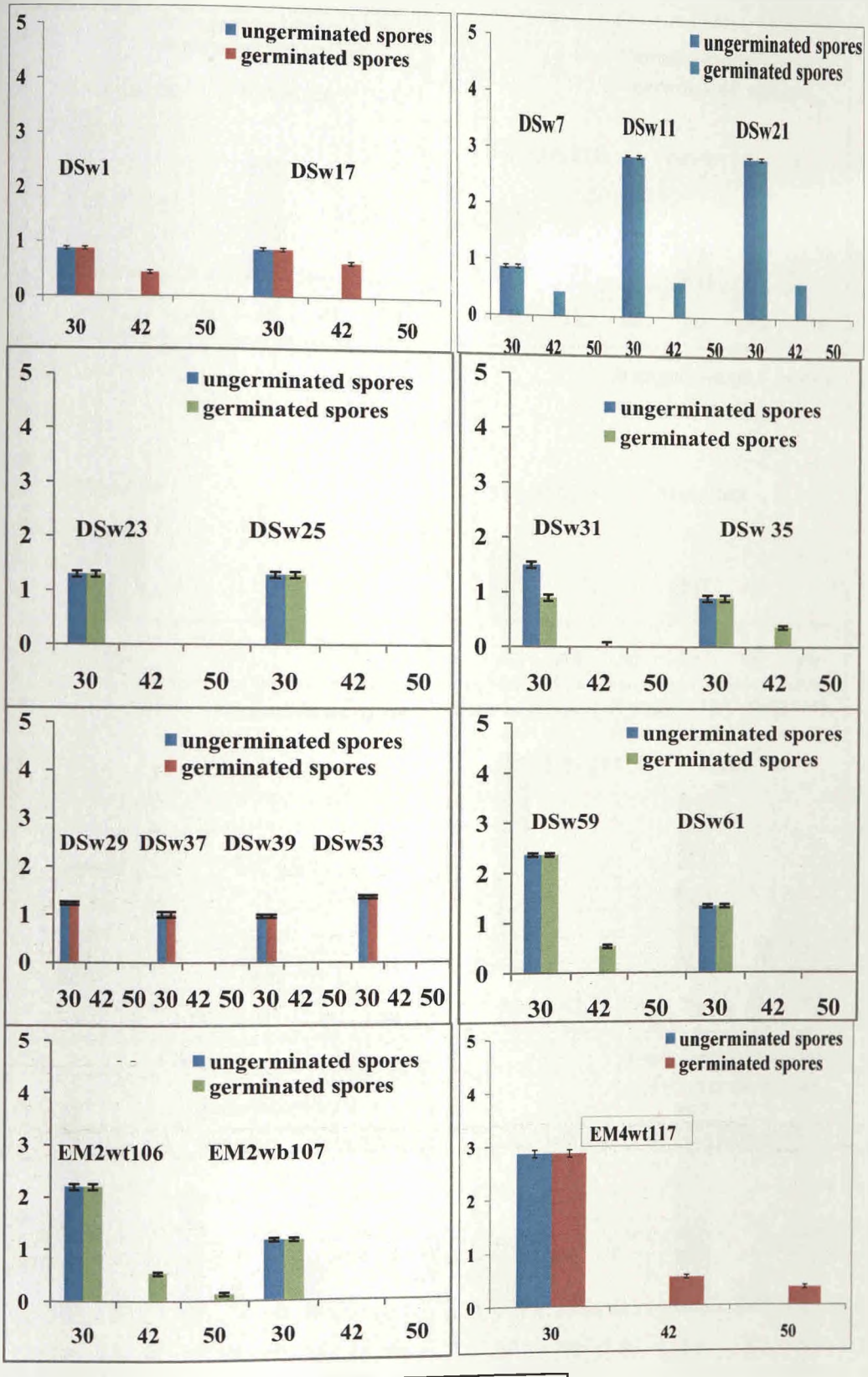


Fig. 3.5a. Growth of germinated and ungerminated spores of isolates at different temperatures.

Contd.....

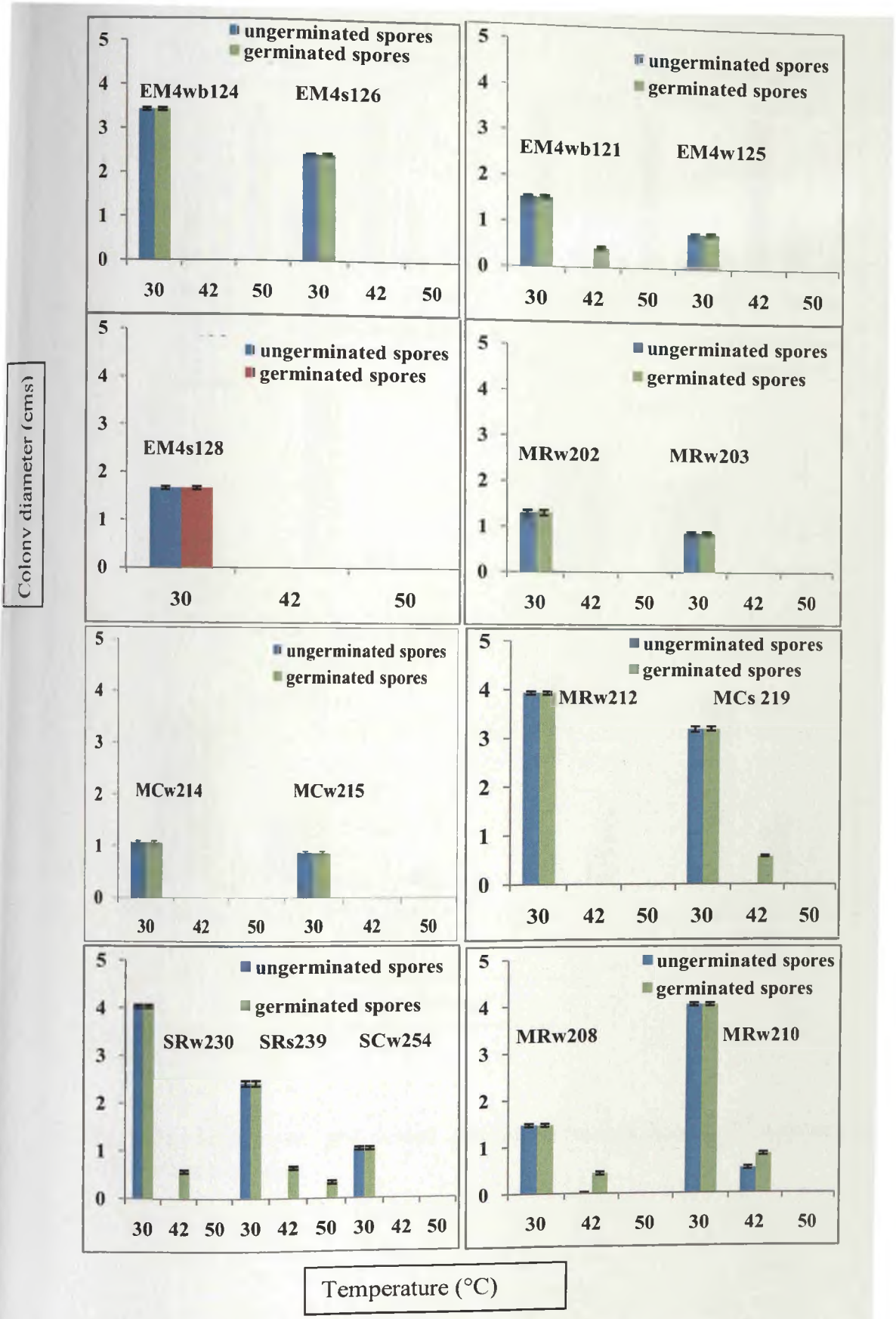


Fig. 3.5a. Growth of germinated and ungerminated spores of isolates at different temperatures.

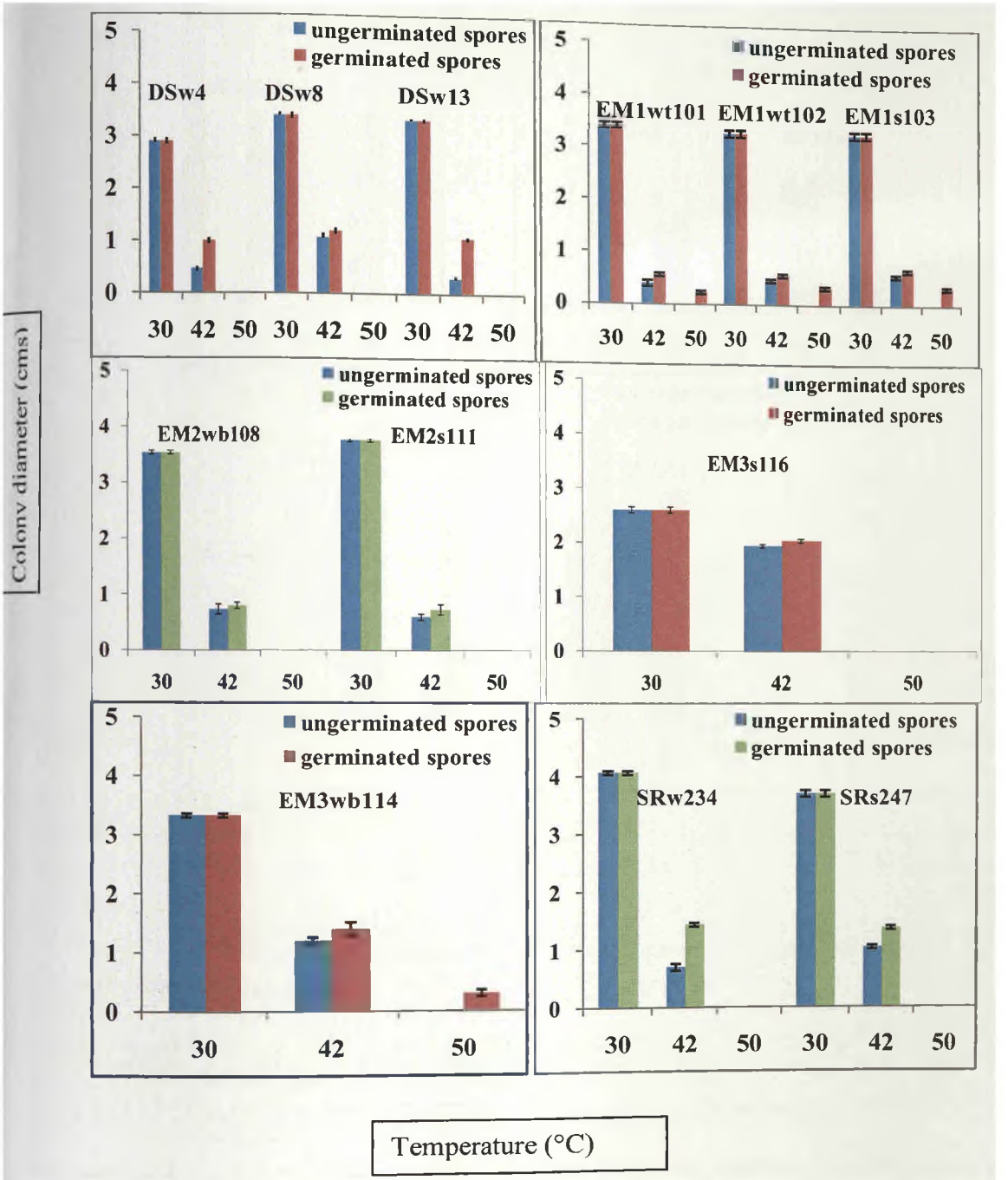


Fig. 3.5b. Growth of germinated and ungerminated spores of isolates at different temperatures.

Contd.....

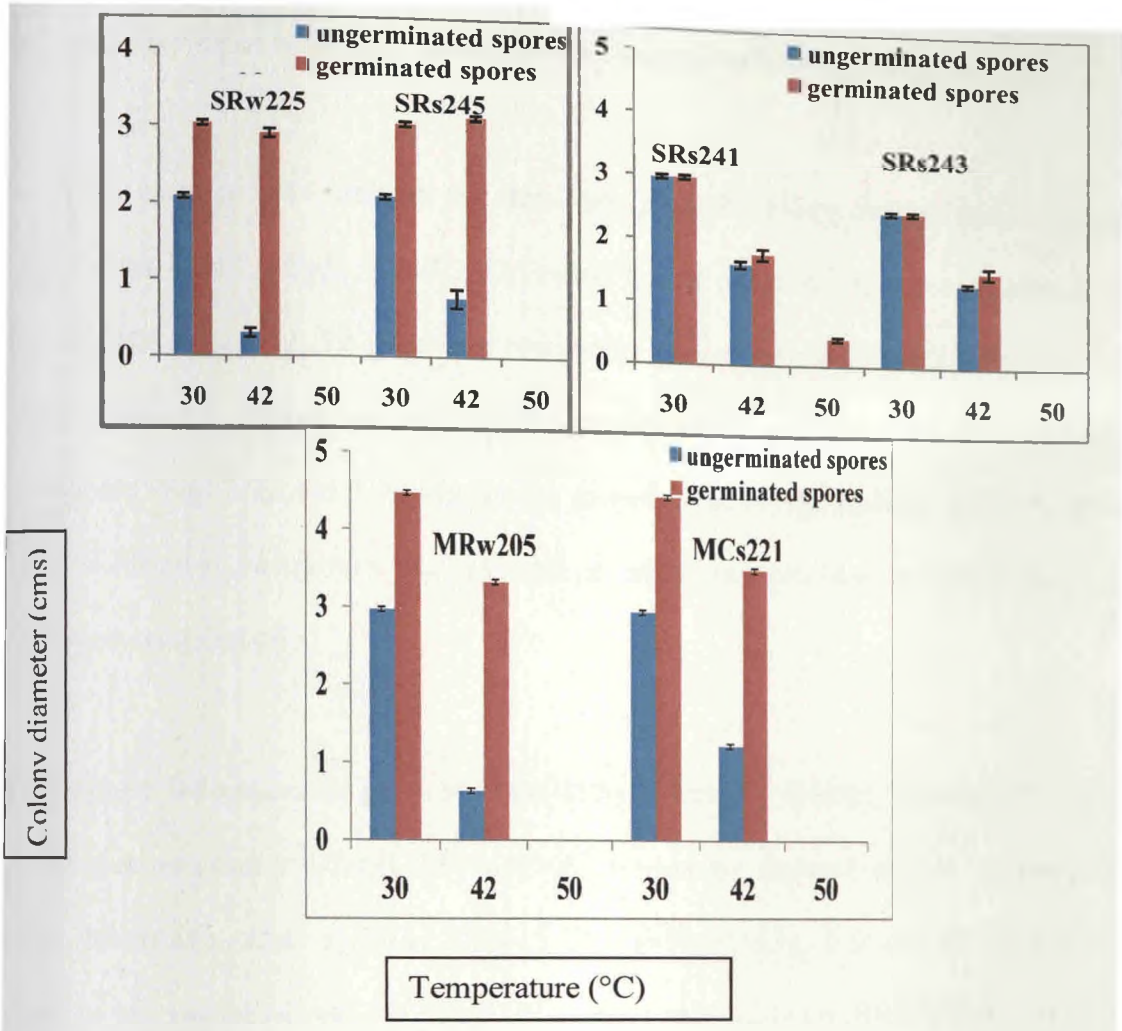


Fig. 3.5b. Growth of germinated and ungerminated spores of isolates at different temperatures.

3.3.3 Growth rate of isolates at varying salt concentrations and temperatures

Not much increase was seen in the day-wise growth among the obligate halophiles *A. penicillioides* DSw1, DSs31, DSs35, EM4wb121. The other isolates showed a steep increase in growth day wise. Fig. 3.6 gives the regression curves for the isolates from the Dead Sea, Mandovi estuary, mangroves and salterns at increased salt concentration and increased temperature. Fig. 3.7a and 3.7b shows the growth rate of the isolates from the Dead Sea, Mandovi estuary, mangroves and salterns at increased salt concentration and increased temperature respectively.

The obligate halophiles *A. penicillioides* DSw1, DSs31, DSs35, EM4wb121, facultative halophile *A. versicolor* DSs59, MCw215 *H. werneckii,* showed almost an flat regression curve, while the other isolates DSw13 *P. corylophilum*, DSw25 *C. cladosporioides*, MRw211 *E. amstelodami*, MCs223 *A. terreus var terreus*, SRw225 *P. chrysogenum* MCs219 *A. flavipes*, SRs245, EM4wb122 *C. cladosporioides* showed a gentle rise in the slope of the line. At increased temperature and increased salt concentration EM4wb121 showed almost an even regression curve.

The ANOVA values were significantly different for the day- wise growth where P value is less than 0.05.

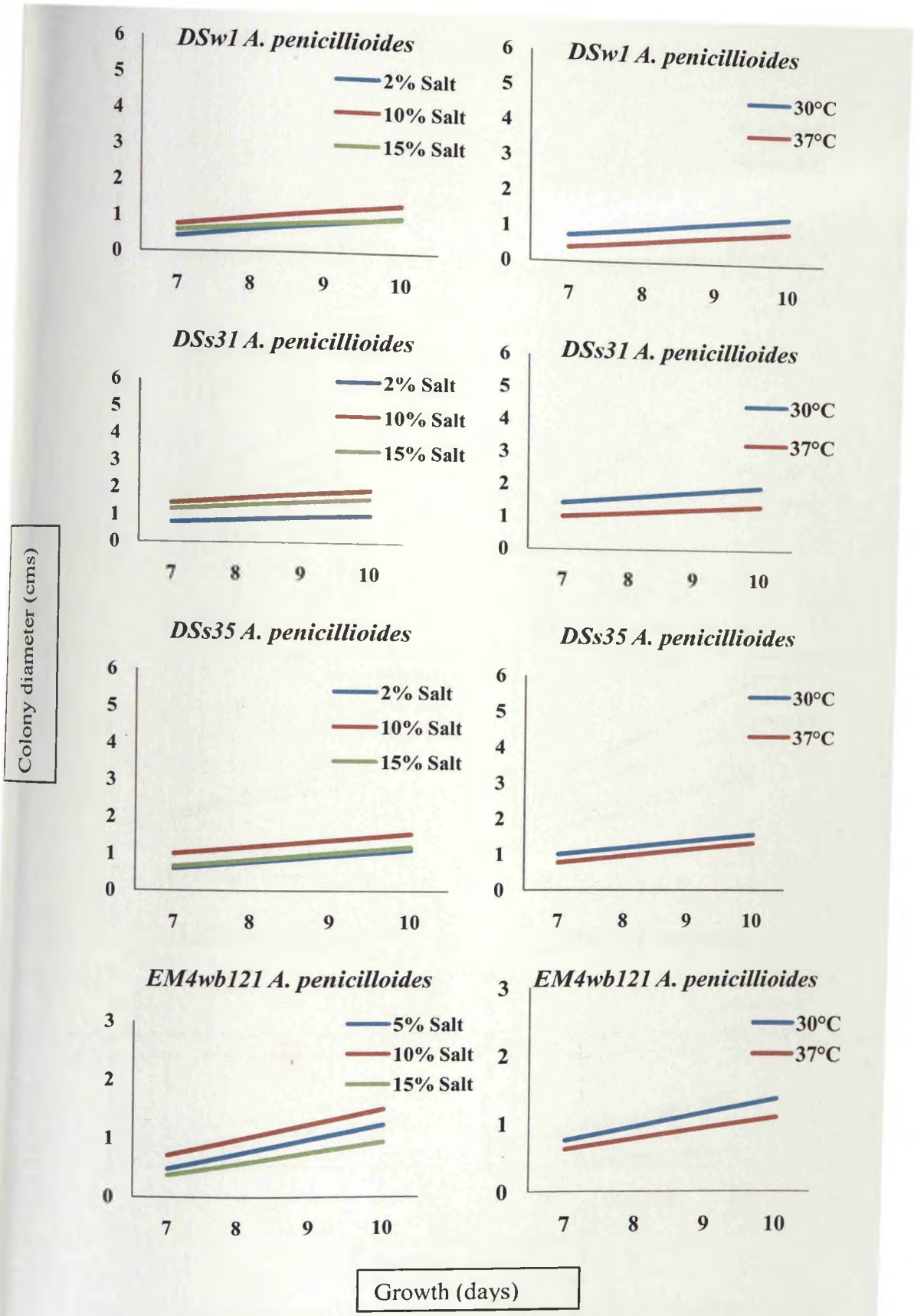


Fig. 3.6 Regression curves of number of days versus colony diameter (cms) with varying salt concentration and increased temperature

Contd.....

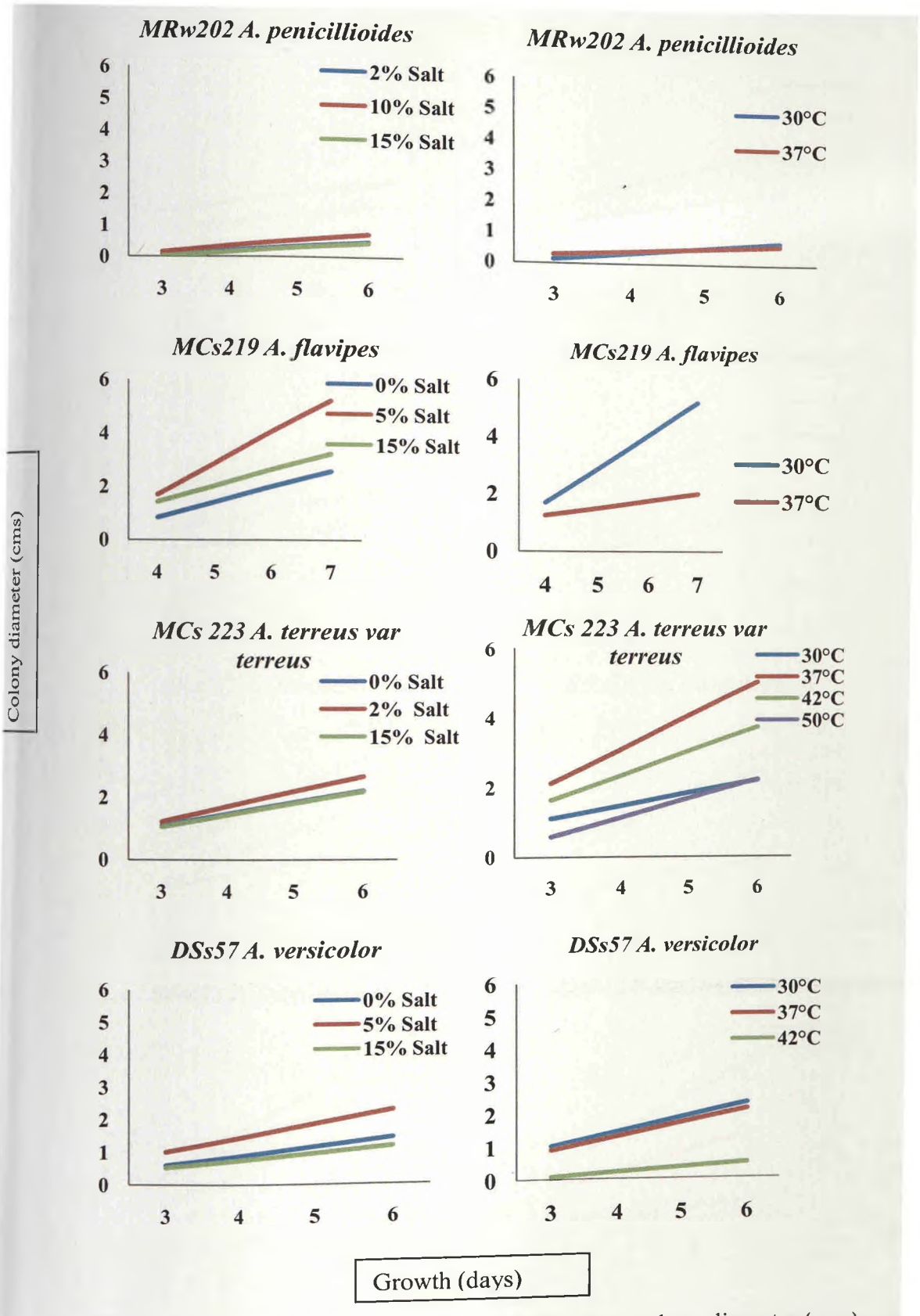


Fig. 3.6 Regression curves of number of days versus colony diameter (cms) with varying salt concentration and increased temperature.

Contd.....

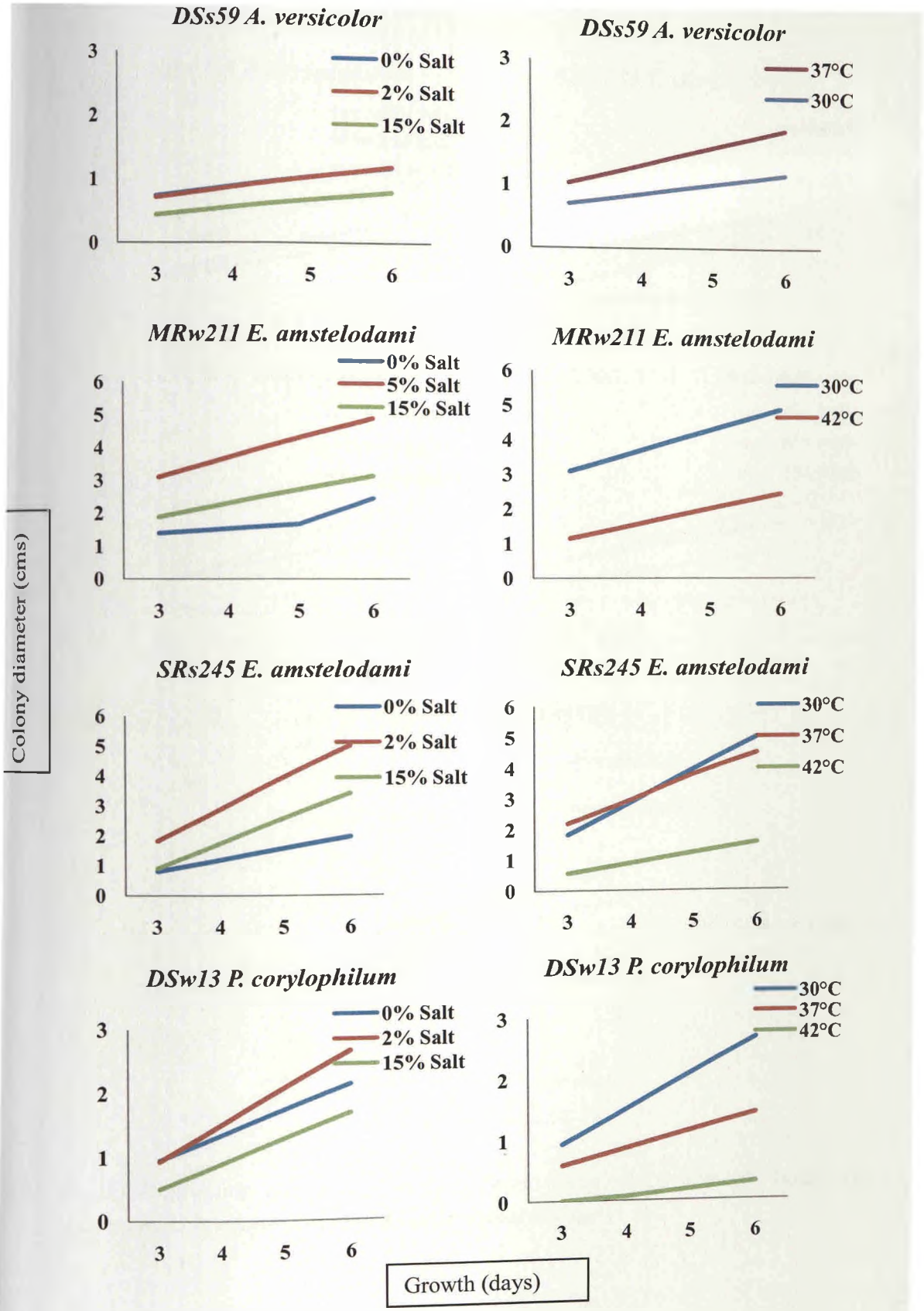


Fig. 3.6 Regression curves of number of days versus colony diameter (cms) with varying salt concentration and increased temperature.

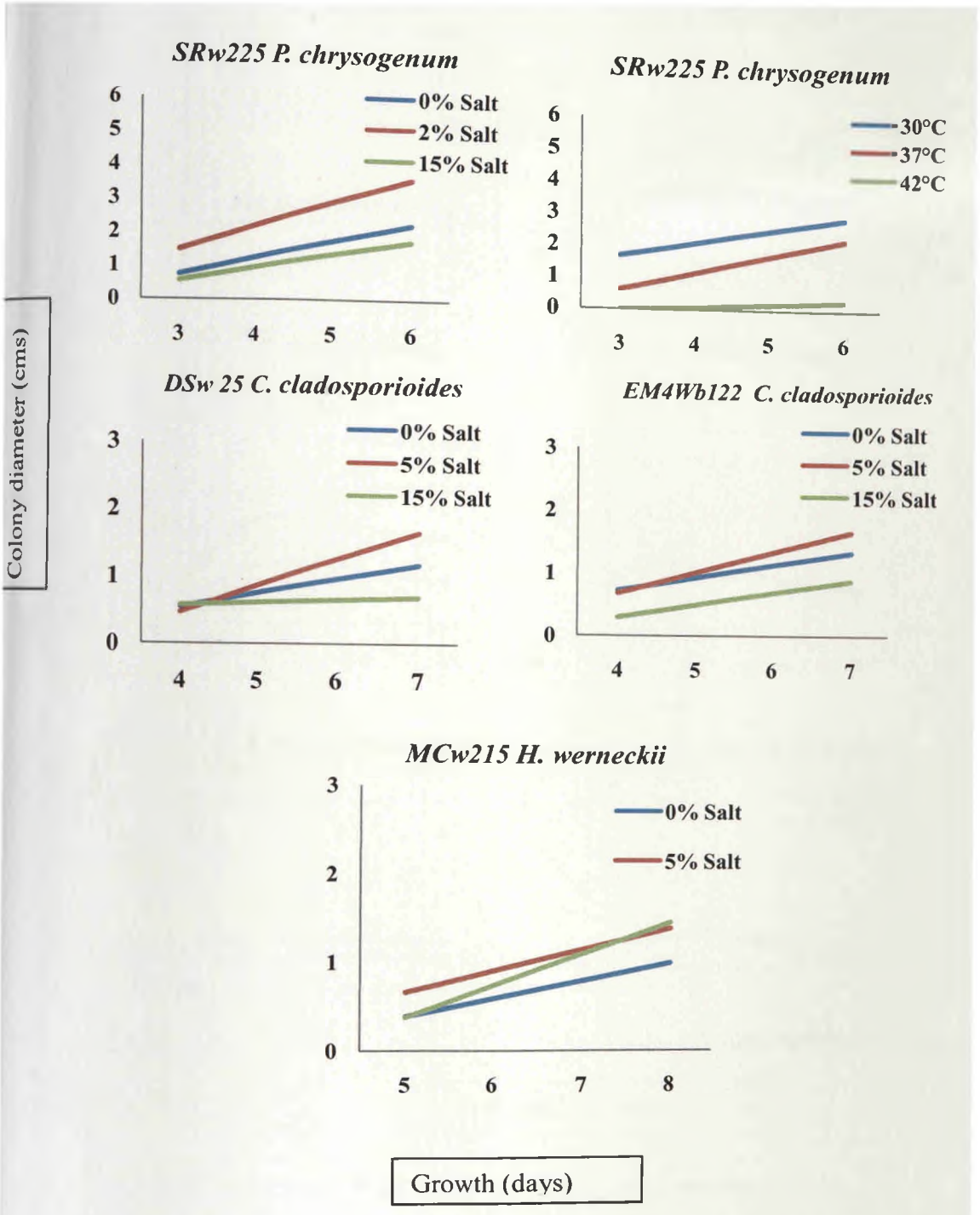
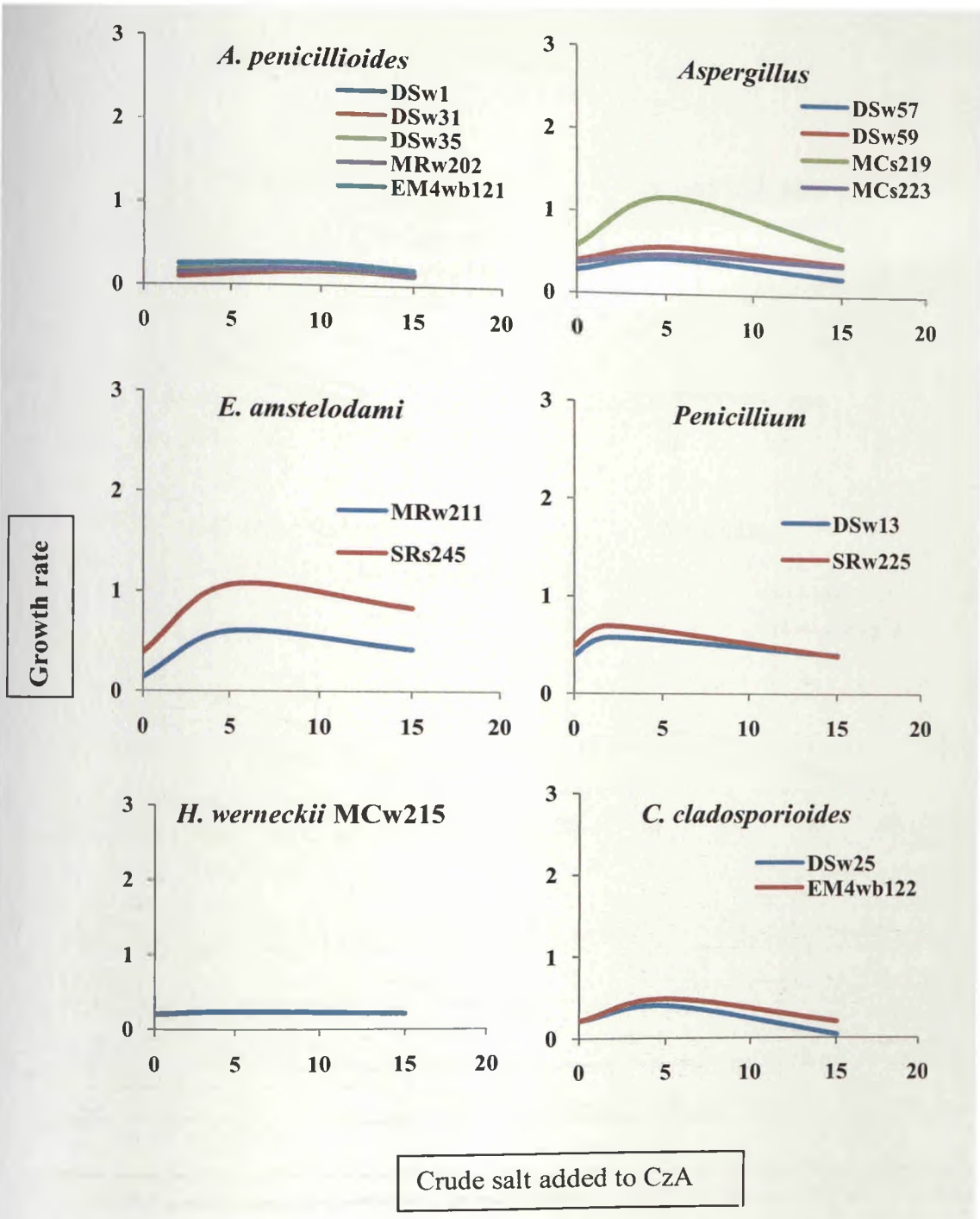
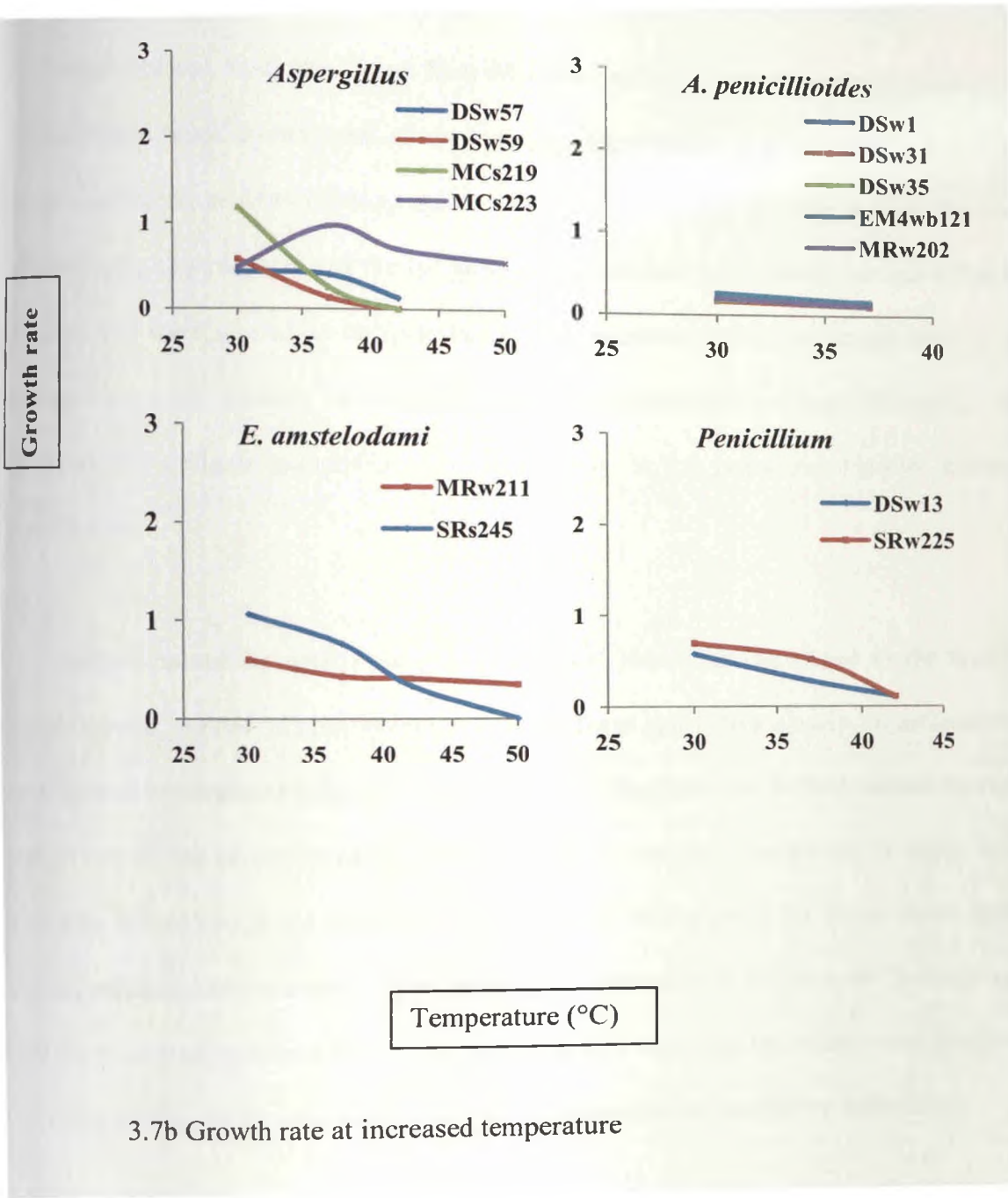


Fig. 3.6 Regression curves of number of days versus colony diameter (cms) with varying salt concentration and increased temperature



3.7a Growth rate of the isolates at varying salt concentration



3.7b Growth rate at increased temperature

3.4 Discussion

The fungi isolated from the Dead Sea, Mandovi estuary, mangroves and salterns were halophilic and some halotolerant; all were euryhaline, capable of growing over a wide range of salt concentration. Most of them were facultative halophiles, growing even in the absence of added salt; however some of the isolates had an absolute requirement for salt added to the medium and were termed as obligate halophiles (Kushner 1978). Although some of these species have been reported earlier, their halophilic nature has not been described. It was observed that obligate halophiles were found only in the genus *Aspergillus*, namely, *A. penicilloides*.

The obligate halophiles which were isolated grew slowly as compared to the facultative halophiles and yielded smaller colonies. Marine fungi grow very slowly on artificial media as compared to terrestrial fungi (Ravishankar 2006). Redkar *et al.* (1996) related the reduced growth rate of salt adapted cultures to the increase in energy demands under stress, wherein cells may utilize energy for compartmentalization or exclusion of Na^+ ions, direct synthesis of compatible osmolytes and synthesis of proteins conferring salt tolerance. To work against such physiological conditions the cells may adapt by reducing its volume and growth rate. The obligate halophiles were few in numbers as compared to facultative halophiles.

Isolates from Dead Sea: *A. versicolor, Penicillium* and *Cladosporium* species, were facultative halophiles and *A. penicillioides* was an obligate halophile. Of the sediment isolates, the majority were obligate halophiles with *A. versicolor* being halotolerant, while those from the water sample were both obligate, as well as facultative halophiles. This could be due to the higher salinity of the sediment as compared to that of water.

Obigate halophiles were isolated from the brackish water of Mandovi estuary contrary to the expectation that hypersaline environment are natural habitats for halophiles, and many of them tolerated up to 25% salt. Systematic seasonal variations of suspended particulate matter along the Mandovi estuary reveals that the concentration of particulate matter is low at river-end stations, increases generally seaward and is highest at sea-end stations of the estuary (Kessarkar *et al.* 2008).

Estuaries are places which have half saline water and half fresh water. Salt water has more minerals, and fresh water has more oxygen. Freshwater fungi are likely to be washed downstream (Tsui and Hyde 2004), therefore seawater environment is a challenge to these fungi. These fungi would eventually have adapted to the gradient saline condition. Many halophiles from Mandovi estuary showed salt tolerance levels till 25%.

Obligate halophiles were isolated from mangroves and none were isolated from the salterns, contrary to the expectation that the salterns would habour more halophiles than the mangroves. However, most of the isolates were facultative halophiles. The isolates were classified according to the definition of Kushner (1978). Most of the isolates obtained from mangroves and salterns were moderate halophiles according to their salt requirement for optimal growth: *A. flavus, A. penicillioides, A. versicolor* and *E. repens*, which required around 10% salt for optimal growth, *E. amstelodami, P. chrysogenum* and *P. griseofulvum* required 5% salt, *H. werneckii,* requiring 5-10% salt and *A. flavipes,* 2 -5% salt, some were slight halophiles *A. sydowii* amd *A. terreus var terreus, A. wentii, P. asymmetrica sec fasiculata* and *P. corylophilum* with optimal growth obtained at 2% added salt, were termed as slight halophiles.

The *Penicillium* isolates were obtained in presence of 20% rather than 10% salt. These showed an extreme tolerance to 20 – 25% crude salt. Jennings and Lysek (1996) have also shown that *Penicillium* species tolerate up to 25% NaCl.

Hortaea werneckii was amongst the first mycobiota to have been isolated from hypersaline environments (Gunde-Cimerman *et al.* 2000, Díaz-Muñoz and Montalvo-Rodríguez 2005) and was found to grow in presence of a wide range of salt concentrations, from complete absence of salt, up to near saturation of 32% with a broad growth optimum of 6-14%, and have been termed as halophilic (Gunde-Cimerman *et al.* 2000, Díaz-Muñoz and Montalvo-Rodríguez 2005) as well as halotolerant (Plemenitaš *et al.* 2008). *H. werneckii* isolates from the Cabo Rojo solar salterns were reported as growing on medium containing 0-25% salt and optimally at 10% (Díaz-Muñoz and Montalvo-Rodríguez 2005). The present findings on isolates of *H. werneckii* also showed their salt tolerance profiles to be 0-25%, with optimal growth at 5-10% salt. Their isolation from salterns in India, Asia, confirm the observation of Butinar *et al.* (2005a) that they are present globally in hypersaline water of man-made salterns. Although hypersaline waters has been named as an ecological niche for *Hortaea* (Gunde-Cimerman *et al.* 2000), this is a first report of the isolation of *H. werneckii* from the waters of mangroves. This work showed that non-melanised filamentous fungi were in greater numbers amongst the isolates obtained from salterns, contrary to earlier reports that melanized fungi have a selective advantage over the other mycoflora in saline environments (Gunde-Cimerman *et al.* 2000) and that mycobiota from highly saline water, are partially replaced by non-melanized fungi at lowered salinities, being detected only occasionally with NaCl concentrations below 5% (Butinar *et al.* 2005a).

All the halophiles were mesophilic having optimal temperature between 25°C-37°C. However some isolates showed variations in the optimal range of growth temperatures: the obligate halophiles, *A. penicillioides,* were more sensitive to heat changes, unable to grow at temperatures above 37°C; the dematiaceous fungi *Cladosporium* grew best at 25°C, while both *Cladosporium* and the black yeast *H. werneckii* could not grow at 37°C. Heat tolerance of the isolates was carried to study the thermotolerant nature of the halophilic fungi. Thermotolerant isolates were mainly from the mangroves, rather than the salterns, contrary to the expectation that the salterns are subject to high intensity of solar radiation and heat, with consequent evaporation in the summer season and would habour more thermotolerant fungi than the mangroves. Notably, two isolates from mangroves, *A. terreus var terreus* and *E. amstelodami* were heat tolerant, able to grow at a temperature of 50°C. Exposure to one stress can increase the acquisition of tolerance against another stress (Schiller *et al.* 1994, Lewis *et al.* 1995, Mager and de Kruijff 1995).

It was seen that although spores of many of the isolates did not germinate, and showed no growth at temperatures of 42°C and 50°C, the pre-germinated spores were able to grow and form colonies, thus indicating that the effect of these temperature on growth was at the germination step (Juniper S and Abbott LK 2006). This was also seen in the growth of spores of those isolates able to germinate at higher temperature, but yielding an enhanced growth when pre-germinated spores were used.

The decrease in growth rate at salt concentrations lesser or higher than the optimal concentration for growth, corroborate the observations of Juniper S and Abbott LK 2006 wherein the growth rate was maximum at optimum concentration of salt and was reduced at

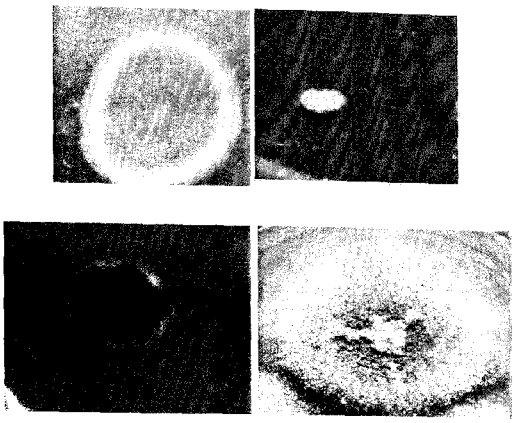
increased or decreased salt concentration, since germination was adversely affected. Similar results were obtained with changes in temperature, wherein the growth rate increased at optimum growth temperature, and decreased at higher or lower temperatures. Ayerst (1969) also reported a delay in growth rates due to delay in germination in *Aspergillus* and *Penicillium* at varying temperature and water activity. In agreement to Ayerst (1969), Magan and Lacey (1984) and Wheeler and Hockings (1988) it was found that spore germination was not always followed by mycelia growth

The results are indicative of the ability of the organism to adapt to the Dead Sea, estuarine ecosystems mangroves and salterns.

3.5 Conclusion

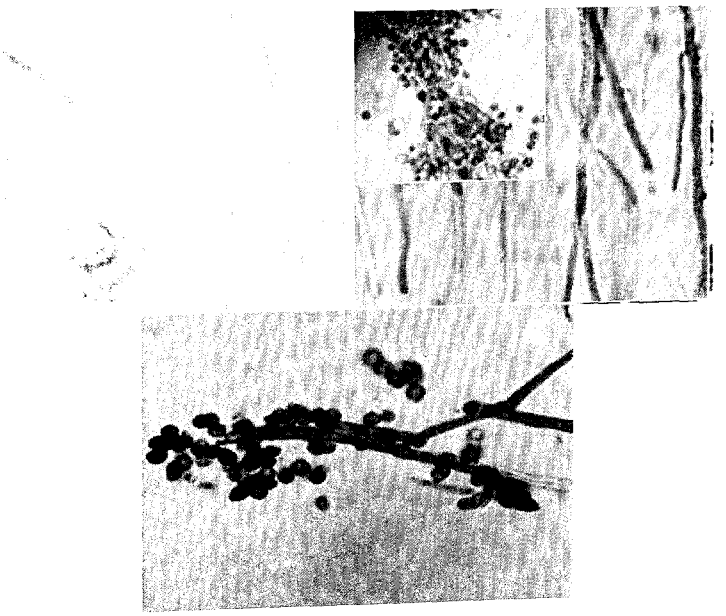
A. penicillioides was the only obligate halophile isolated. All the other isolates: *Aspergillus Penicillium* and *Cladosporium* were facultative halophiles. The *Penicillium* isolates from mangroves and saltern were from water sample. *Cladosporium* was not isolated from mangroves and salterns.

All the isolates obtained were mesophilic. *A. penicillioides* did not grow above 37°C while *H. wernekii, C. cladosporides, A. glaucus* grew only up to 30°C. Most of the isolates had optimum growth at 30°C while few grew optimally at 37°C. *Eurotium* appeared to have a range of 25°C-42°C, and tolerated 42°C. Some *Aspergillus* species also tolerated a temperature of 42°C. Most of the *Penicillii* tolerated 37°C. *P. chrysogenum* tolerated temperature 42°C whereas others tolerated 37°C. *E. repens* from salterns and mangroves tolerated temperature of 42°C and even 50°C. *E. repens* from Mandovi tolerated upto 37°C.



CHAPTER 4

CULTURAL AND MORPHOLOGICAL CHANGES TO VARIED LEVELS OF SALT AND HEAT STRESS



CONTENTS

4.1 Introduction

This chapter deals with the cultural and morphological changes produced by the isolates as a growth response to varying concentration of salt and heat stress. In nature, microorganisms are exposed to a constantly changing physical and chemical environment. However, microorganisms have evolved a variety of means by which they survive adverse stress conditions.

Various species of *Mucor, Rhizopus, Absidia, Cunninghamella*, and other Mucorales developed as yeast like colonies on media containing 10 to 15% NaCl. Brightly coloured pigments, normally not evident, were produced commonly by certain of the aspergilii and penicillii; intensification of pigmentation frequently also occurred with other organisms at particular NaCl levels. Enhanced mycelial growth was occasionally observed. Increased sporulation sometimes was encountered, especially among the penicillii and aspergilli at 5 to 10% NaCl. Zygospore formation was greatly stimulated in *Sporodinia grandis* by 5% NaCl.

Kuncic *et al.* (2010) showed an increased size of mycelial pellets at high salinities, with an increase in cell wall thickness *in W. muriae* and *W. sebi*. Supression of conidiation at low salt concentration and increase at optimum salt was observed by Kane and Summerbell (1987). For most of the micro-organisms, temperature is a critical environmental signal for regulating developmental and physiological process. However, at higher temperature growth was reduced drastically, pigment yield increased remarkably. It has been reported that following exposure to elevated temperature, many organisms rapidly synthesize a highly

conserved set of proteins termed heat shock proteins and their induction appears to correlate with the adaptation of the organism to hypothermic stress condition (Schlesinger 1990).

The work deals with the study of cultural and morphological changes under salt and heat stress.

4.2 Materials and Methods

4.2.1 Effect of salt

Spore suspensions were made as described earlier (Chapter 3) and spot-inoculated on CzA supplemented with low concentration (0% / 2%), optimum concentration (5% / 10%), high concentration (15%) crude salt; the plates were incubated at 30°C. Cultural characteristics such as nature of growth, colour of the spores and the mycelia, presence of pigmentation and morphological characteristics such as septation of the hyphae, structure of the fruiting body and the arrangement of spores were noted on the 4th day for the facultative halophiles and 7th day for the obligate halophiles.

4.2.2 Effect of heat

Isolates were inoculated as above on two sets of plates on CzA + S_o; one set was incubated at 30°C and one at increased temperature of 37°C/42°C/50°C according to the tolerance level. Cultural and morphological characteristics were noted on the 7th day.

4.3 Results

Cultural and morphological characteristics of the selected isolates with varying salt concentrations are shown in Fig. 4.1 a-p. At salt concentrations yielding optimal growth; isolates grew well in comparison to the isolates at salt concentrations lower or higher than that required for optimal growth which grew with a lag and at slow rate; there was also decrease in the number of fruiting bodies and sporulation. The fruiting bodies were well formed and many in number, with dense sporulation at optimal salt concentrations for growth.

The particular differences in the species are as follows. At high concentration *A. penicillioides* DSw1, DSw35, MRw202 showed no sporulation and showed distortion in the fruiting body while at low concentration there was a decrease in sporulation with thin mycelium and small fruiting body. *A. penicillioides* DSs31 showed decrease in sporulation with slight change in the colour of spores and showed distortion in the fruiting body at increased concentration of salt. *A. penicillioides* showed thickening of the mycelium at increased concentration of salt. At high concentration of salt EM4wb121 showed no sporulation, mycelium showed thickening and had beaded appearance with distorted fruiting body.

At low concentration of salt *A. versicolor* DSs57 produced a diffusible brown pigment and showed more sporulation than that at high concentration. There was thickening of the mycelium at optimum salt and high concentration of salt. Halotolerant *A. versicolor* DSs59 produced a non-diffusible pigment at low concentration of salt. At high salt concentration there was less pigmentation, thick mycelium with distorted fruiting body.

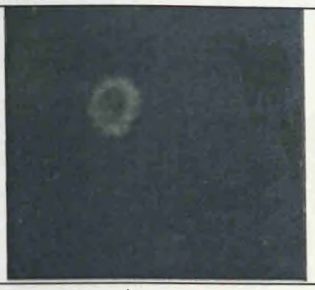
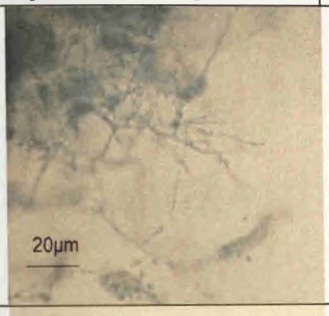
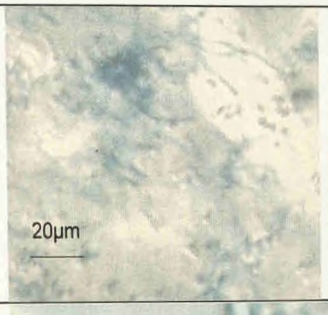
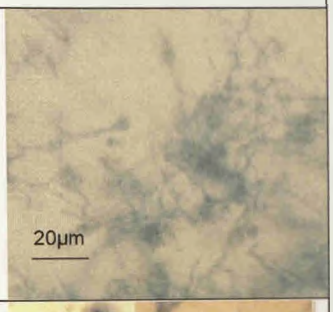
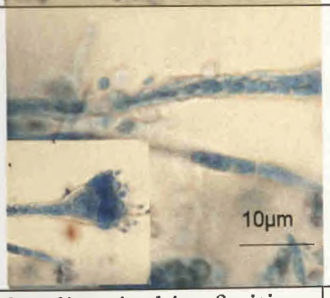
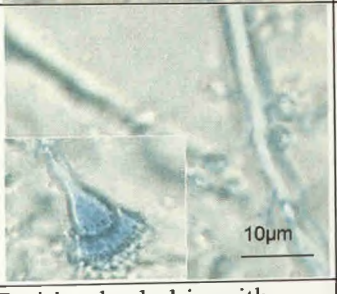
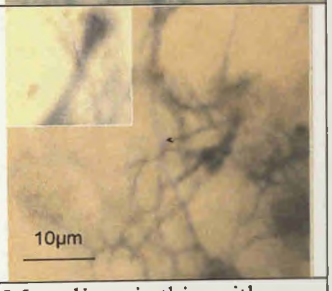
Salt concentration in CzA for growth			
Culture No	2%	10%	15%
DSw1 *A. penicillioides*			
	Decrease in spores as copared to the optimum	Colony elevated, increased spores	Decrease in sporulation
40x			
100x			
	Mycelium is thin; fruiting body is conical and small	Fruiting body big with round vesicle, thin mycelium	Mycelium is thin with distorted fruiting body

Fig 4.1a Cultural characteristics at increased salt concentration

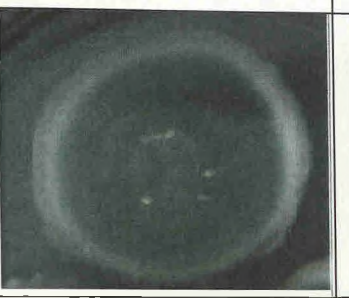
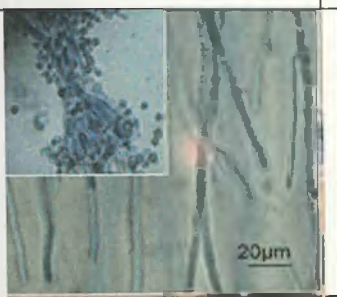
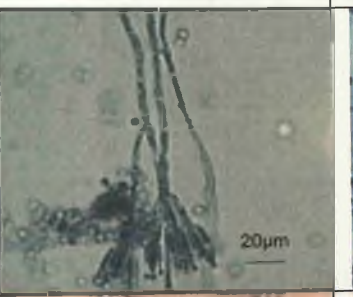
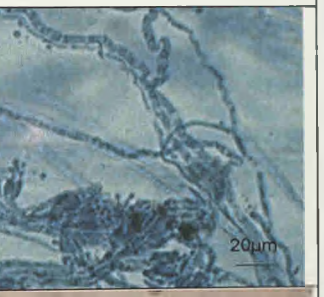
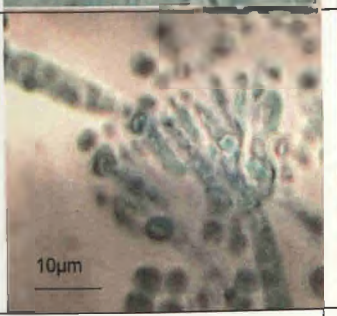
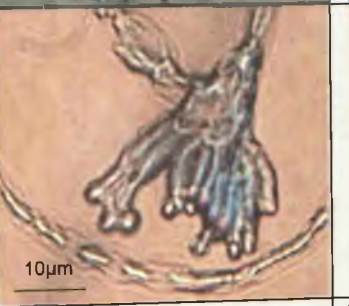
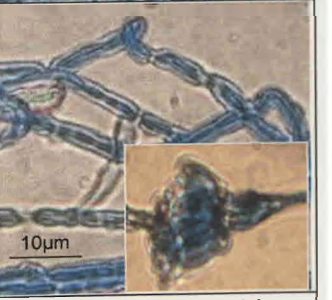
Salt concentration in CzA for growth			
Culture No	0%	2%	15%
DSw13 *P. corylophilum*			
	Colony elevated, pale exudates present	More spores present undulated, pale exudates present	Less number of spores present exudates lacking
40x			
100x			
	Mycelium and the fruiting body with long conidiophore	Mycelium with cytoplasmic constrictions and the fruiting body with long conidiophores	Mycelium is thick with cytoplasm constrictions, the fruiting body is distorted and clumped

Fig 4.1b Cultural characteristics at increased salt concentration

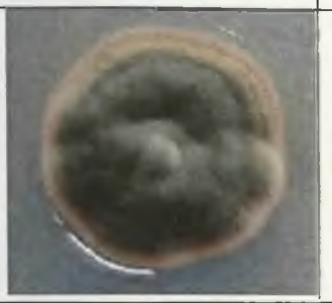
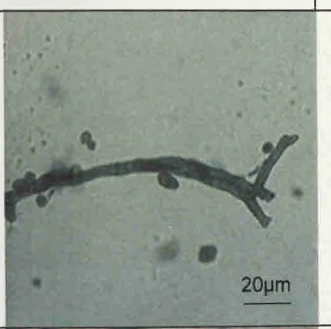
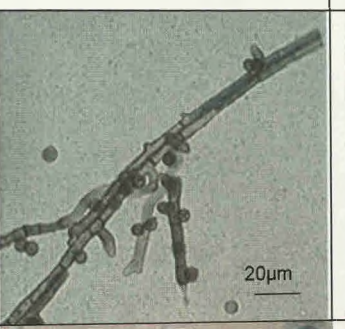
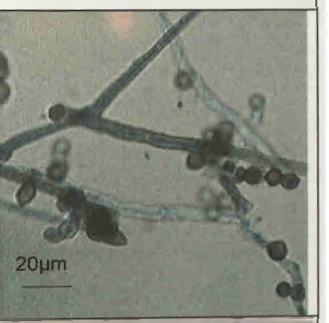
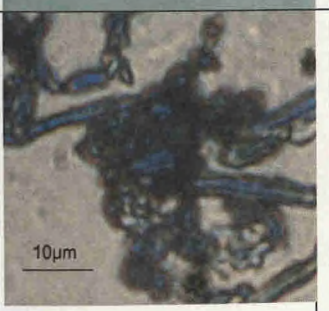
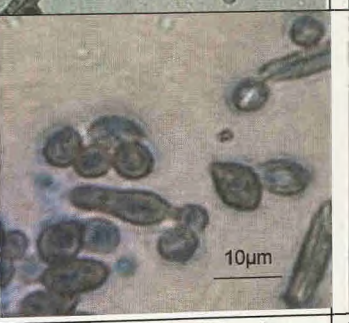
Salt concentration in CzA for growth			
Culture No	0%	5%	15%
DSw25 *C. clasporioides*			
	Colony elevated, reverse brown	Concentric rings on the surface of the colony	Slow growth and less spores
40x			
100x			
	Spores are smaller in size as compared to optimum and high concentration of salt	Spores are bigger in size.	Spores are bigger in size

Fig 4.1c Cultural characteristics at increased salt concentration

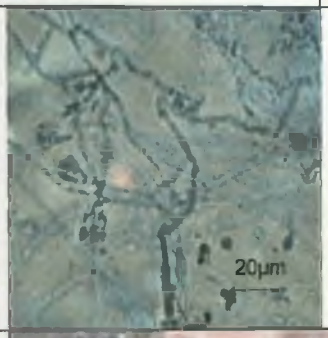
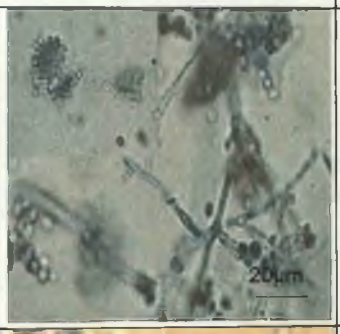
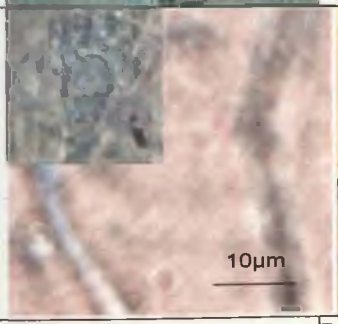
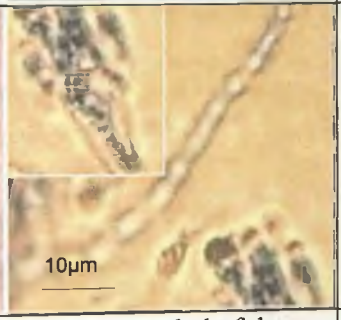
Salt concentration in CzA for growth			
Culture No	2%	10%	15%
DSs31 *A. penicillioides*			
	Few spores present	More amount of spores present	Decrease in sporulation with change in the colour of spores
40x			
100x			
	Thin mycelium and the with small fruiting body.	Broad and beaded of the mycelium, big fruiting body	Mycelium was thick and fruiting body distorted.

Fig 4.1d Cultural characteristics at increased salt concentration

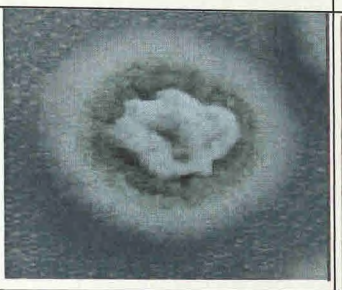
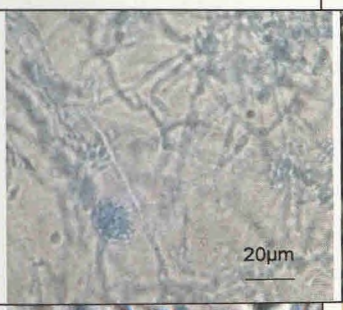
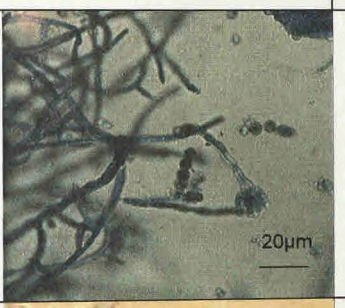
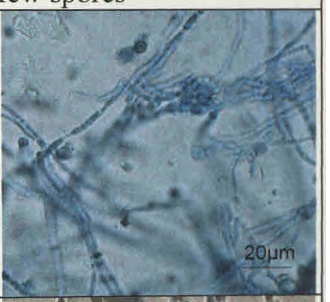
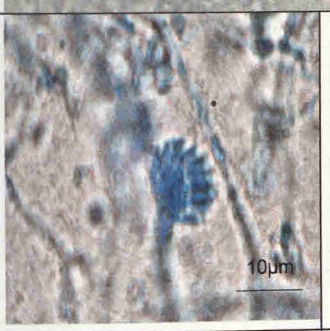
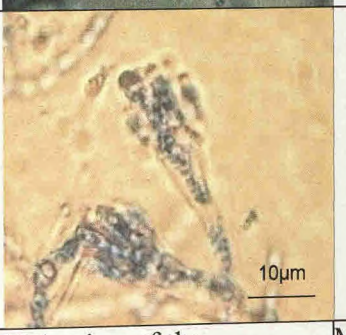
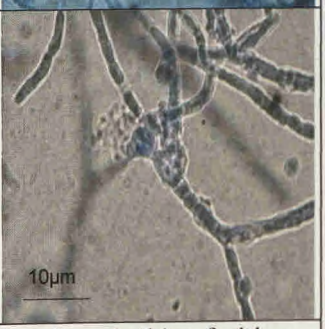
Salt concentration in CzA for growth			
Culture No	2%	10%	15%
DSs35 *A. penicillioides*			
	Decrease in sporulation	Increase in sporulation	Colony with a furrow, few spores
40x			
100x			
	Mycelium is thin	Thickening of the mycelium	Mycelium is thin, fruiting body distorted with fewer philades

Fig 4.1e Cultural characteristics at increased salt concentration

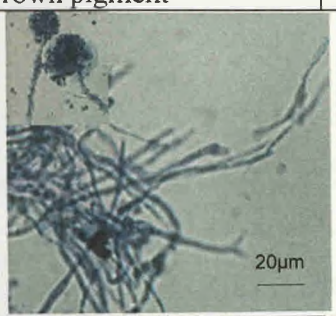
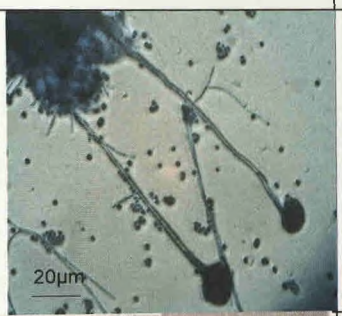
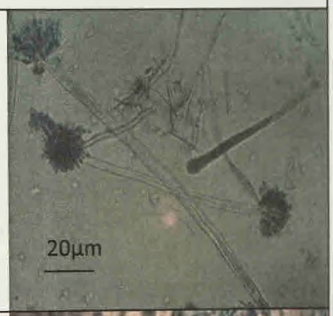
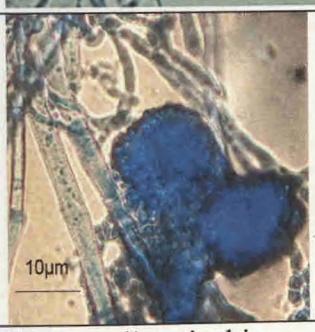
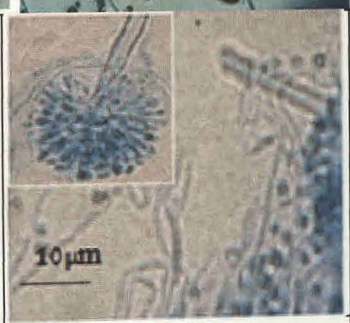
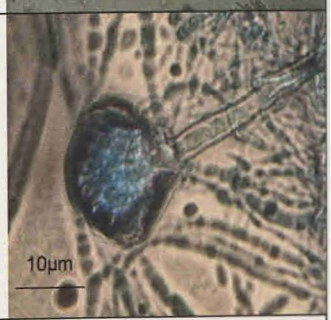
Salt concentration in CzA for growth			
Culture No	0%	10%	15%
DSs57 *A.versicolor*			
	Colony with a furrow, dark brown, diffusible brown pigment	Dark brown, diffusible brown pigment increases	No pigment, few spores
40x			
100x			
	The mycelium is thin	Thickening of the mycelium	Mycelium is thick and beaded , distorted fruiting body

Fig 4.1f Cultural characteristics at increased salt concentration

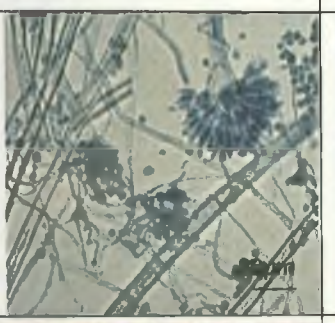
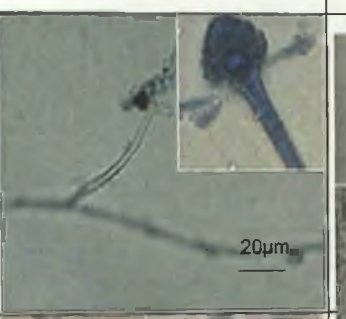
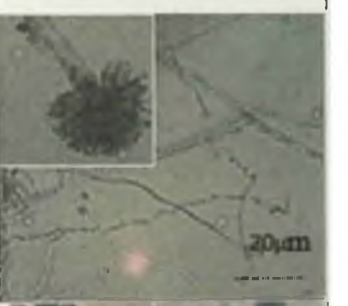
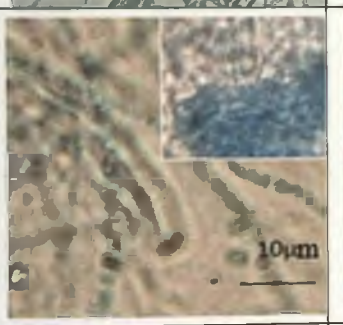
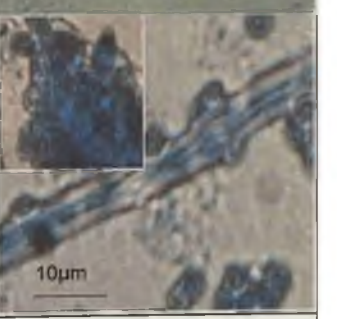
Salt concentration in CzA for growth			
Culture No	0%	5%	15%
DSs59 *A.versicolor*			
	Colony with concentric rings. pigment	Colony concentric, increase in pigment	Decrease in sporulation, no pigment
40x			
100x			
	Mycelium is thin	The mycelium is thick	Thick mycelium with distorted fruiting body

Fig 4.1g Cultural characteristics at increased salt concentration

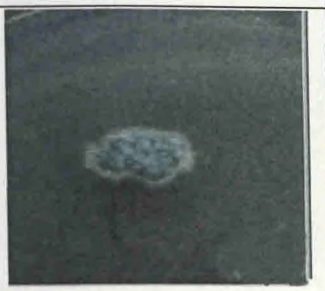
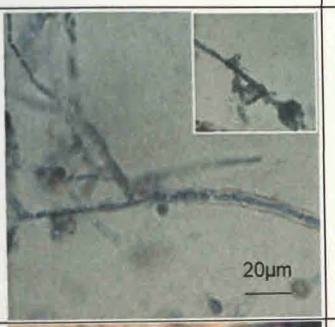
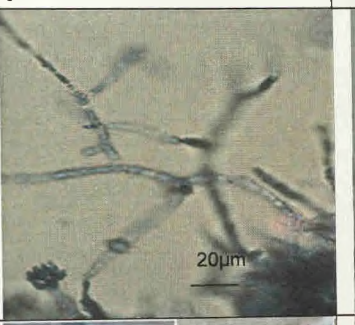
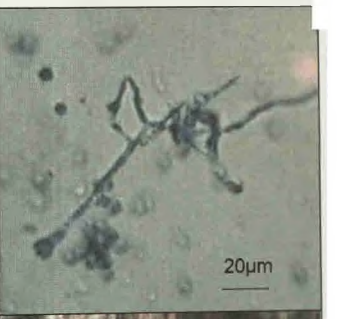
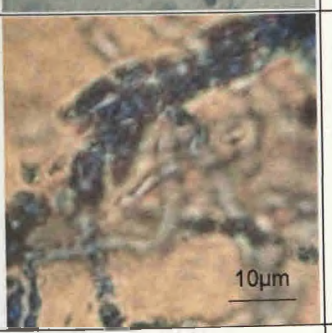
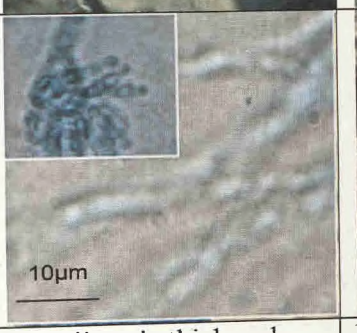
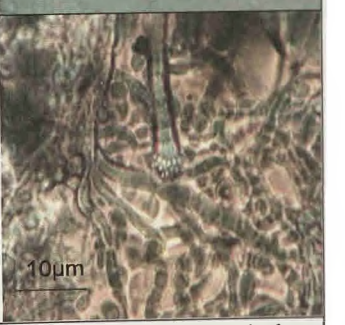
Salt concentration in CzA for growth			
Culture No	5%	10%	20%
EM4wb121 *A. penicillioides*			
	Less number of spores	Increase in number of spores	Decrease in sporulation
40x			
100x			
	Mycelium is thin	Mycelium is thick and beaded	Mycelium thick, beaded, fruiting body distorted with phialades clumped together

Fig 4.1h Cultural characteristics at increased salt concentration

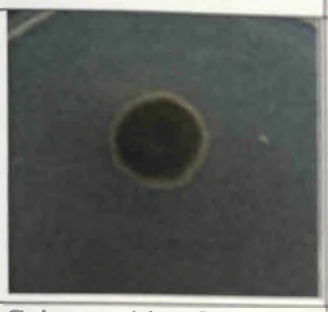
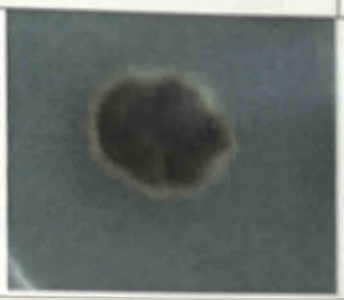
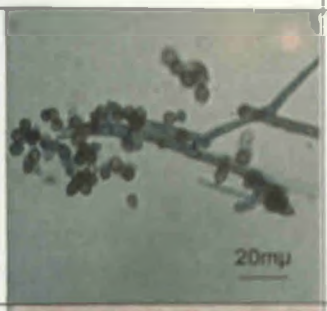
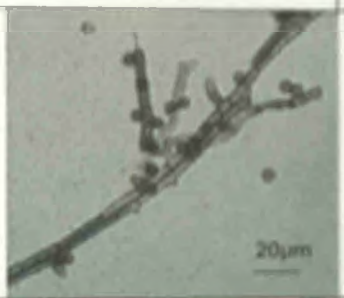
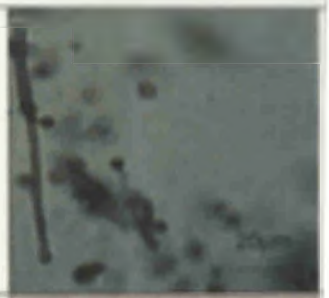
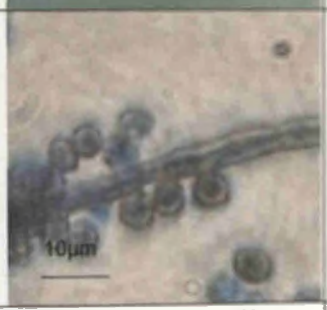
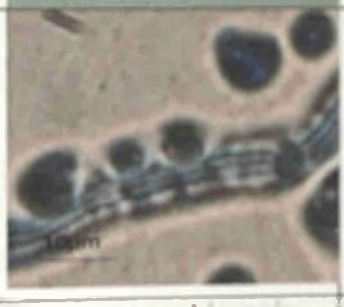
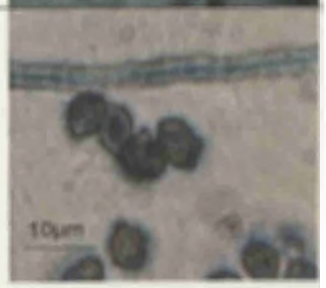
Salt concentration in CzA for growth			
Culture No	0%	2%	15%
EM4wb122 *C. cladosporioides*			
	Colony with a furrow black at the reverse	Colony with concentric rings, reverse black	Colony undulated, reverse black
40x			
100x			
	The spores are smaller	The spores are bigger in size.	Spores are bigger in size

Fig 4.1i Cultural characteristics at increased salt concentration

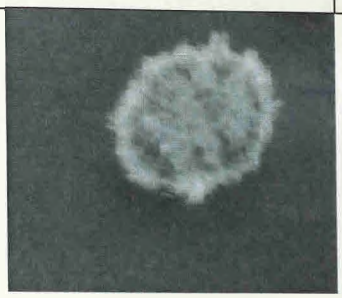
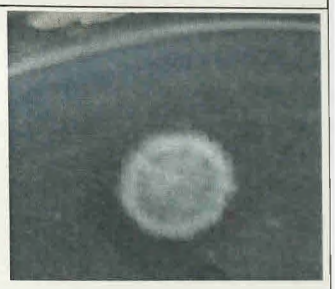
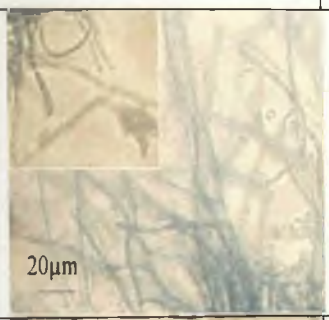
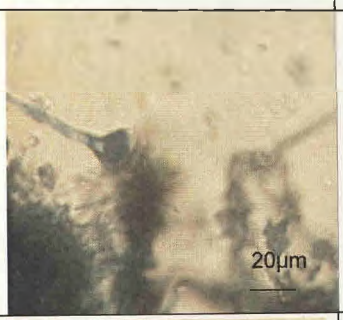
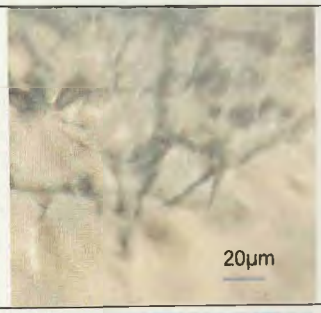
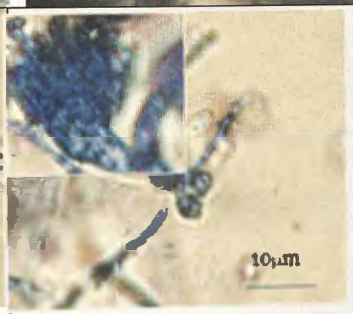
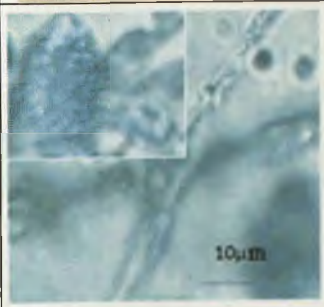
Salt concentration in CzA for growth			
Culture No	2%	10%	15%
MRw202 *A. penicillioides*			
	Decrease in sporulation	More spores present	Decrease in sporulation
40x			
100x			
	The mycelium is thin	Mycelium is thick	Mycelium is thick and fruiting body is distorted

Fig 4.1j Cultural characteristics at increased salt concentration

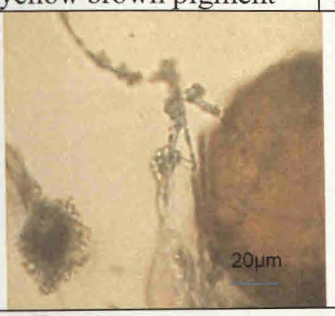
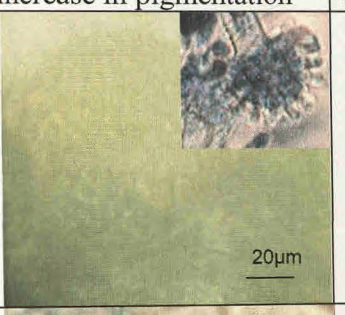
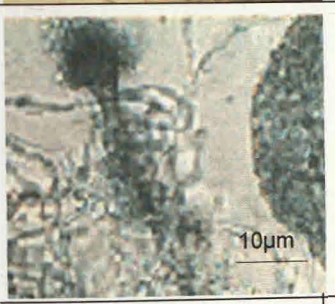
Salt concentration in CzA for growth			
Culture No	0%	5%	15%
MRw211 *E. amstelodami*			
	Green spores at the sides yellow brown pigment	Green spores at the sides, increase in pigmentation	Colony elevated at the centre, Green spores
40x			
100x			
	Cleistothecia more in number, few conidial heads.	Cleistothecia more,few conidial heads, thick mycelium	Cleistothecia more with few conidial heads only structure, mycelium is thick

Fig 4.1k Cultural characteristics at increased salt concentration

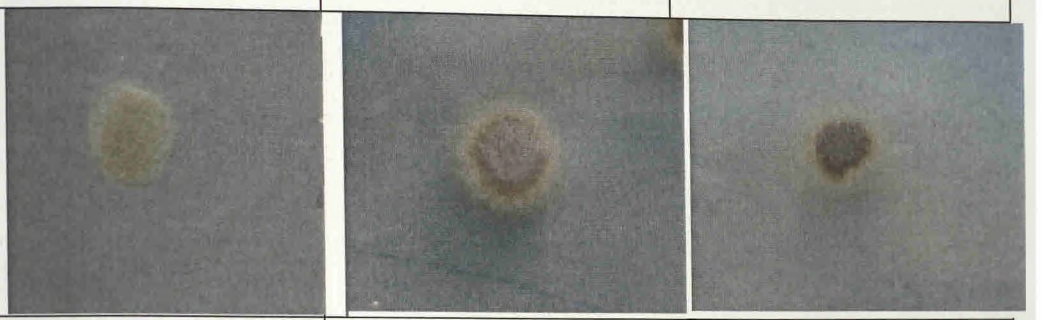
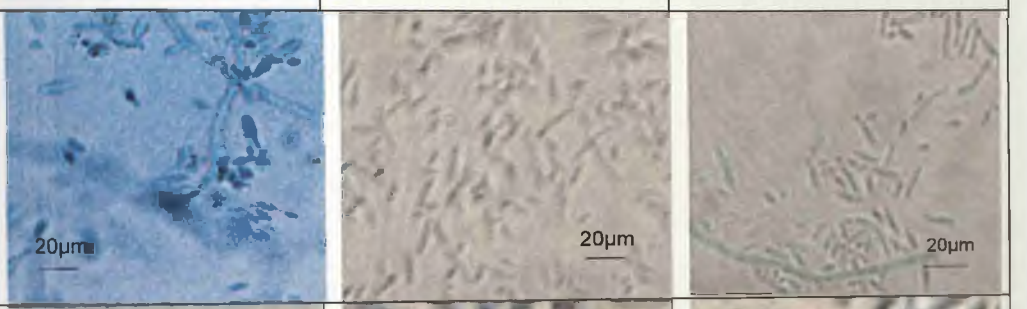
Salt concentration in CzA for growth			
Culture No	0%	5%	20%
MCw215 *H.werneckii*			
	Less spores seen	Increase in sporulation	Decrease in sporulation
40x			
100x			
	Mycelium along with few yeast like cells	They are seen as yeast-like cells and are long	They are seen as yeast like cells and are long

Fig 4.11 Cultural characteristics at increased salt concentration

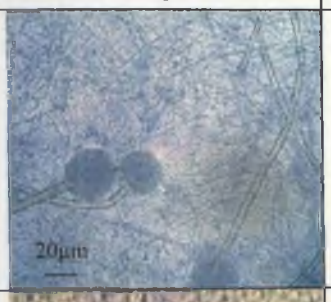
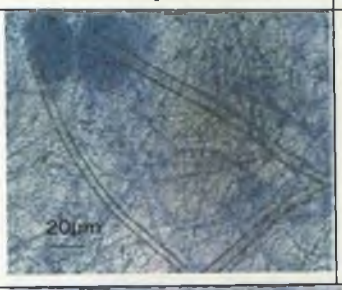
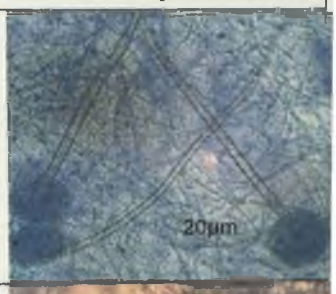
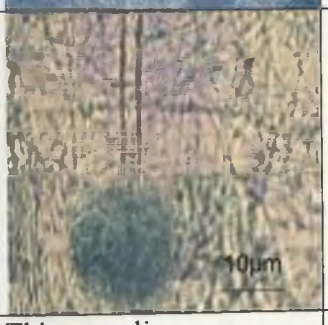
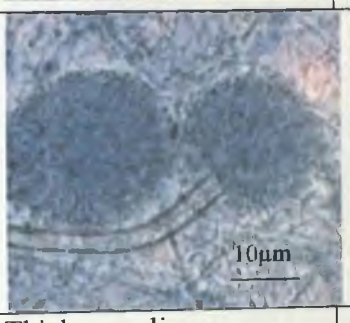
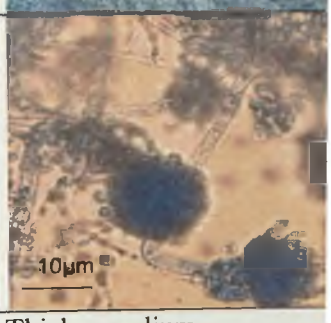
Salt concentration in CzA for growth			
Culture No	0%	5%	15%
MCs219 *A.flavipes*			
	Decrease in sporulation	Increase in sporulation	Decrease in sporulation
40x			
100x			
	Thin mycelium	Thick mycelium	Thick mycelium

Fig 4.1m Cultural characteristics at increased salt concentration

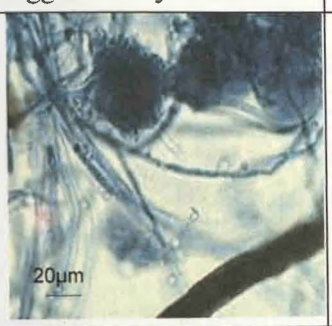
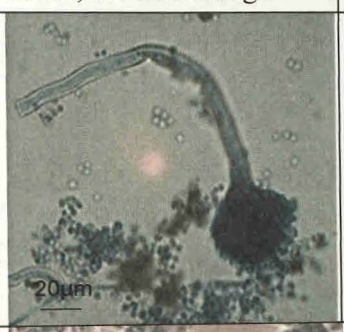
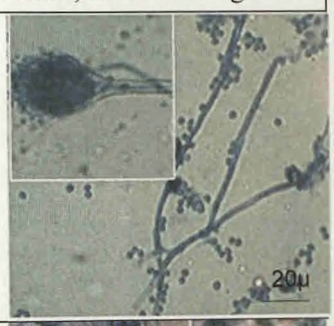
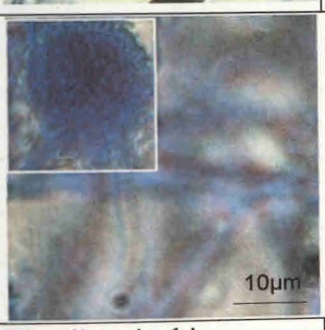
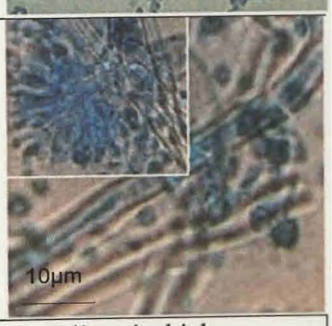
Salt concentration in CzA for growth			
Culture No	0%	2%	15%
MCs223 *A.terreus*			
	Decrease in spores, but bigger colony diameter	Colony elevated at the centre, reverse orange	Colony elevated at the centre, reverse orange
40x			
100x			
	Mycelium is thin	Thick mycelium	Mycelium is thick

Fig 4.1n Cultural characteristics at increased salt concentration

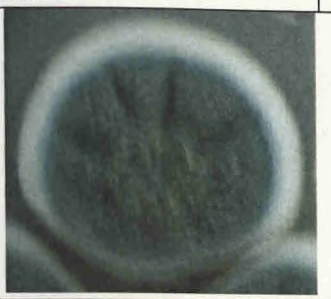
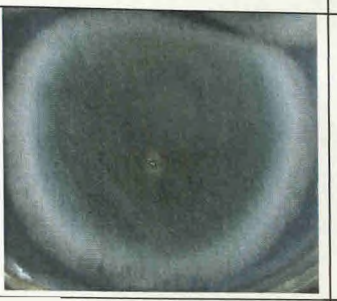
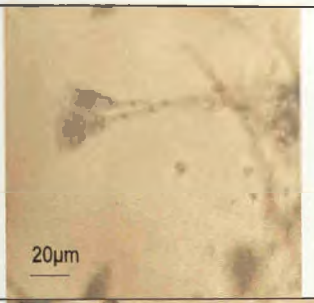
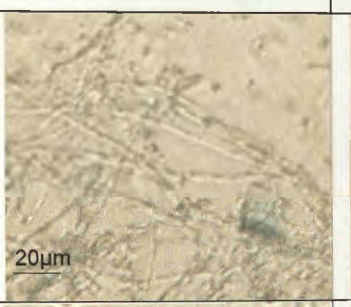
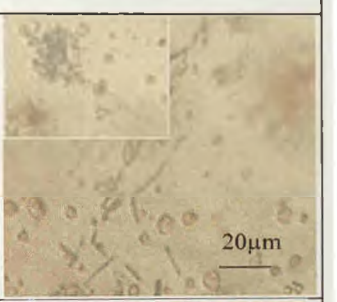
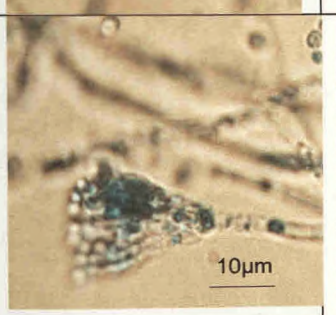
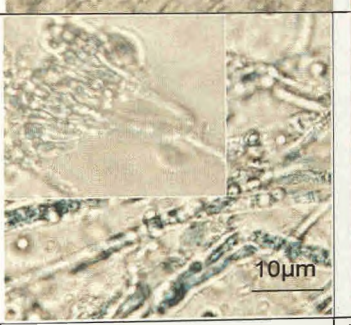
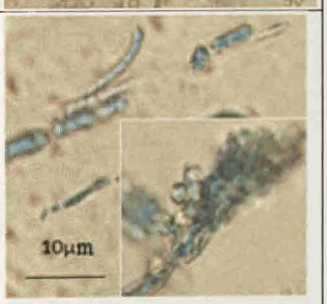
Salt concentration in CzA for growth			
Culture No	0%	5%	15%
SRw225 *P. chrysogenum*			
	Colony undulated, colourless exudate	Colony concentric Reverse white	Colony concentric Reverse white
40x			
100x			
	The mycelium is thin and the fruiting body is without branches	The mycelium is thick and the fruiting body is branched	The mycelium is beaded in between and the fruiting body is distorted

Fig 4.1o Cultural characteristics at increased salt concentration

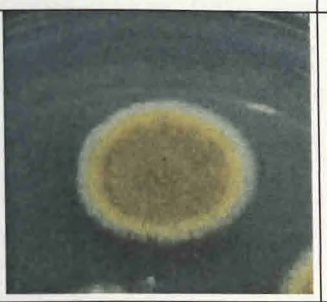
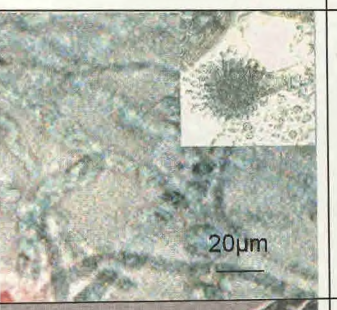
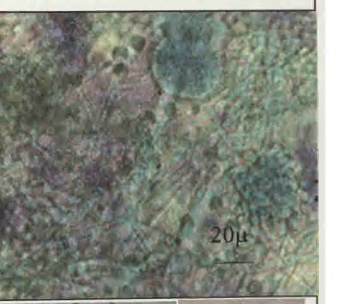
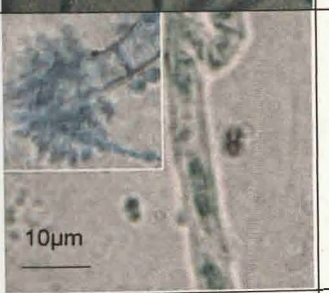
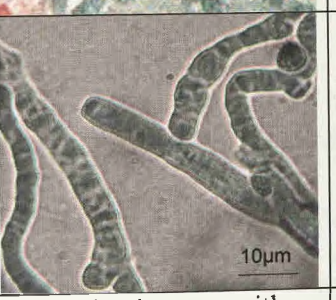
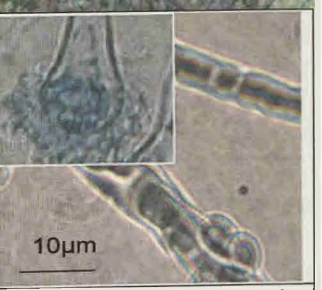
Salt concentration in CzA for growth			
Culture No	0%	5%	15%
SRs245 *E. amstelodami*			
	Yellow brown pigment	Increase in pigmentation	Green spores, , Reverse brown
40x			
100x			
	Conidial head with narrow base along with cleistothecia	Cleistothecia more with few conidial heads, mycelium is thick	Conidial head with broad base, along with cleistothecia, thick mycelium

Fig 4.1p Cultural characteristics at increased salt concentration

MCs219 *A. flavipes* showed thick mycelium at high concentration of salt. At high salt concentration the colony of MCs223 *A. terreus var terreus* was elevated at the centre, orange at the reverse. Mycelium was thick at optimum salt concentration while thick at high salt concentration.

At low concentration of salt the colonies of DSw25 and EM4wb122 *C. cladosporioides* showed decrease in sporulation Optimum concentration and high salt concentration showed spores which were bigger in size.

MRw211 *Eurotium amstelodami* at low concentration of salt showed more cleistothecia and few conidial heads which were small while at high salt concentration, mycelium was thick with few conidial heads and rare cleistothecia.

At low concentration of salt SRs245 *E. amstelodami* showed conidial heads along with cleistothecia while at optimum concentration there were few conidial heads only cleistothecia. At increased salt concentration cleistothecia were seen with few conidial heads. The colony of MCw215 *H. wernekii* was black and slimy. At low salt concentration, mycelium was seen along with few yeast cells. Optimum and high concentration showed only yeast cells and was cylindrical and thick.

In DSw13 *P. corylophilum* mycelium and fruiting body could be seen with long conidiophores at optimum concentration of salt. At high salt mycelium was thick and beaded, the fruiting body was distorted. At optimum concentration the mycelium of SRw225 *P. chrysogenum* was thick, at high concentration the mycelium was beaded with

distortion in the fruiting body. *Penicillium* isolates showed the formation of pale exudates at low salt concentration.

Cultural and morphological characteristics of the selected isolates at increased temperature are shown in Fig. 4.2 a-h. At temperatures yielding optimal growth; isolates grew well in comparison to the isolates at increased temperature than that required for optimal growth which grew with a lag and at slow rate; there was also decrease in the number of fruiting bodies and sporulation. The fruiting bodies were well formed and many in number, with dense sporulation at optimal temperature for growth.

At 30°C *A. penicillioides* DSw1 had thin mycelium while at 37°C the fruiting body was distorted. At 37°C the colonies of *A. penicililoides* DSw31, DSw35 showed thick mycelium and distorted fruiting structure while EM4wb121 MRw202 showed beaded mycelium and the fruiting body was distorted.

At 42°C the mycelium of DSw13 *P. corylophilum* was thick with distorted fruiting structure. SRw225 *P. chrysogenum* had short conidiophores and less fruiting body at 42°C.

A. versicolor DSs57 at 42°C had less philiades with beaded mycelium. The mycelium of DSs59 *A. versicolor* was thick at 42°C. MCs219 *A. flavipes* showed not much change at 37°C with exception of reduced number of spores at high salt concentration.

MCs223 *A. terreus var terreus* at 42°C had a thick mycelium while at 50°C no fruiting body was seen along with thick mycelium.

Morphologically MRw211 *E. amstelodami* at 42°C had few cleistothecia and more conidial heads with round and broad vesicle while at 37°C cleistothecia was seen with few conidial heads. Morphologically at 30°C MRw245 *E. amstelodami* had cleistothecia with few conidial heads, at 42°C few cleistothecia was seen with conidial heads.

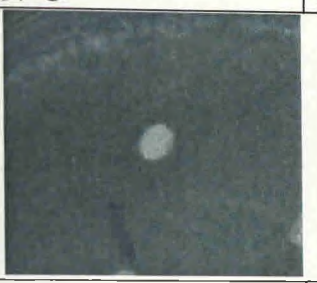
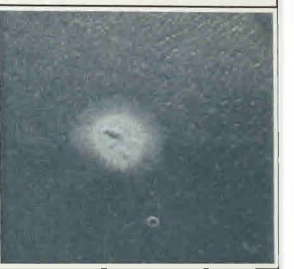
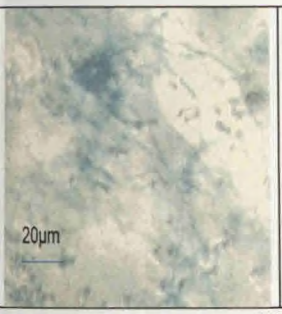
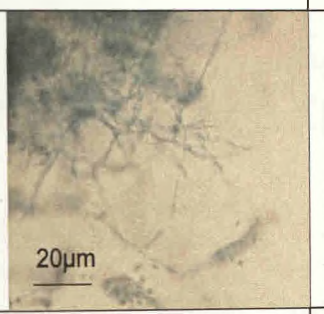
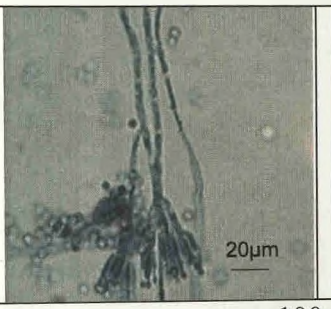
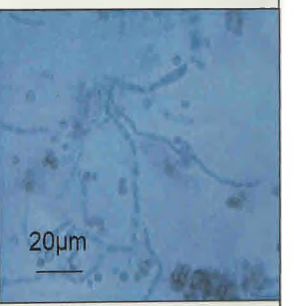
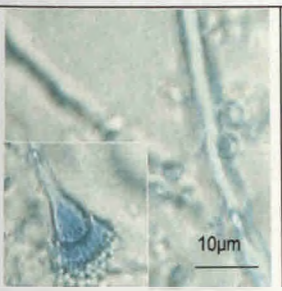
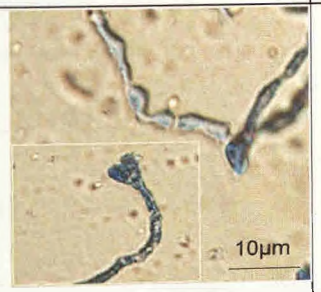
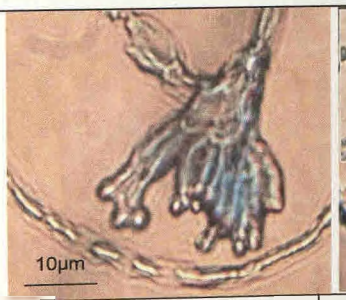
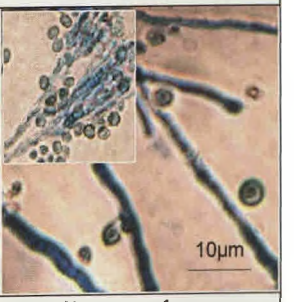
Culture No			
DSw1 *A. penicillioides*		DSw13 *P. corylophilum*	
30°C	37°C	30°C	42°C
			
Colony elevated, increased spores	Dark green, reverse green	More spores present undulated, pale exudates present	Colony elevated at the centre, undulated
40x		40x	
			
100x		100x	
			
The fruiting body is big, the mycelium is thin	The mycelium and the fruiting body is distorted	The mycelium and the fruiting body with long conidiophore	Mycelium and spores are thick, fruiting body is distorted

Fig. 4.2a Cultural characteristics at increased temperature

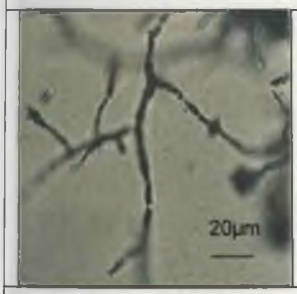
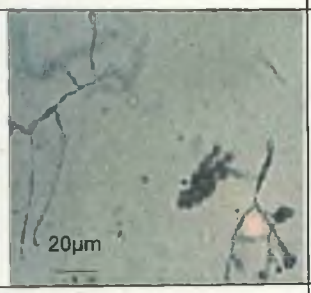
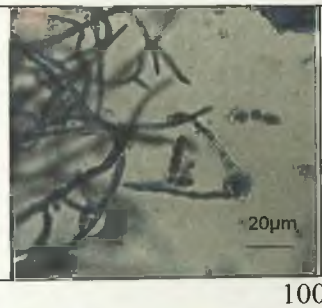
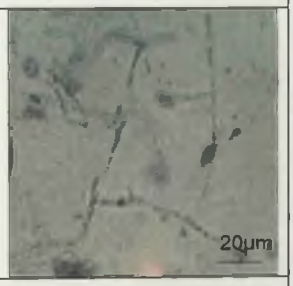
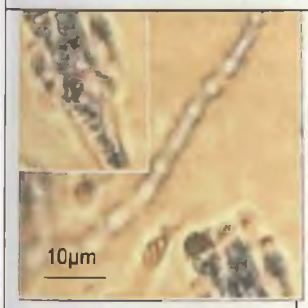
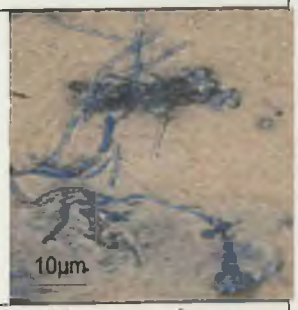
Culture No			
DSs31 *A .penicillioides*		DSs35 *A .penicillioides*	
30°C	37°C	30°C	37°C
			
More amount of spores present	Slight change in the colour of spores	Increase in sporulation,	Few spores seen
40x		40x	
			
100x		100x	
			
Thick and beaded mycelium, the fruiting body is big	Fruiting body is distorted	There is a broadening of the mycelium	Fruiting body is distorted

Fig. 4.2b Cultural characteristics at increased temperature

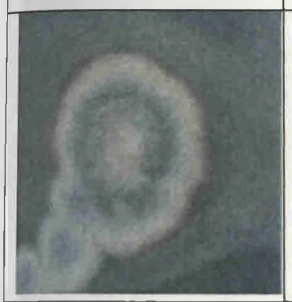
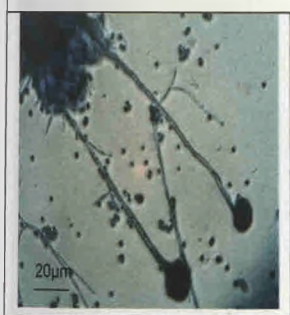
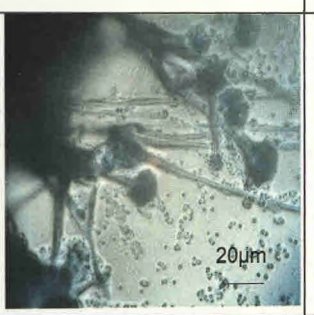
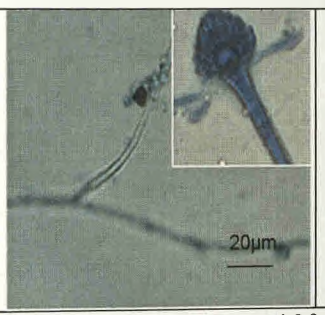
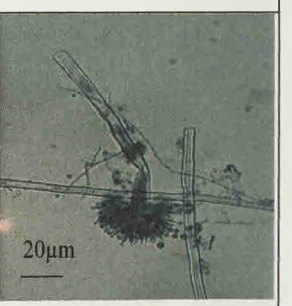
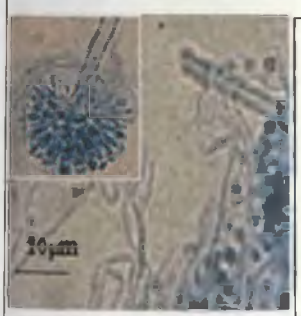
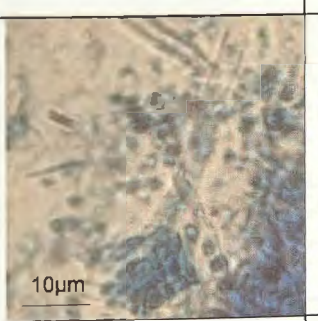
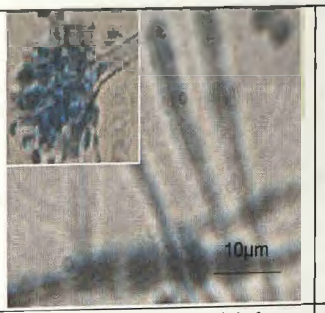
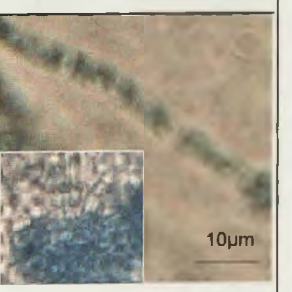
Culture No			
DSs57 *A. versicolor*		DSs59 *A.versicolor*	
30°C	42°C	30°C	37°C
			
Colony concentric undulated reverse yellow white	Colony with concentric rings	Colony with concentric, rings	Colony elevated
40x		40x	
			
100x		100x	
			
Thickening of the mycelium	Less philades and the mycelium is beaded	The mycelium is thick	The mycelium is thick

Fig. 4.2c Cultural characteristics at increased temperature

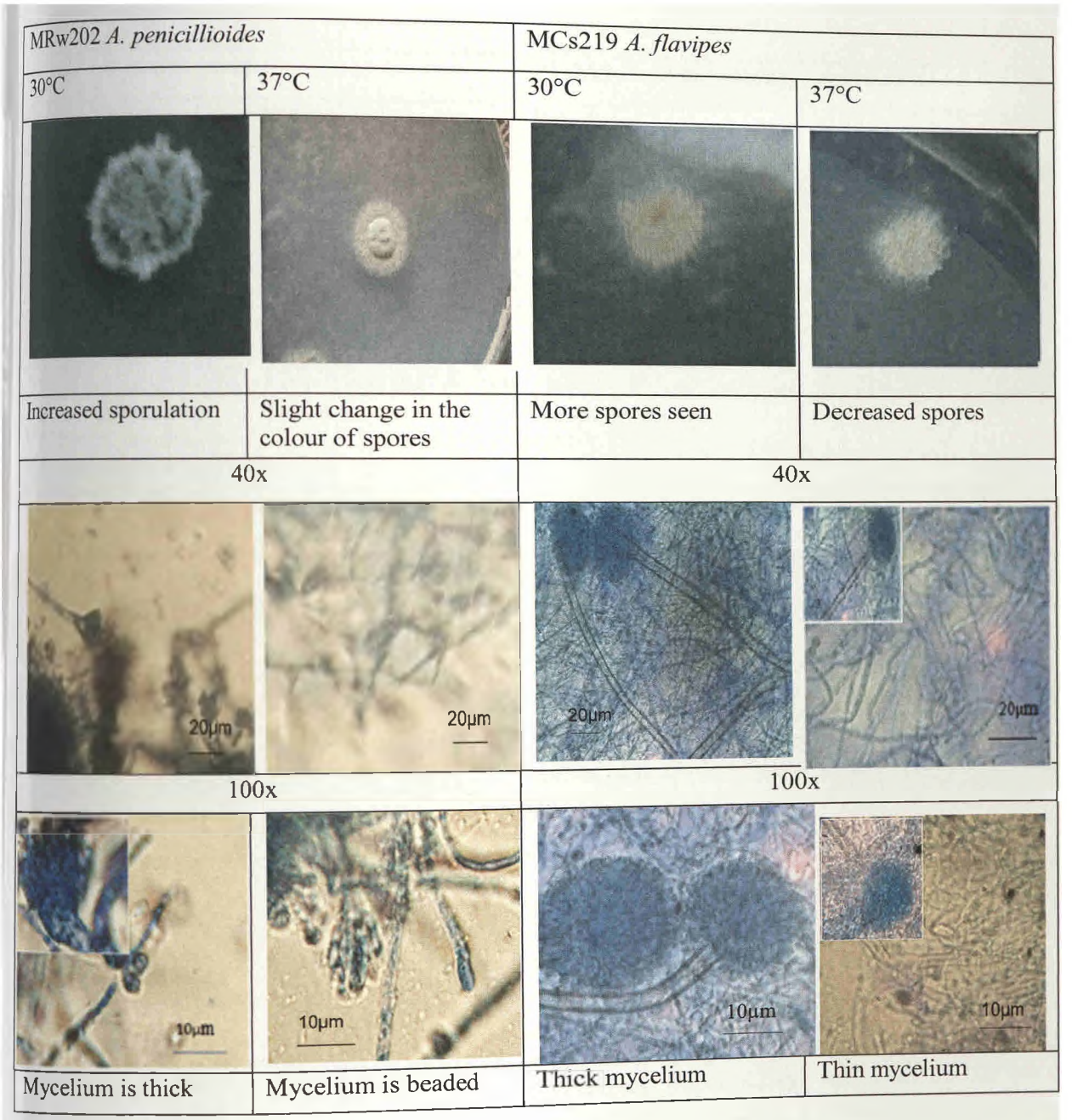


Fig. 4.2d Cultural characteristics at increased temperature

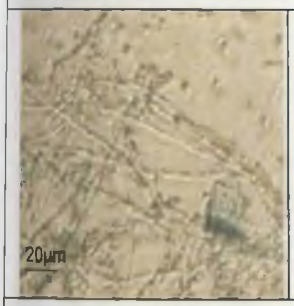
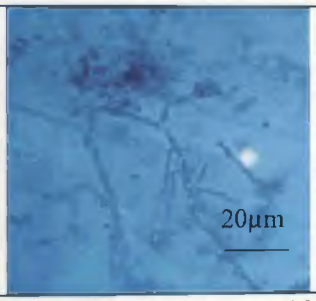
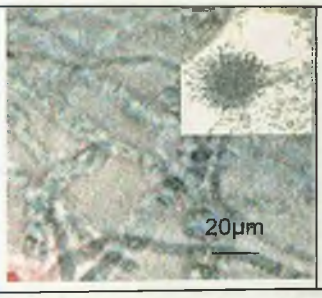
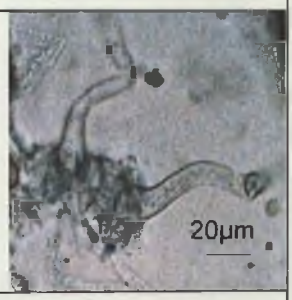
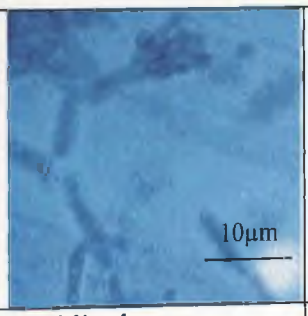
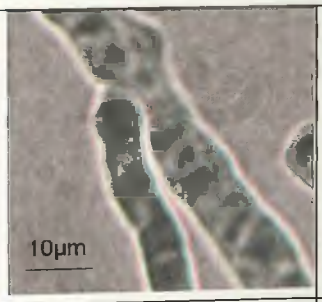
SRw225 *P. chrysogenum*		SRs245 *E. amstelodami*	
30°C	42°C	30°C	37°C
			
Colony concentric Colourless droplets, Reverse pale yellow	Decreased spores	Green spores at the centre.	Decreased spores
40x			
			
100x			
			
Mycelium and the fruiting body	Conidiophores are short.	Cleistothecia were seen with few conidial heads.	Cleistothecia were few in number, conidial heads were seen

Fig. 4.2e Cultural characteristics at increased temperature

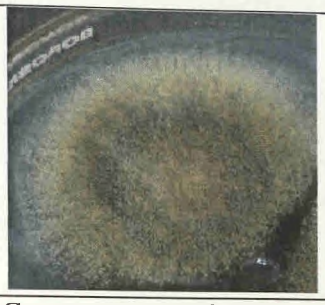
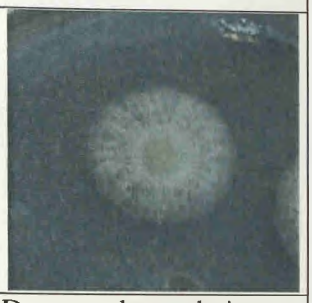
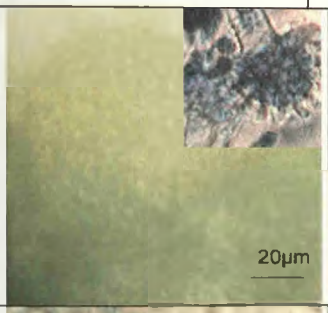
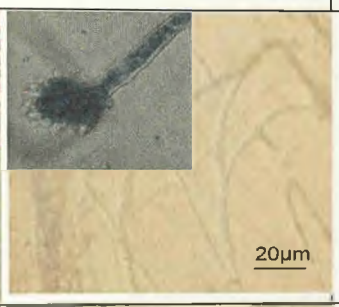
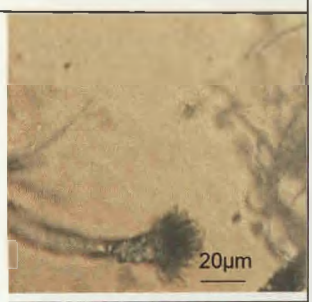
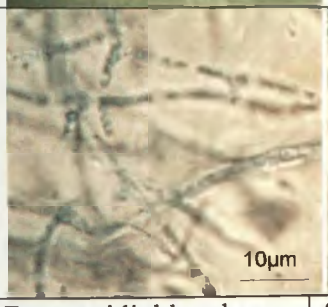
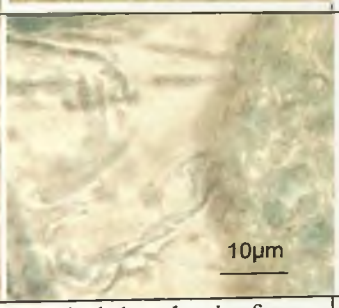
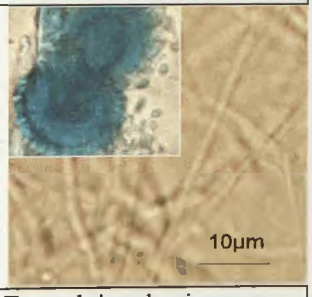
Culture No	30°C	37°C	42°C
MRw211 *E.amstelodami*			
	Colony elevated, green spores at the sides.	Green spores at the sides.	Decreased sporulation
40x			
100x			
	Few conidial head more cleistothecia was seen	Small cleistothecia, few conidial head	Few cleistothecia, conidial head

Fig. 4.2f Cultural characteristics at increased temperature

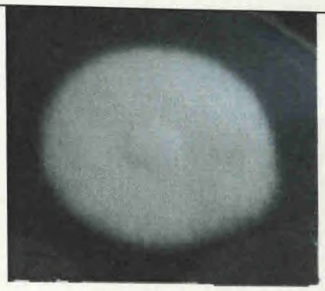
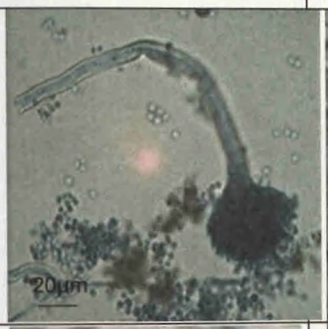
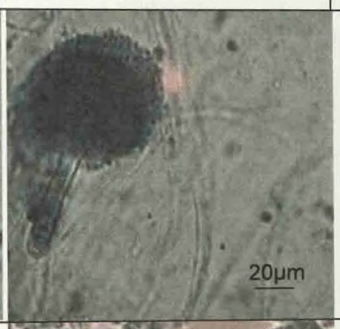
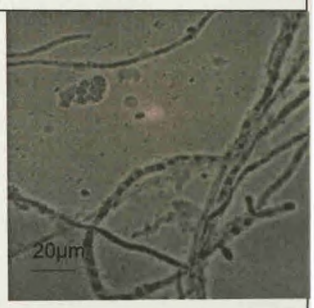
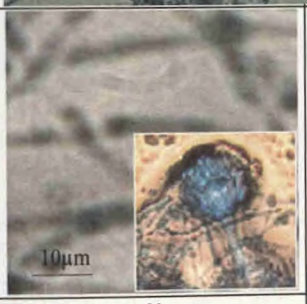
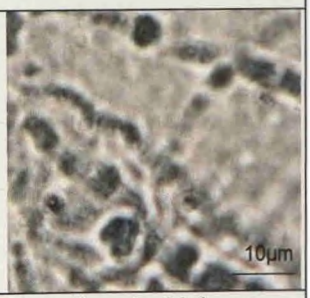
Culture No	30°C	42°C	50°C
MCs223 *A. terreus var terreus*			
	Colony elevated, brown spores, , orange at the reverse	Colony increased in diameter, few spores present	Colony increased in diameter, no sporulation
40x			
100x			
	Thick mycelium	Mycelium is thin	Mycelium is thick, no fruiting body

Fig. 4.2g Cultural characteristics at increased temperature

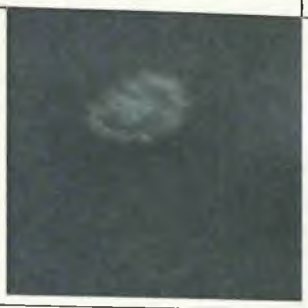
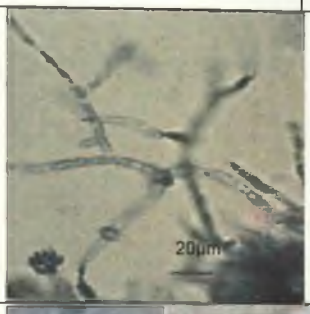
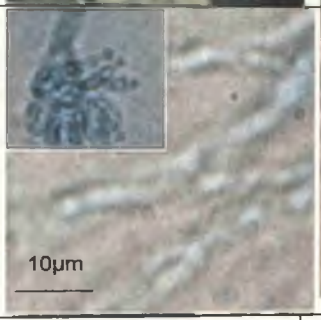
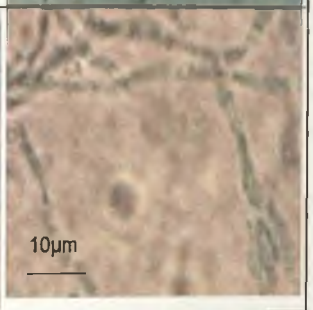
Culture No	30°C	37°C
EM4wb121 *A. penicillioides*		
	Colony undulated, elevated, green spores	Decreased sporulation.
40x		
100x		
	Mycelium is thick The spores are smaller In size	Mycelium is thin and is beaded

Fig. 4.2h Cultural characteristics at increased temperature

4.4 Discussion

Fungal growth increased with increasing NaCl concentration up to a point supporting maximal growth and sporulation, beyond which there was a decrease in growth. Thus, salt concentrations more than or less than that supporting a good growth and sporulation, resulted in a slower growth and reduction in biomass. Stress can imbalance the osmotic potential in fungal cells, thus generating a water scarcity, and the influx of sodium may lead to metabolic toxicity which could be the reason for the drastic reduction in biomass (Adler *et al.* 1985, Larsson and Gustavsson 1993, Neves *et al.* 1997, Obuekwe *et al.* 2005). It is known that microorganisms under stress are less efficient in the conversion of substrates into microbial biomass synthesis (Bardgett and Saggar, 1994).

Colony characteristics showed that in some isolates there is hardening of the colony and pigment production increased. Similar stimulating effect of NaCl on pigment production was reported by Vigants *et al.* (1995). However, a delayed pigment production has also been reported by Alder *et al.* (1985), Larsson and Gustavsson (1993), Neves *et al.* (1997), Obuekwe *et al.* (2005). Babitha *et al.* (2007) reported that when *Monascus* cells suffer from hydrolysis, they overproduce hydrophobic substances such as pigments for blocking these enzymatic attacks. In the present study, since the organisms were subjected to extreme saline stress, thick walled mycelium and overproduction of hydrophobic pigments could be a possible way to encounter the loss of water from the cytoplasm to the surrounding medium.

Increased concentrations of salt also resulted in increase in the width of the mycelium, thickening of cell wall, formation of bulbous structures and distortion in the fruiting structure. Kunic *et al.* (2010) reported that thick cell wall might be important as armor

against changes of osmotic pressure, since it provides a mechanical protection against hyposaline stress at dilution conditions. Electron micrograph of *Trichoderma pilluliferum halophila* showed rough hyphae, flask shaped conidiophores with smooth and rough conidia, by increasing salt concentration to 15%(w/v) sodium chloride showing conidiophores branching more or less symmetrically near the tips and with short stipes. *A. restrictus* was unable to produce phialides or conidia and appeared as sterile heads, these heads become spathulate to globose in shape. Electron micrograph of *A. restrictus* grown at 10% sodium chloride showing conidiophores, long hyaline and smooth-walled with hemispherical or flask shaped vesicle lacking metulae. Conidial heads columnar, conidia ellipsoidal, echinulate, while at 20% sodium chloride showing flask shape phialides with chain of globose rough conidia (El Meleigy *et al.* 2010).

Bartnicki-Garcia *et al.* (2000) reported that hyper-branched hyphal tips under glycerol stress may be the result of the inability of the organism to maintain positive turgor pressure, thus inducing premature branching. Kuncic *et al.* (2010) correlated increase in cell wall thickness with successful growth at low a_w. Shortening and thickening of the hyphal compartments in halotolerant species has been observed (Clipson *et al.*, 1989). Babich and Stotzky (1982) correlated the extended log phase of cells under metal stress, to the period required by the cells to repair the damage caused by metals.

Spore formation has been generally accepted as a response to environmentally adverse conditions (Shin *et al.*1998, Suh and Shin 2000). Conidiation was suppressed at the 3% salt level in *Fonsecaea compacta* and *Phialophora verruscosa*, at 5% salt in *Fonsecaea pedrosoi* and at 7 to 9% salt in *Exophiala jeanselmei*. Similar results were observed in this study where condiation of the obligate halophiles was maximum at 10% salt and suppressed at

lower concentrations of 2%-5% salt and at higher concentrations of 20%-30% salt. Facultative halophiles showed maximum conidiation at 2%-10% salt whereas conidiation was suppressed at 0% and also at 20%-30% salt. This corroborates the results of Kane and Summerbell (1987) wherein few conidia were formed by *Alternaria* on standard laboratory medium but was increased in presence of salt, being maximum at 9% salt concentrations, after which point conidiation decreased. Other alterations noted included suppression of aerial mycelium, conversion from filamentous to moniliform growth, secretion of oily substances, enhanced roughness of verrucose cell walls, and suppression of cell wall melanization.

For most of the microorganisms, temperature is a critical environmental signal for regulating developmental and physiological process. However, at higher temperature growth was reduced drastically, pigment yield increased remarkably under a heat shock. It has been reported that following exposure to elevated temperature, many organisms rapidly synthesize a highly conserved set of proteins termed heat shock proteins and their induction appears to correlate with the adaptation of the organism to hypothermic stress condition (Schlesinger 1990).

Bamford and Heath (2011) investigated the effect of environmental temperature on spore germination in *Ascosphaera apis* and reported that activation and enlargement of the spore was independent of temperature within a range of 10 to 40°C, and 25 to 40°C, respectively. Germ-tube production occurs within the range 25 to 37°C, with an optimum between 31°C and 35°C and was temperature related.

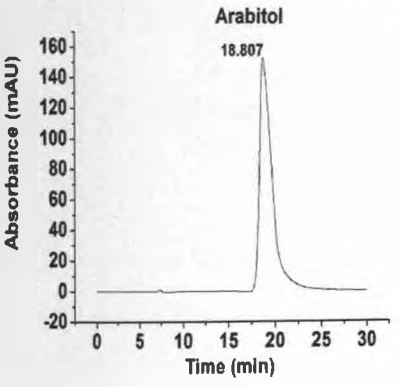
Changes seen in the colony and morphological characteristics such as compact growth with reduced number of spores, distortion in fruiting structure during growth at elevated temperatures could be attributed to the adverse effect of heat on the growth of the fungi which therefore resulted in a decreased growth. Morphological changes as a result of thermal stress were observed in mesophilic fungus *M. purpureus* with optimum growth temperature below 35°C. Thickening of the cell wall could transiently protect the cells by absorbing thermal energy (Rosasa and Casadevall 1997, Han *et al.* 2004).

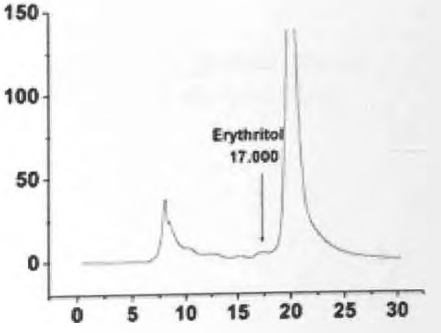
Nikolaou *et al.* (2009) reported that microbes must sense environmental stresses, transduce these signals and mount protective responses to survive in hostile environment. Significant changes in fungal colony and morphology were observed in response to stress of varying salt concentrations and of higher temperatures, resulting in a decrease in growth and consequently in sporulation, which could be an expression of the changes occurring in their natural habitats.

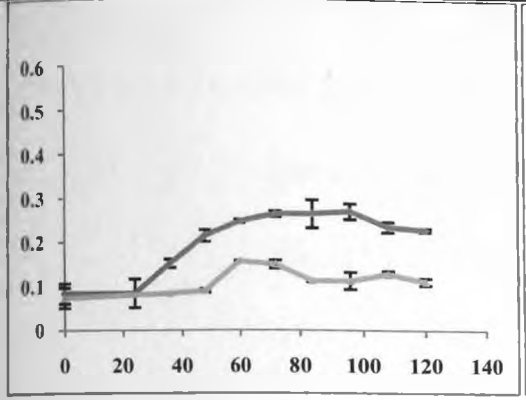
4.5 Conclusion

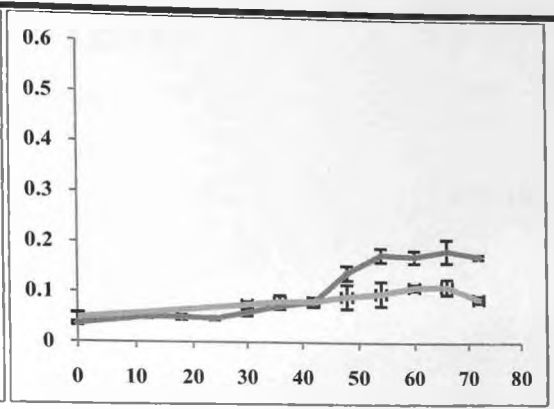
Cultural and morphological changes were induced in the presence of increasing amount of salt. Decrease in sporulation, delayed growth, thickened and bulbous mycelium with frequent septation, distortion in fruiting body was observed in the presence of salt. Cultural characteristics like decrease in sporulation, delayed growth with prolonged lag phase was observed. With increase in temperature the isolates of *Aspergillus* and *Penicillium* showed distortion in fruiting bodies.

OSMOLYTE PRODUCTION






0.6
0.5
0.4
0.3
0.2
0.1
0
0 20 40 60 80 100 120 140


0.6
0.5
0.4
0.3
0.2
0.1
0
0 10 20 30 40 50 60 70 80

CHAPTER 5

CONTENTS

CHAPTER 5: OSMOLYTE PRODUCTION

5.1 Introduction

This chapter deals with the osmolyte production at different growth phases under optimum salt concentration and at increased temperature. Microorganisms are adapted for optimum functioning in their normal physiological environments. Any extreme change in environmental conditions from the optimum inflicts a stress on an organism. The extent of the change will determine whether the organism is killed, ceases growth, or has an increased lag time and reduced growth rate (Ray 1986, Russell *et al.* 1995).

Stress conditions imposed on yeast can be diverse as entry into stationary phase, nutrition starvation, high and low temperature, high and low osmolarity, high ethanol concentration and presence of heavy metals or oxidative compounds. It is well known that glycogen and trehalose accumulates in yeast under nutrient starvation or entering into the stationary phase of growth, and high levels of trehalose are found in heat-shocked cells. The filamentous fungus *Aspergillus niger* produces a number of different polyols, including glycerol, erythritol, and D-mannitol. The intracellular concentrations of the individual polyols in *A. niger* depend on growth conditions and developmental stage, suggesting that polyols have important functions in fungal physiology (Witteveeen and Visser 1995).

Tolerance of heat shock has been often associated with an increased intracellular trehalose concentration (Hallsworth and Magan 1994). Trehalose can enhance the resistance of cellular components against adverse conditions such as extreme temperature, dehydration or osmotic stress (Van Laere 1989, Miekle *et al.* 1991, Piper 1993). Miekle *et al.* (1991) found that yeasts accumulated greater amounts of intracellular glycerol along with trehalose, arabitol and ribitol when grown with NaCl.

The present study has been carried out to find out the osmolytes produced at high salt concentration with respect to the exponential and stationary growth phases, and the effect of temperature on osmolyte production.

5.2 Materials and Methods

Osmolytes were detected at mid-exponential phase and at stationary phase. For increased temperature the osmolytes were detected at mid-exponential phase.

5.2.1 Growth of the culture

Growth curve was studied by inoculating (10^6) spores in 26 flasks containing CzB supplemented with 2% or 5% or 10% salt, as required for optimal growth of the respective isolate and incubating at 30°C, 150 rpm. Two flasks were taken out at each interval of 0, 12, 15, 18h and thereafter at every 6h intervals up to 72h and the mycelia filtered through a pre-weighed Whatman filter paper No.1. The biomass was dried in the oven at 65°C till constant weight was obtained.

The growth curves of the selected isolates were also carried out at 37°C/ 42°C in similar manner.

Those isolates which did not grow initially at 42°C were inoculated as above and incubated at 30°C, 150rpm, till they germinated. The period for germination was determined by examining microscopically, 0.5µl of suspension at 3h intervals, using lactophenol cotton blue stain. After germination the flasks were then incubated at 42°C and readings were obtained as above.

Statistically significant difference ($P<0.05$) of growth of the isolates at optimum salt concentrations and increased temperature was analysed by two-way ANOVA.

5.2.2 Detection of Osmolytes

Osmolytes were detected by thin layer chromatography and confirmed by HPLC.

5.2.2.1 Thin layer chromatography

5.2.2.1A Preparation of plates

The plates used for thin layer chromatography (TLC) were prefixed with leucoplast on either side to obtain the thickness of the silica gel layer as well as to fix the plates in position. Silica gel slurry was prepared in water and spread evenly onto the plate, with the help of rod. After air-drying, the plates were activated in the oven at 110°C for 1h prior to use.

5.2.2.1B Preparation of the chambers

The walls of the solvent chamber were covered with Whatman filter paper to facilitate saturation of the chamber with the solvent vapours. The solvent used as the mobile phase was ethyl acetate : butanol : water (6:3:1). The solvent was poured in to the chamber and kept overnight for saturation, prior to use.

5.2.2.1C Preparation of Standards

Standards (2%) were prepared with distilled water. Standards used were glucose, trehalose, arabitol, erythritol, glycerol, inositol, mannitol, sorbitol and xylitol.

5.2.2.1D Preparation of Samples

Samples were prepared according to the method of Hallsworth and Magan 1994 as described by Ramirez *et al.* (2004). Fresh mycelia (10–50mg) at mid-exponential phase and stationary phase of cultures grown at 30°C from all the samples were weighed in 2ml eppendorf microtubes. Deionised water (1ml) was added to each sample. The mixture was kept at -20C for 1h, then crushed with mortar and pestle, after which it was sonicated with 10 pulses of 150mv for 20 seconds each at an interval of 10 seconds (B-BRAUN LABSONIC). The period of sonication required was determined by sonicating at pulse of 1min at 28µm for increasing periods of time and examining an aliquote microscopically at 1min intervals between 3-6min, for the complete disruption of mycelia. The samples were then boiled in water bath 5.5min and allowed to cool. Samples were shaken vigorously and then centrifuged at 13000rpm in an eppendrof centrifuge 5417R for 15min. The resulting supernatant was used to run TLC.

Mycelial extracts of cultures at mid-exponential phase when grown at 37°C/42°C were obtained in similar manner.

5.2.2.1E Sample spotting

The samples along with standards were spotted on the activated plate, taking care to keep the sample spot to a minimum size; the plate was then placed in the saturated chamber. The solvent was allowed to run ¾ of the plate, which was then removed and allowed to dry before spraying with the reagent.

5.2.2.1F Spraying reagent

The spraying agent consisted of 0.5%KMnO4 in 1N NaOH.

5.2.2.2 HPLC

5.2.2.2A Extraction of osmolytes

Samples were prepared as that used for TLC analysis, using deionised water; to the samples that were boiled and then cooled, was added Acetonitrile:water (650µl; 40:60). Samples were shaken vigorously and then centrifuged at 13000rpm in an eppendrof centrifuge 5417R for 15 min. The resulting supernatant was taken up into a sterile 1 ml syringe and filtered through a 0.2µm, 13mm diameter, nylon syringe filter (Whatman) directly into HPLC vials and sealed. HPLC was carried out using WATERS HPLC, Sugar-PAK column (6.5x300mm) and refractive index detector (WATERS 2410). Statistically significant difference ($P<0.05$) of the osmolytes produced at different growth phase and at increased temperature was analysed by two-way ANOVA.

5.3 Result

5.3.1 Growth Curve at 30°C

The growth curves of the different isolates are shown in Fig. 5.1. The obligate halophiles showed a similar pattern of growth. *A. penicillioides* DSw1, DSs31, DSs35, EM4wb121 and MRw202 grew as small pellets after a prolonged lag phase and entered the stationary phase at 60-84h: DSs31, DSs35 and MRw202 at 60h, EM4wb121 and DSw1 at 72h and 84h respectively.

The facultative halophiles grew in the form of medium-size pellets. *A. versicolor* DSs57 and DSs59 entered the stationary phase at 84h and 54h respectively. *A. flavipes* MCs219, *A. terreus* MCs223 entered the log phase at 48h and had the stationary phase from 60h. *Eurotium* showed filamentous type of growth. *E. amstelodami* SRs245, MRw211 entered the log phase after 18hrs and 15hrs respectively while the stationary phase at 60h and 30h respectively. The *Penicillium* isolates entered the log phase after 18hr; DSw13 *P. corylophilum* reached stationary phase at 66h and SRw225 *P. chrysogenum* at 30h. *C. cladosporioides* DSw25 entered the log phase after 12h and the stationary phase at 24h; EM4wb122 entered the log phase after 24h and stationary phase from 30h. *H. werneckii* MCw215 had a log phase from 12h and stationary phase at 42h.

5.3.2 Growth Curve at 42°C

The lag phase was longer than that at 30°C and the log phase was short, growth was in the form of small pellets. In the halophiles *A. penicillioides* DSw31, DSw35 log phase was from 48-60h while *A. penicillioides* DSw1, EM4wb121 log phase was from 36-84h after which they entered the stationary phase. *A. flavipes* MCs219, *A. terreus var terreus* MCs223 and *A. versicolor* DSw57 had a log phase from 54-60h, 18-54h, 24-84h respectively, after which they entered the stationary phase.

MRw211, SRs245 *E. amstelodami* had a log phase up to 60h after which they entered the stationary phase. *P. corylophilum* DSw13 and *P. chrysogenum* SRw225 had an exponential phase of 42-54h and 18-42h respectively thereafter which they entered the stationary phase. *A. penicillioides* Dsw1, EM4wb121 MCs219 *A. flavipes* took 35h, 36h and 24h respectively for germination of 50% of spores. Fig. 5.1 shows the growth curve of the isolates at 30°C and 42°C.

The ANOVA values were significantly different for the growth curve among the same genus as P value is less than 0.05.

5.3.3 Osmolytes produced in relation to growth phase.

Osmolytes produced by the isolates at different growth phases or at varied temperatures detected by TLC, are shown in Fig. 5.2a and 5.2b. These results were confirmed by HPLC analysis. Osmolytes produced in relation to growth phase are shown in Fig. 5.3a, 5.3b, 5.3c.

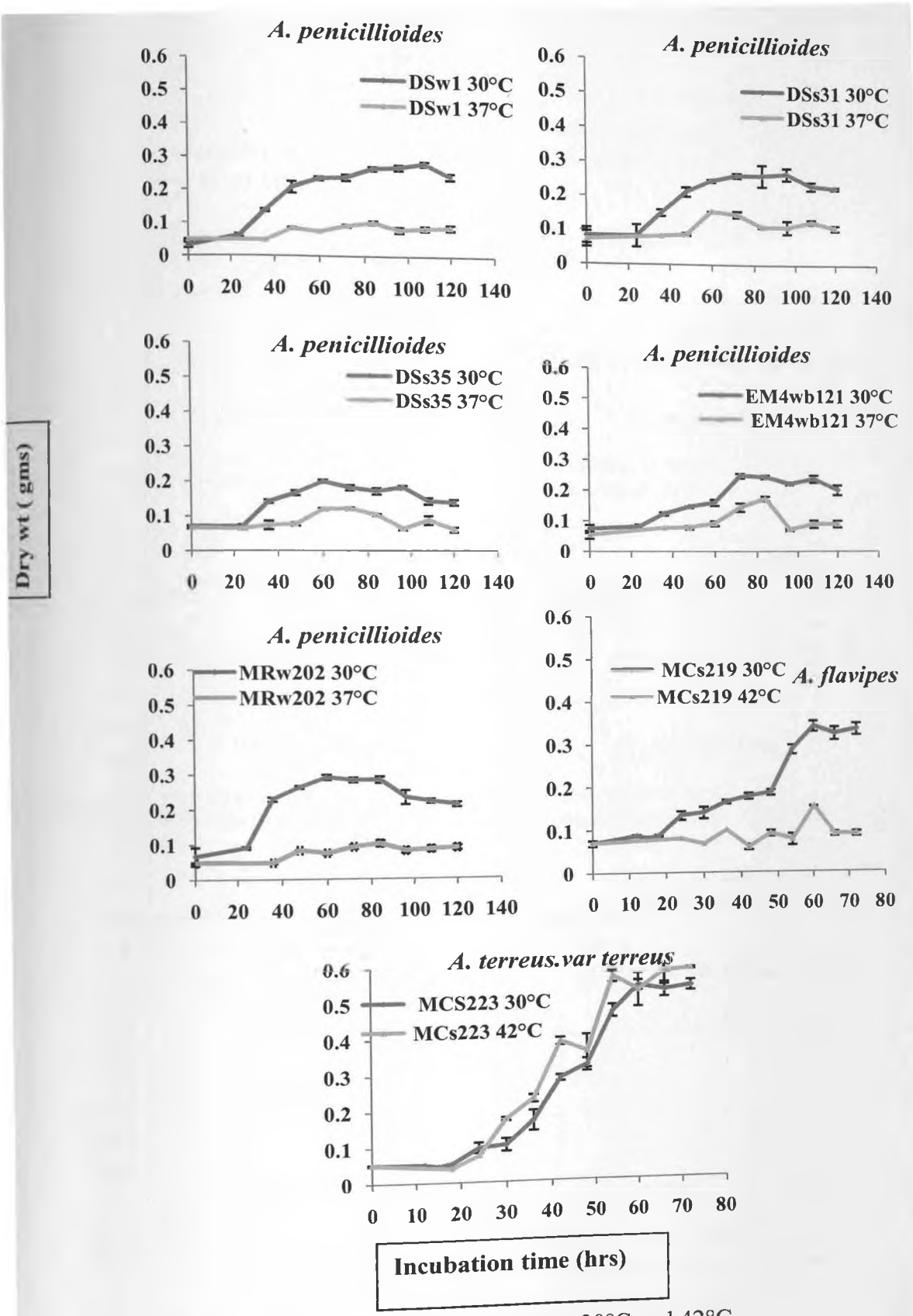


Fig. 5.1 Growth Curves of the isolates at 30°C and 42°C

Contd.....

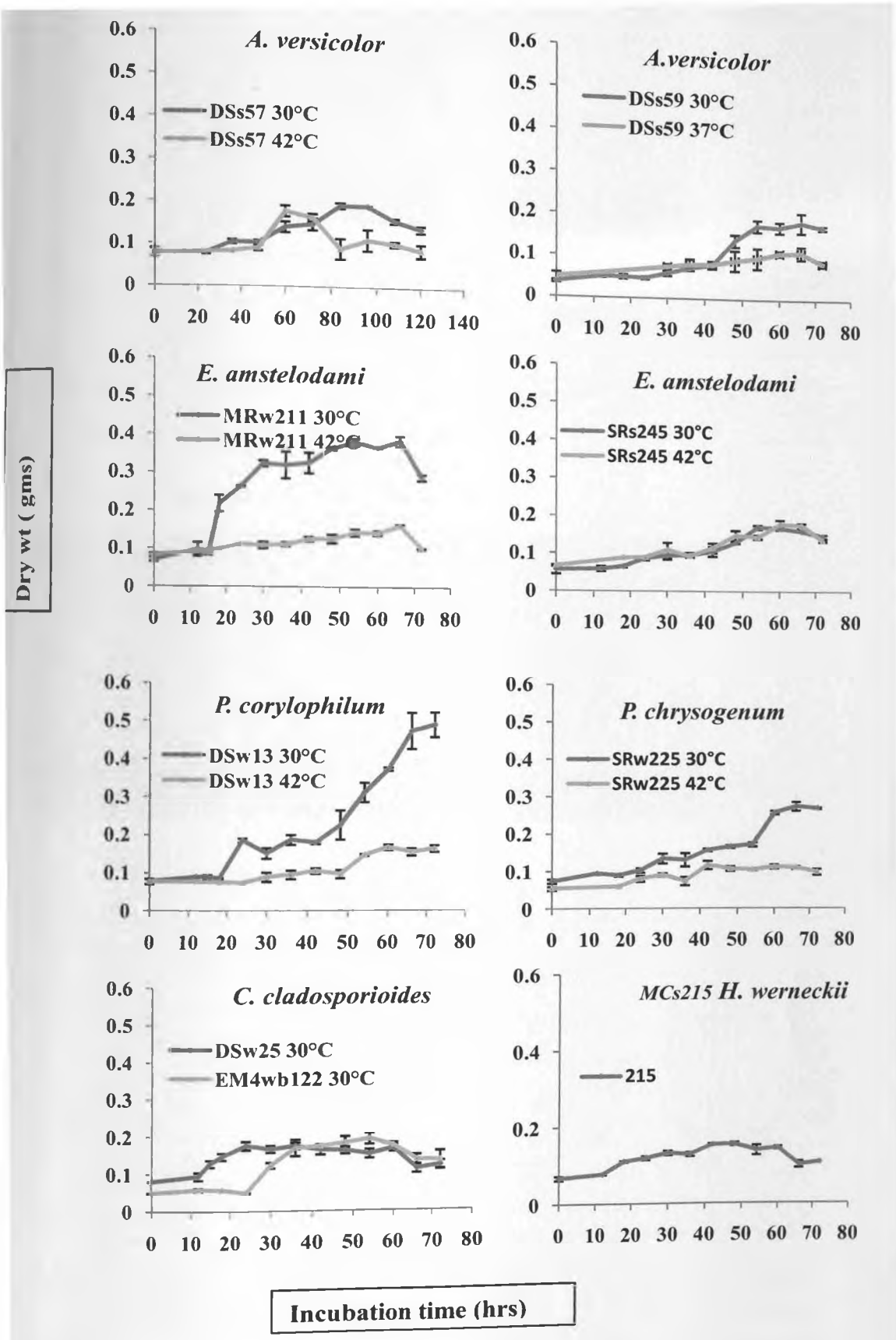
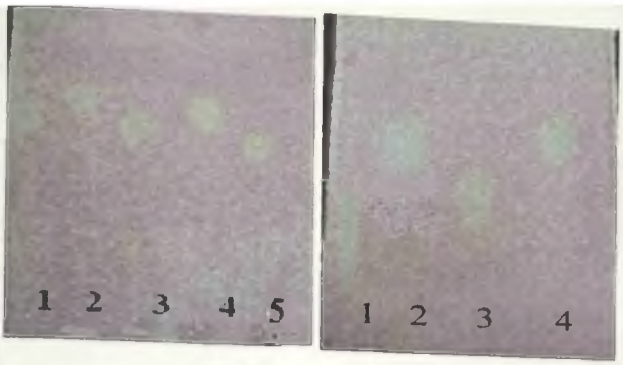


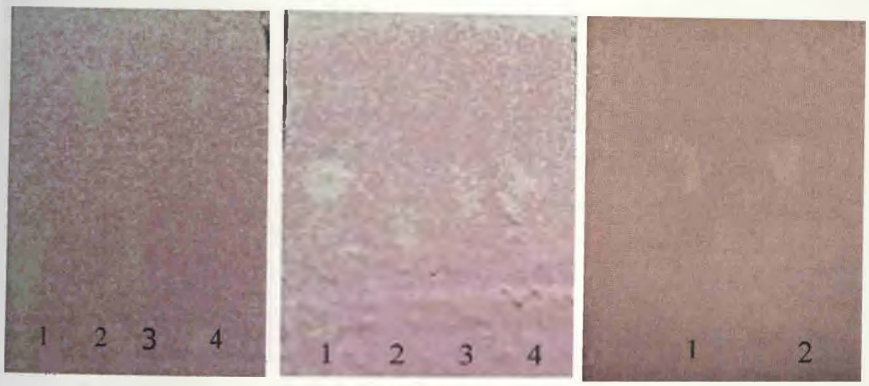
Fig. 5.1 Growth Curves of the isolates at 30°C and 42°C



a b

a	1	2	3	4	5
Standards	Arabitol	Erythritol	Glucose	Glycerol	Mannitol
Rf	0.68	0.72	0.7	0.74	0.65
b	1	2	3	4	
Standards	Trehalose	Sorbitol	Inositol	Xylitol	
Rf	0.60	0.52	0.27	0.59	

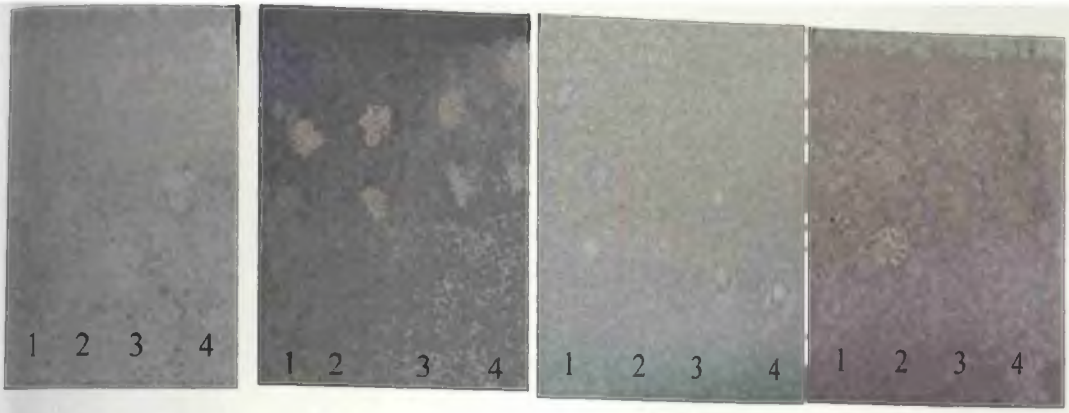
Fig 5.2a TLC of standards



a b c

a	1	2	3	4
Sample	35ME	35St	35 37°C	13ME
	Inositol	Glycerol	Inositol	Trehalose
Rf	0.27	0.74	0.27	0.60
		Glucose	Glucose	Erythritol
Rf		0.7	0.7	0.72
b				
Sample	13sta	13 37 °C	25ME	25Sta
	Arabitol	Trehalose	Trehalose	Trehalose
Rf	0.68	0.60	0.60	0.60
	Inositol			
Rf	0.27			
c				
Sample	215ME	215Sta		
	Glycerol	Glycerol		
Rf	0.74	0.74		

Fig 5.2b TLC of samples



d e f g

d	1	2	3	4
Sample	57ME	225 37	225Sta	225ME
	Glycerol	Trehalose	Trehalose	Trehalose
Rf	0.74	0.60	0.60	0.60
		Erythritol	Erythritol	Erythritol
Rf		0.72	0.72	0.72
e				
Sample	57Sta	57 37 °C	122ME	122Sta
	Glucose	Trehalose	Trehalose	Trehalose
Rf	0.7	0.60	0.60	0.60
	Erythritol			
Rf	0.72			

f	1	2	3	4
Sample	223ME	223St	223 42°C	223 50°C
	Erythritol	Erythritol	Trehalose	Trehalose
Rf	0.72	0.72	0.6	0.6
		Glucose	Glucose	
Rf		0.7	0.7	
g				
Sample	211ME	211st	211 42°C	211 50°C
	Glucose	Trehalose	Inositol	Trehalose
Rf	0.7	0.6	0.27	0.6
		Glucose	Glucose	
Rf		0.7	0.7	

Fig 5.2 b TLC of samples

Contd…

h i

h	1	2	3	4
Sample	35ME	35St	35 37°C	13ME
	Inositol	Glycerol	Inositol	Trehalose
Rf	0.27	0.74	0.27	0.60
		Glucose	Glucose	Erythritol
Rf		0.7	0.7	0.72
i				
Sample	13sta	13 37 °C	25ME	25Sta
	Arabitol	Trehalose	Trehalose	Trehalose
Rf	0.68	0.60	0.60	0.60
	Inositol			
Rf	0.27			

Fig 5.2b TLC of samples

The halophile *A. penicillioides* DSw35 produced trehalose as the major osmolyte followed by glucose during its mid-exponential phase while glucose was the major osmolyte during the stationary phase followed by inositol, glycerol, galactose and arabitol.

DSs57 *A. versicolor* produced erythritol as the major osmolyte followed by glucose, inositol, glycerol during its mid-exponential phase while erythritol was the major osmolyte followed by trehalose, , arabitol, glucose, glycerol was produced during its stationary phase. Erythritol was produced by MCs223 *A. terreus var terreus* during its mid-exponential phase and stationary phase.

MRw211 *E. amstelodami* produced inositol as the major osmolyte followed by erytritol and glycerol during its mid-exponential phase while trehalose was produced during its stationary phase.

The osmolytes produced by *P. chrysogenum* SRw225 during mid-exponential phase were trehalose and erythritol while galactose was produced in addition to trehalose and erythritol during the stationary phase. Erythritol was the major osmolyte during both the growth phases. The osmolytes produced by *P. corylophilum* DSw13 during mid-exponential phase were galactose, trehalose, inositol and erythritol while glucose, inositol and erythritol were produced during the stationary phase. Erythritol was the major osmolyte during both the growth phases.

The osmolytes produced by *C. cladosporioides* EM4wb122 during the mid-exponential phase was glucose, trehalose, galactose, inositol while glucose, trehalose, inositol was produced during the stationary phase. Glucose was the major osmolyte produced during

mid-exponential and stationary phase. *C. cladosporioides* DSw25 produced trehalose as the major osmolyte followed by glycerol, inositol and galactose during its mid-exponential phase while erythritol was the major osmolyte followed by trehalose and during its stationary phase. *C. cladosporioides* DSw25 that was isolated from Dead Sea on CzA+10% salt showed fewer amounts of osmolytes as that of EM4wb122 isolated from Mandovi on CzA+20% which showed trehalose throughout. Glucose was absent in DSw25 at mid-exponential phase. MCw215 *H. werneckii* produced glucose in the mid-exponential phase as well as stationary phase. Erythritol the major osmolyte was also produced in the stationary phase. *A. versicolor* and *A. penicillioides* showed glucose throughout.

Table 5.1a shows the amount of osmolytes at different growth stages. Fig. 5.4a shows the osmolytes present at different growth stages. Fig. 5.5a shows the amount of sugars and sugar alcohols present at different growth stages.

5.3.4 Osmolytes produced at increased temperature.

Osmolytes produced at increased temperature are shown in Fig. 5.3c.

The halophile DSs35 *A. penicillioides* produced trehalose as the major osmolyte followed by glucose at 30°C while glucose trehalose and inositol was produced at an increased temperature of 37°C.

The osmolytes produced by DSs57 *A. versicolor* at 30°C were inositol which was the major osmolyte followed by glucose, erythritol, glycerol while trehalose was produced at 42°C. MCs223 *A. terreus var terreus* produced erythritol at 30°C; erythritol was the major osmolyte followed by trehalose at 42°C. Trehalose was the only osmolyte produced at 50°C. Inositol, erythritol glycerol was produced by MRw211 *E. amstelodami* at 30°C,

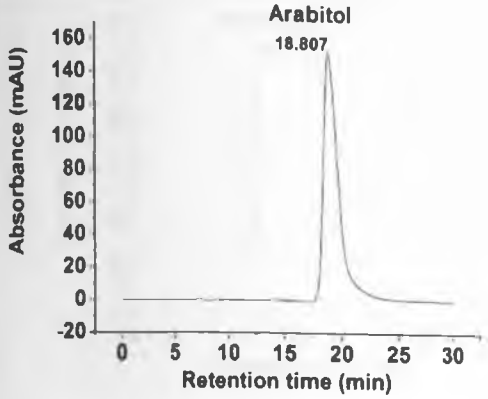
inositol was the major osmolyte followed by glucose at 42°C while trehalose was the only osmolyte at 50°C.

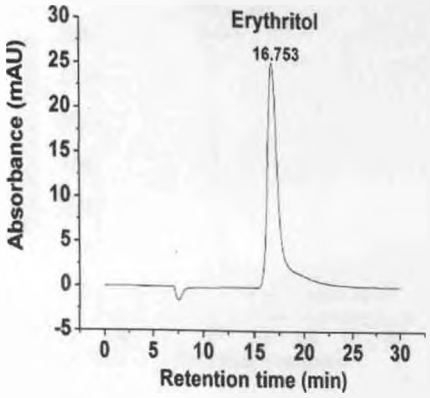
At 30°C *P. chrysogenum* SRw225 produced trehalose and erythritol while trehalose was produced at 42°C. *P. corylophilum* DSw13 produced galactose, trehalose, inositol and erythritol at 30°C and trehalose at 42°C. Erythritol was the major osmolyte produced by *P. corylophilum* and *P. chrysogenum* at 30°C. As compared to the sugars more amount of sugars alcohols were produced by *Penicillium* during mid-exponential phase and stationary phase as well.

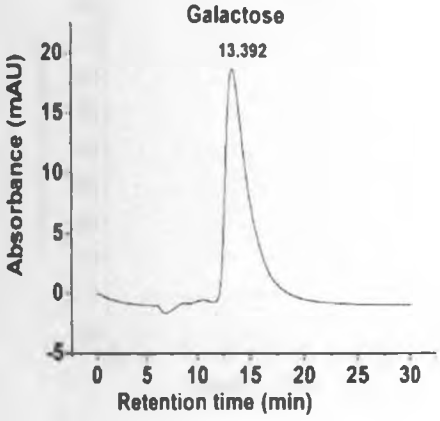
Amount of different osmolytes at increased temperature is shown in Table 5.1b Osmolytes present at increased temperature are shown in Fig. 5.4b Amount of sugars and sugar alcohols present at increased temperature is given in Fig. 5.5b.

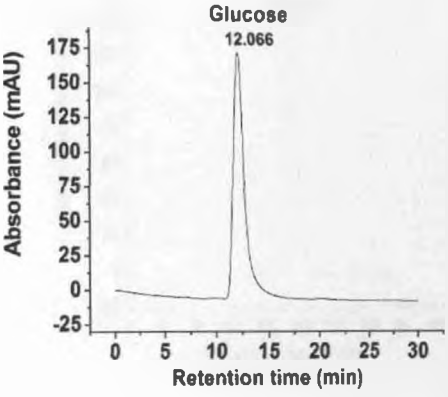
At increased temperature only sugars were produced in all the cultures, sugar alcohols were not produced. *A. penicillioides* produced sugar alcohols.

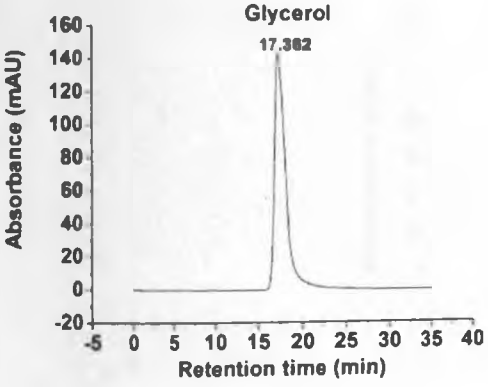
The ANOVA results of sugar and sugar alcohols were significantly different as the P value is less than 0.05.











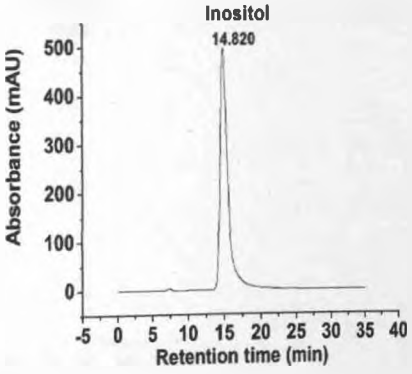


Fig 5.3a HPLC of standards

Contd.....

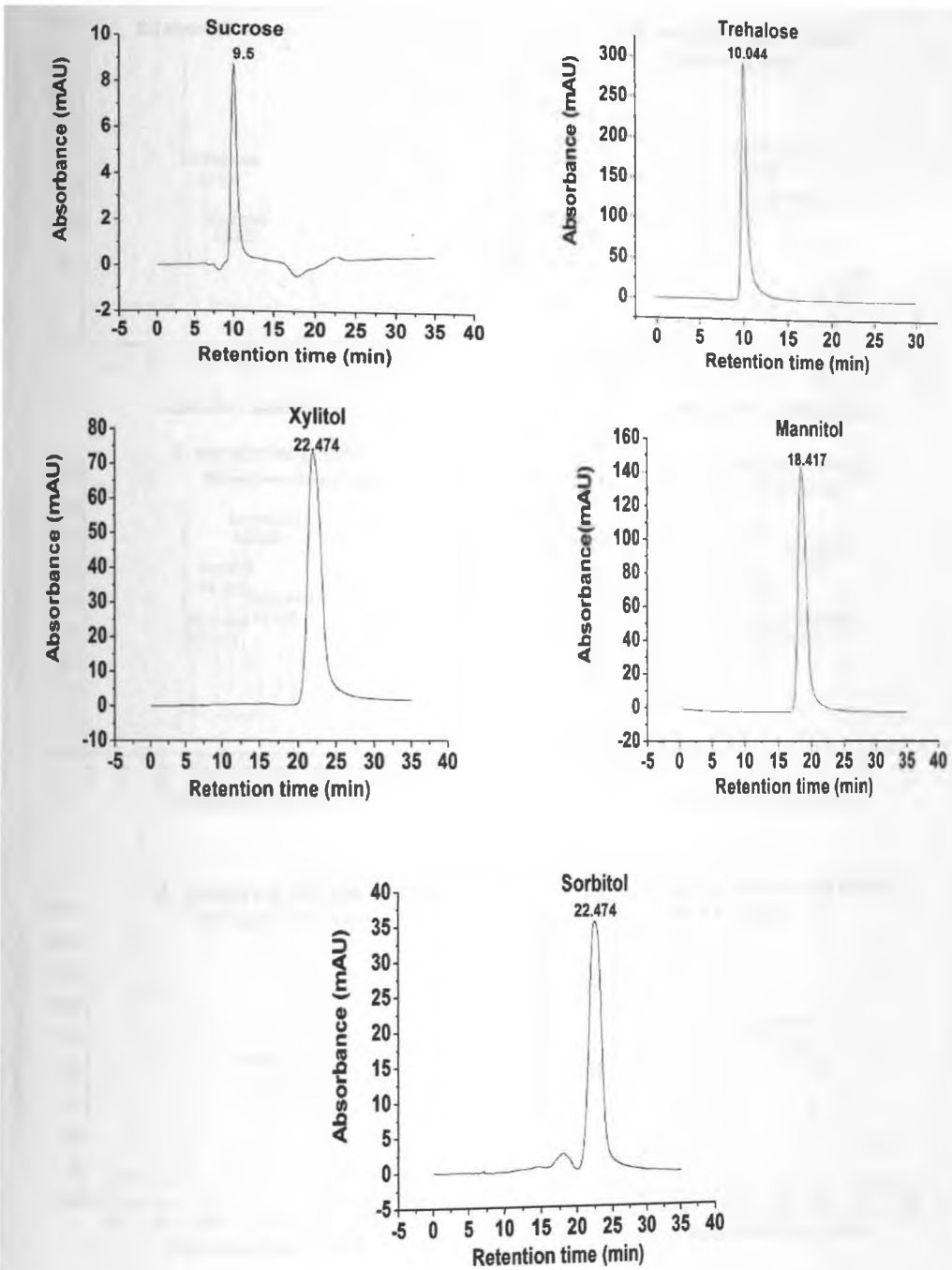


Fig 5.3a HPLC of standards

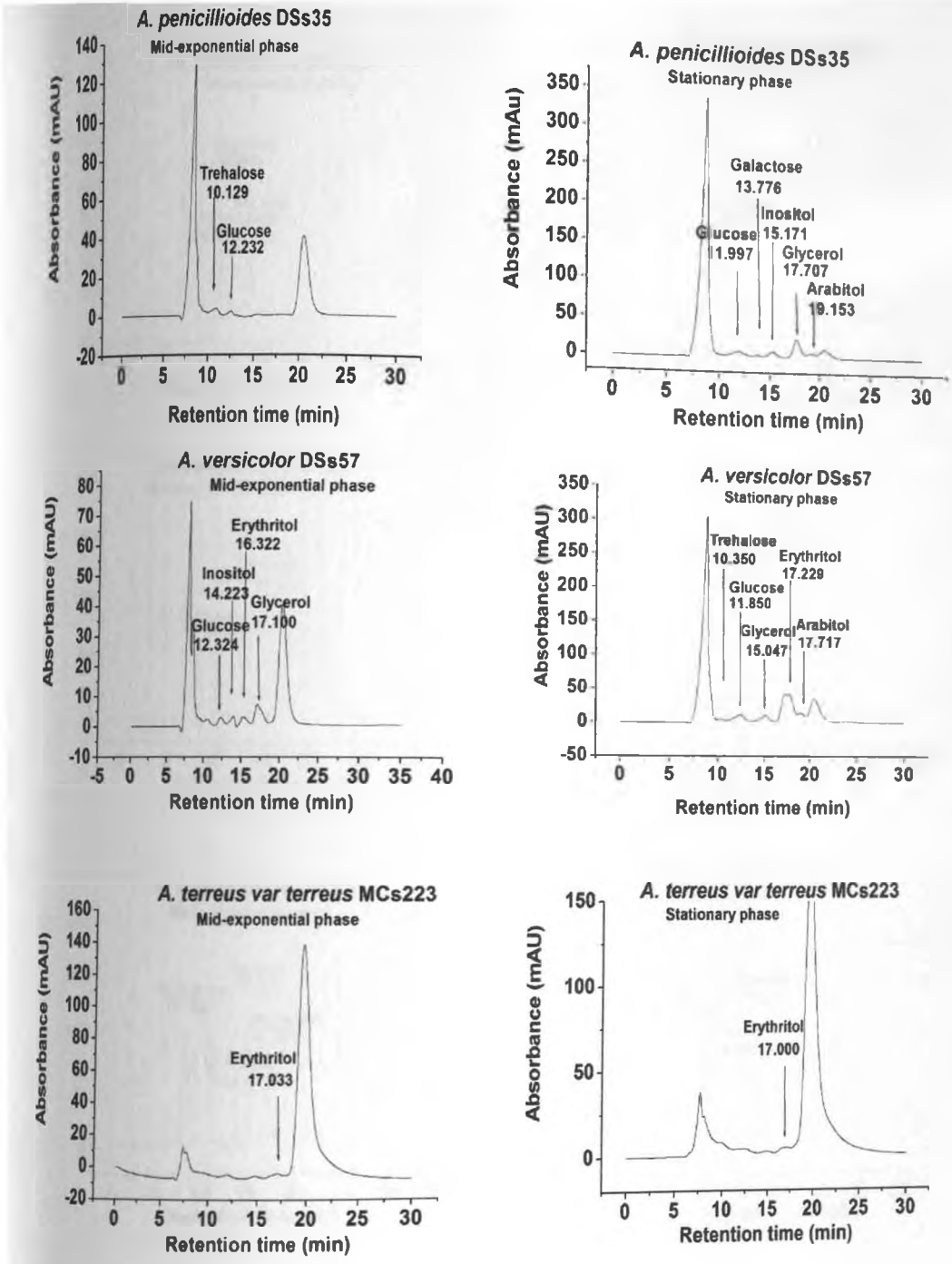


Fig 5.3b HPLC of the isolates at different growth phases

Contd.....

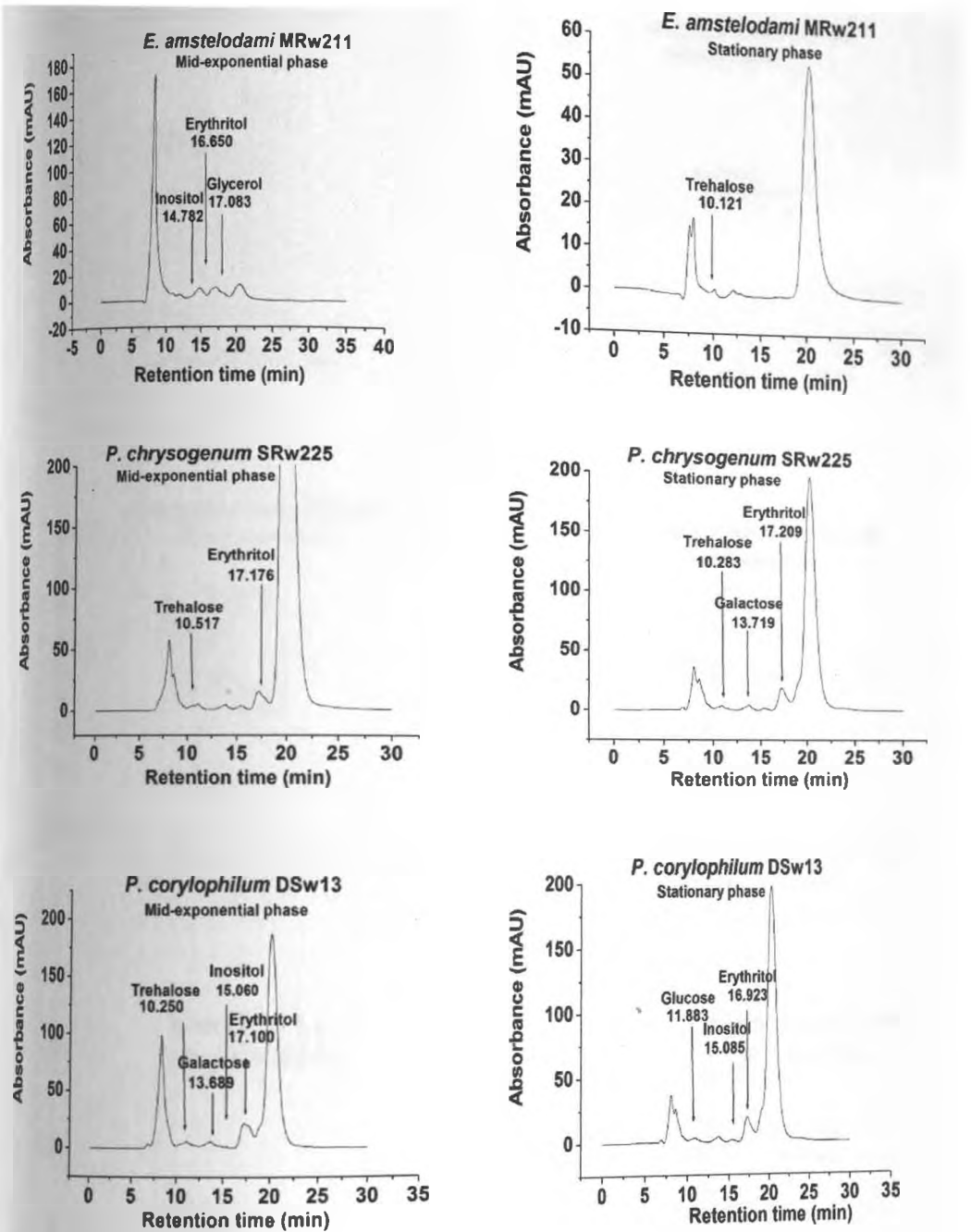


Fig 5.3b HPLC of the isolates at different growth phases

Contd.....

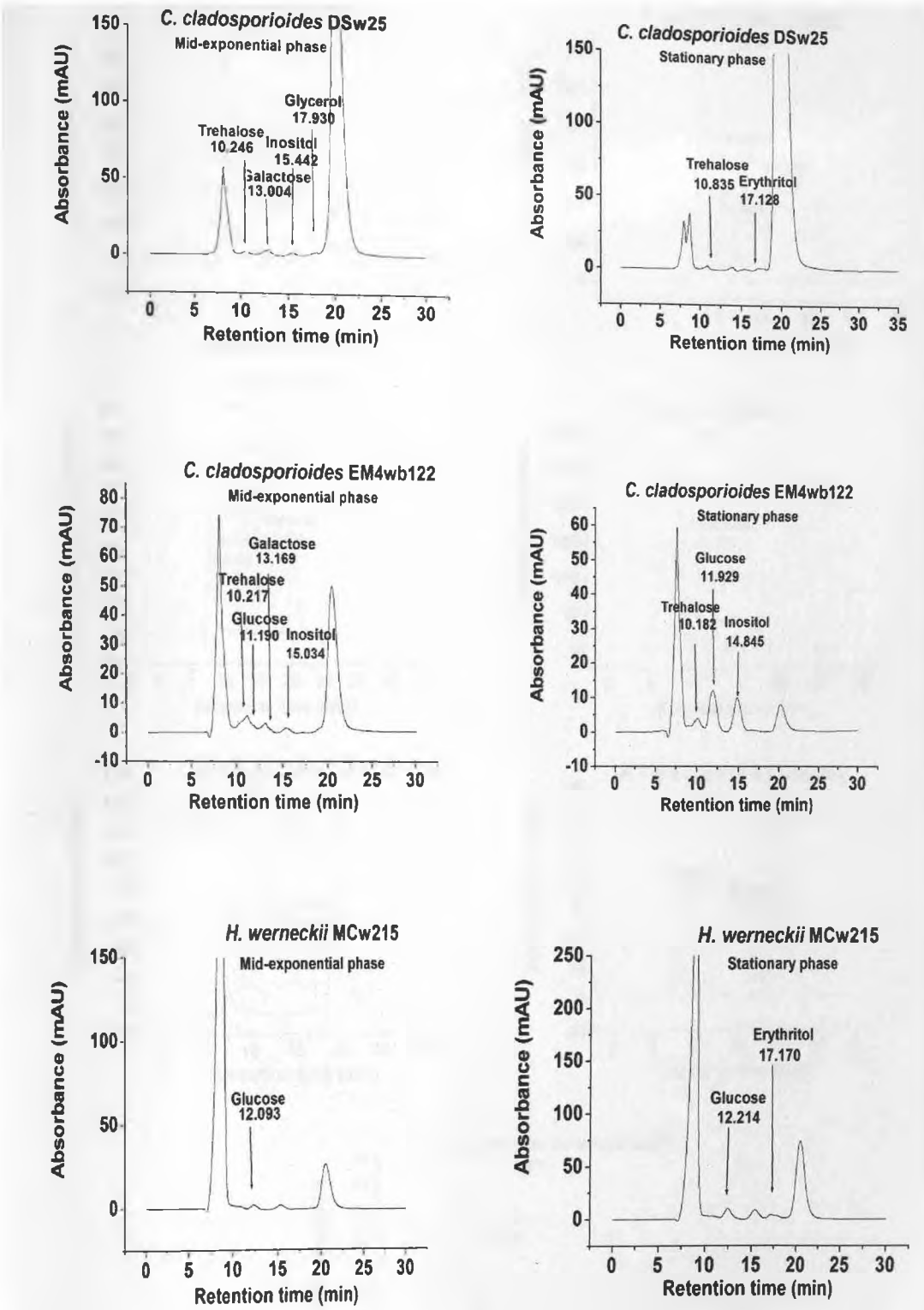


Fig 5.3b HPLC of the isolates at different growth phases

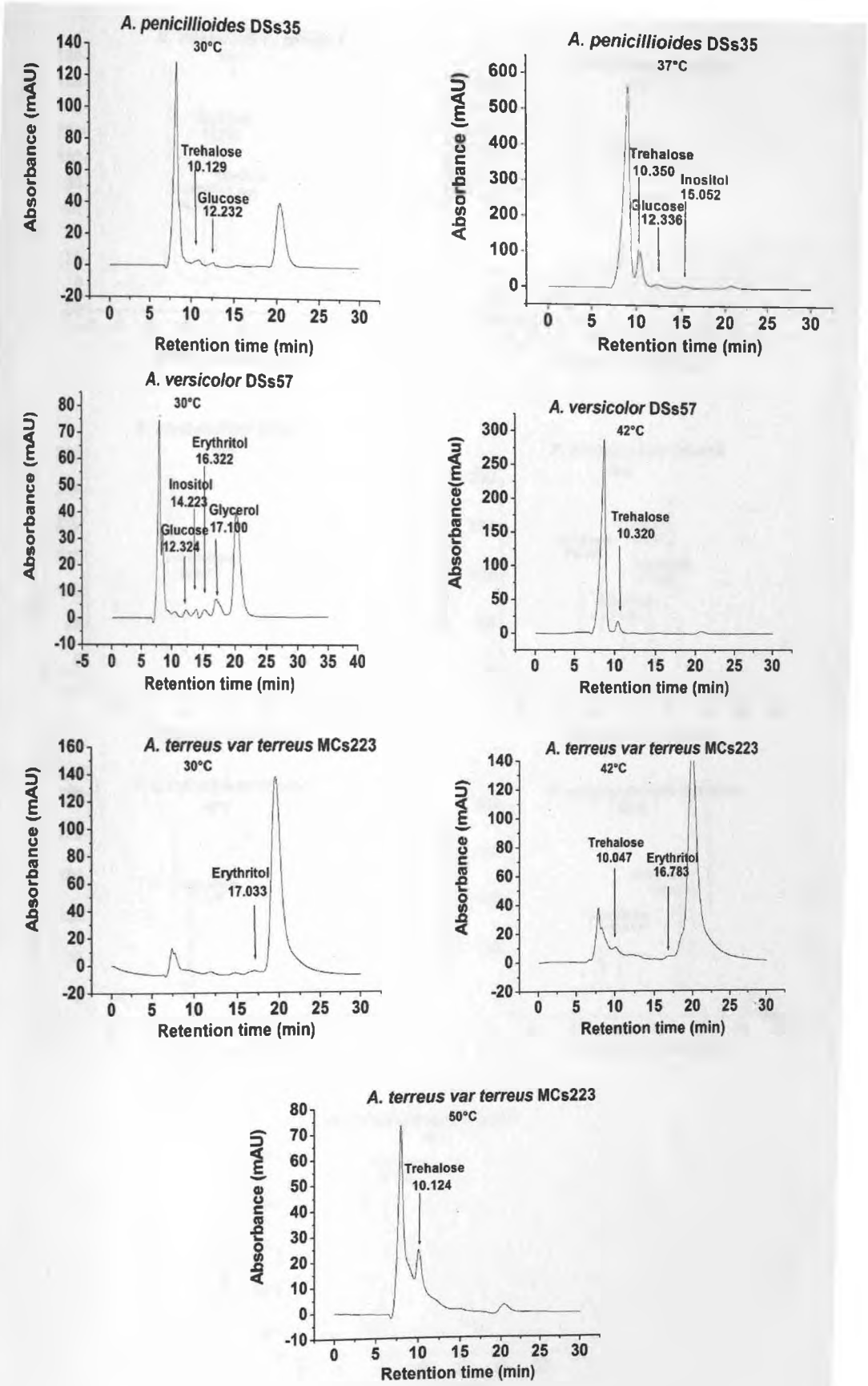


Fig 5.3c HPLC of the isolates at increased temperature

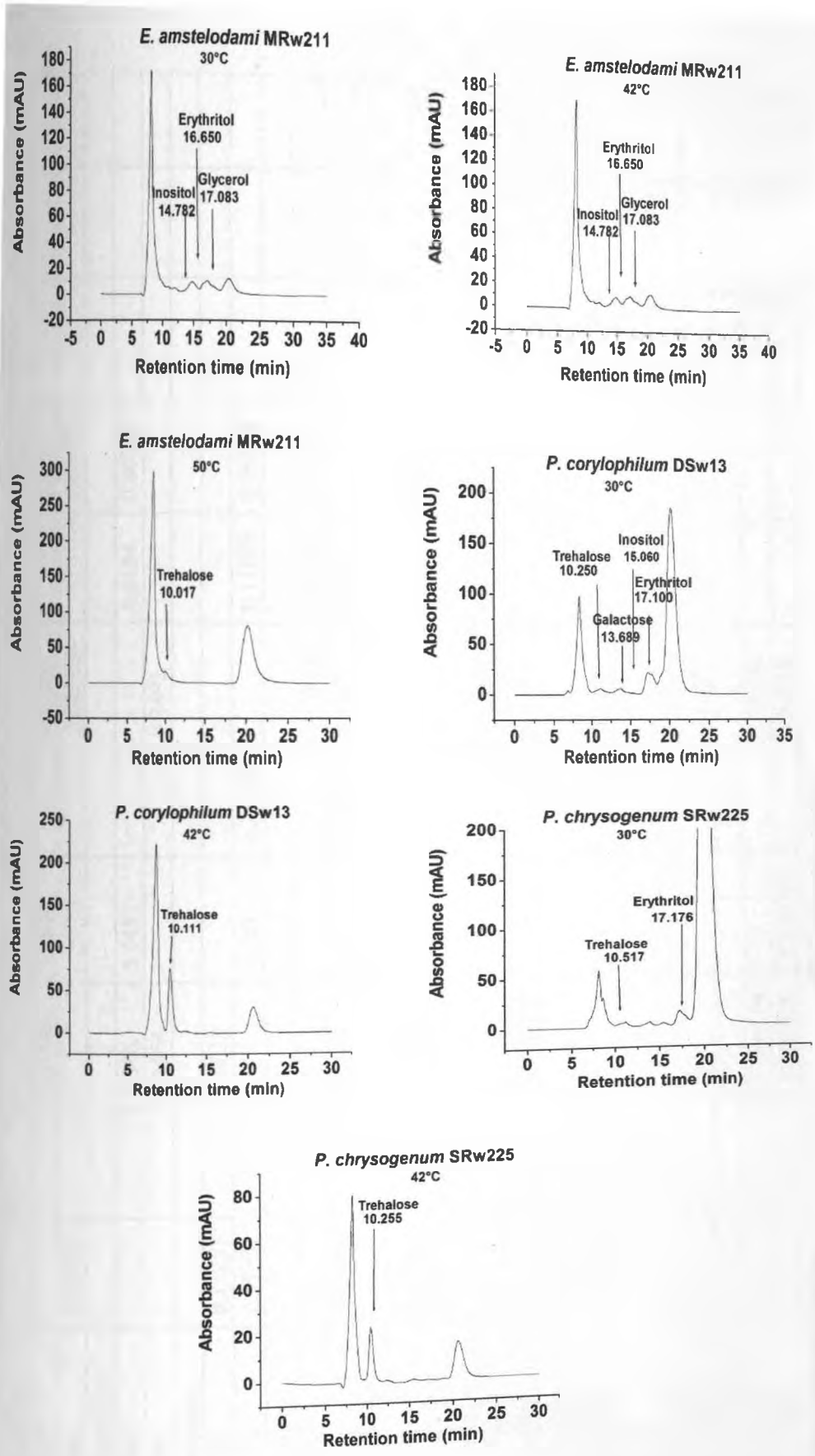


Fig 5.3c HPLC of the isolates at increased temperature

Table 5.1a Amount of osmolytes present at growth stages

Culture No	Sugar/Sugar alcohol mg/ml										
	Glucose	Sucrose	Trehalose	Galactose	Arabitol	Erythritol	Glycerol	Inositol	Mannitol	Sorbitol	Xylitol
P.corylophilum											
DSw13ME			0.01646	3.4806		0.10266		0.012			
DSw13Sta	0.32335					2.242800		0.03421			
C.cladosporium											
DSw25ME			0.038	3.4452			0.0154	0.00769			
DSw25Sta			0.1554			0.3492					
A.penicilloides											
DSs35ME	0.11253		0.5632								
DSs35 Sta	0.57303			*3.265*	0.23748		0.11688	0.26549			
A.versicolor											
DSs57 ME	0.2					0.448286	0.08123	0.1571			
DSs57Sta	0.09758		0.1426		1.85512	8.6688	0.688				
C.cladosporioides											
EM4wb122ME	0.260		0.0320	0.7209				0.040			
EM4wb1222sta	2.12136		0.28592					0.38030			
E.amstelodami											
MRw211ME						0.519385	0.08352	0.92			
MRw211Sta			0.045158								
H.werneckii											
MCw215ME	0.31624										
MCw215 Sta	1.3875					4.5692					
A.terreus											
MCs 223ME						0.1325					
MCs 223Sta						0.2313					
P.chrysogenum											
SRw 225ME			0.046286			1.21590					
SRw 225Sta			0.007728	2.930783		1.53032					

ME- Mid-exponential phase Sta- Stationary phase

Table 5.1b Amount of osmolytes present at increased temperature.

Culture No	Sugar/Sugar alcohol mg/ml										
	Glucose	Sucrose	Trehalose	Galactose	Arabitol	Erythritol	Glycerol	Inositol	Mannitol	Sorbitol	Xylitol
P.corylophilium											
DSw13 30°C			0.01646	3.4806		0.10266		0.012			
DSw13 42°C			2.6609								
A.penicilloides											
DSs35 30°C	0.11253		0.5632								
DSs35 37°C	0.4599		0.4167					0.55008			
A.versicolor											
DSs57 30°C	0.2					0.448286	0.08123	0.1571			
DSs57 42°C			1.820091								
E.amstelodami											
MRw211 30°C						0.519385	0.08352	0.92			
MRw211 42°C	0.6048							0.6096			
MRw211 50°C			1.9308								
A.terrus var terrus											
MCs 223 30°C						0.1325					
MCs 223 42°C			0.229931			1.2545					
MCs 223 50°C			1.003								
P.chrysogenum											
SRw 225 30°C			0.046286			1.21590					
SRw 225 42°C			1.94022								

ME- Mid-exponential phase Sta- Stationary phase

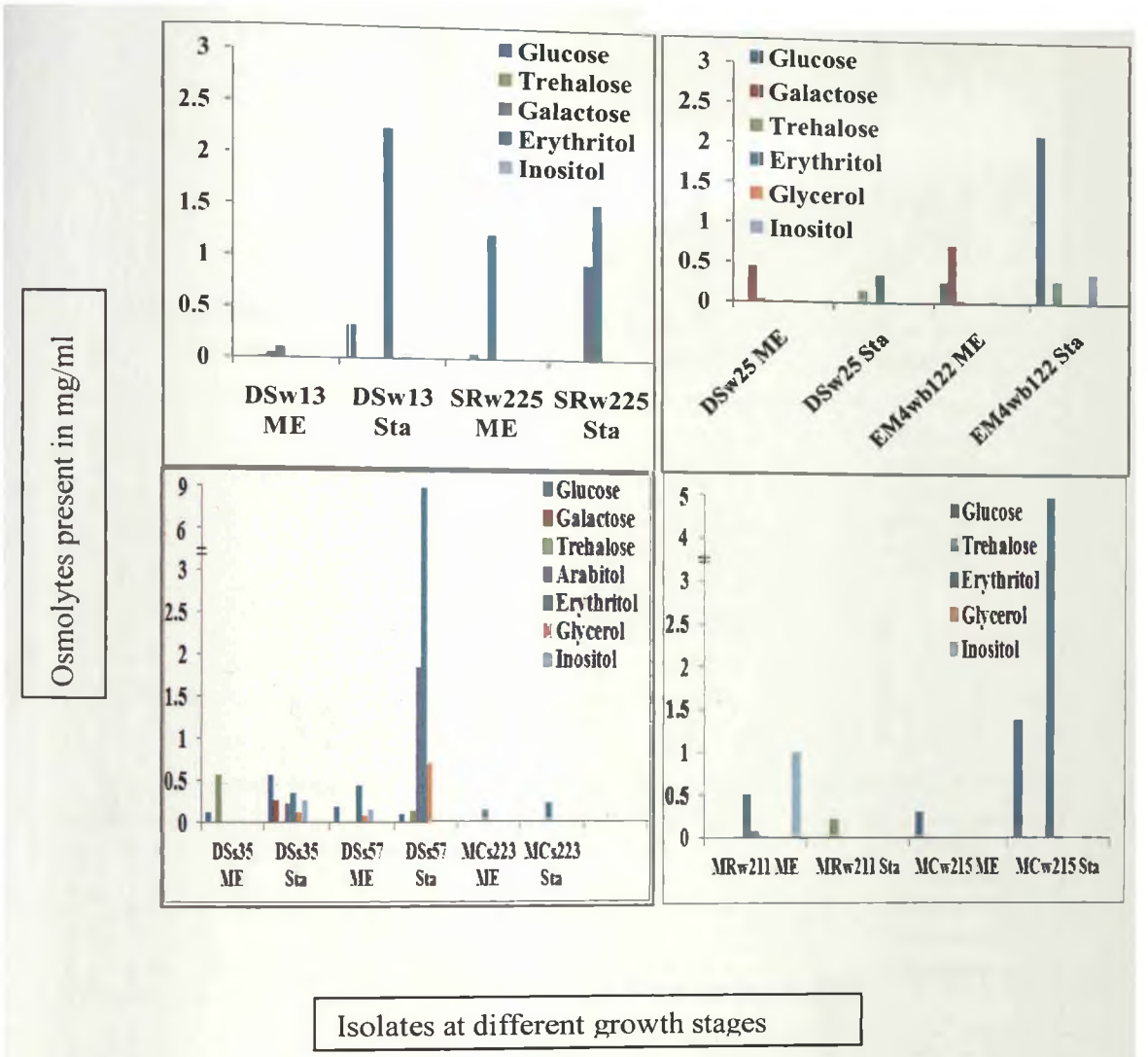
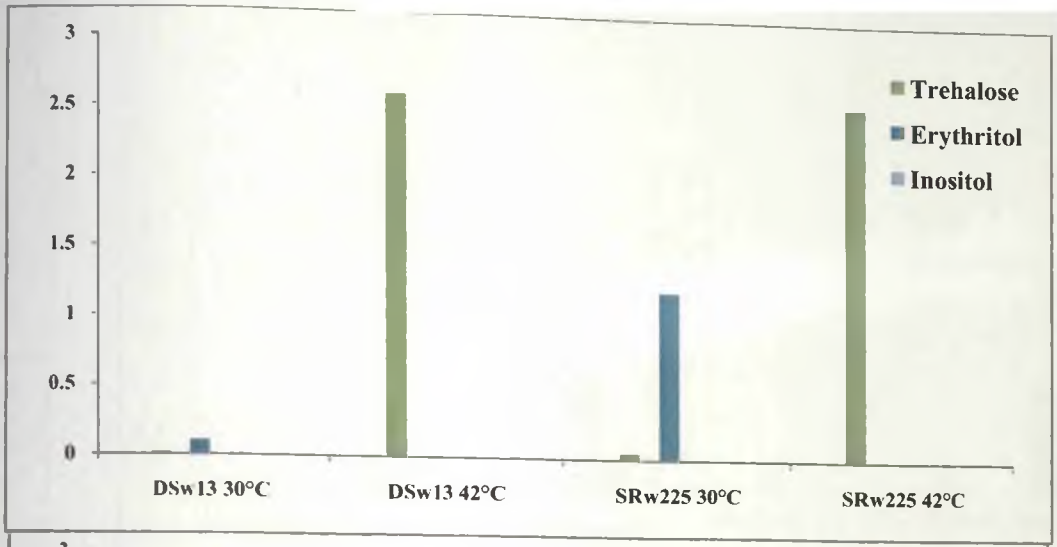
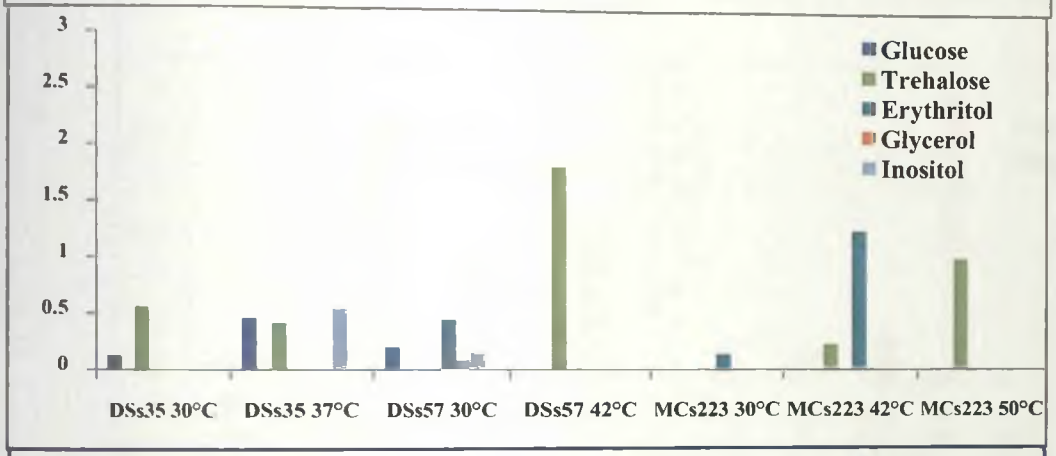
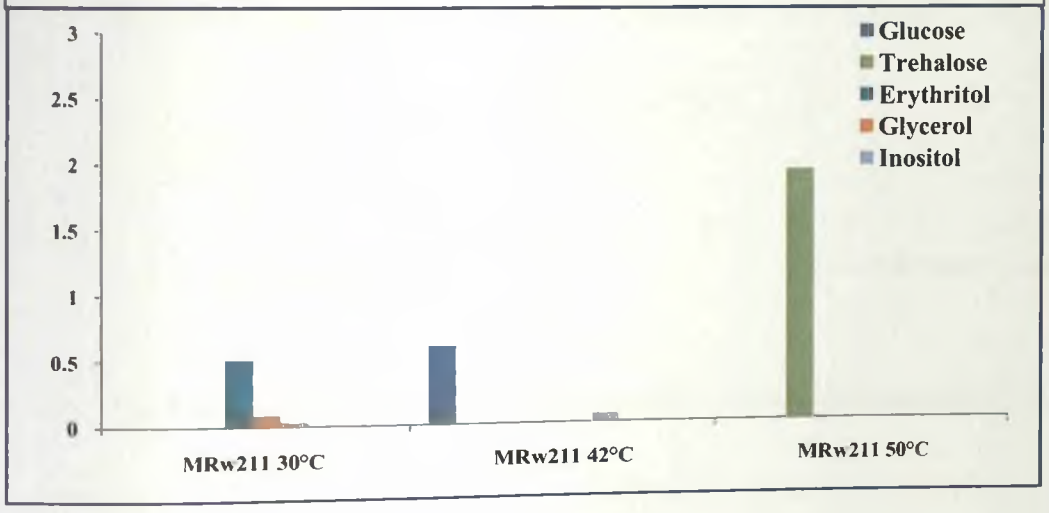


Fig. 5.4a. Osmolytes present at different growth stages



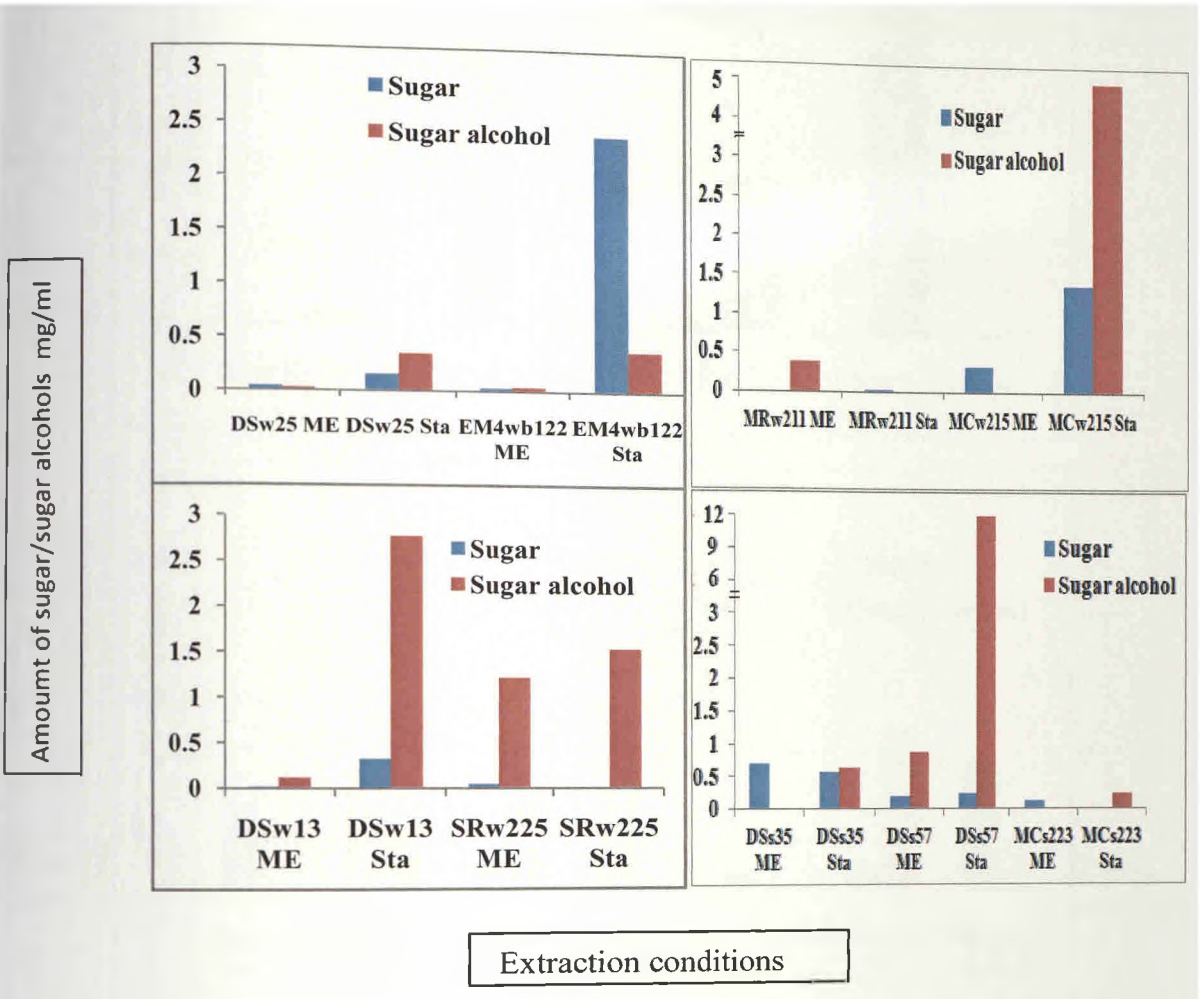




Osmolytes present in mg/ml

Isolates at different temperature

Fig. 5.4b. Osmolytes present at increased temperature



ME mid-exponential phase

Sta Stationary phase

Fig. 5.5a. Amount of sugars and sugar alcohols present at different growth stages

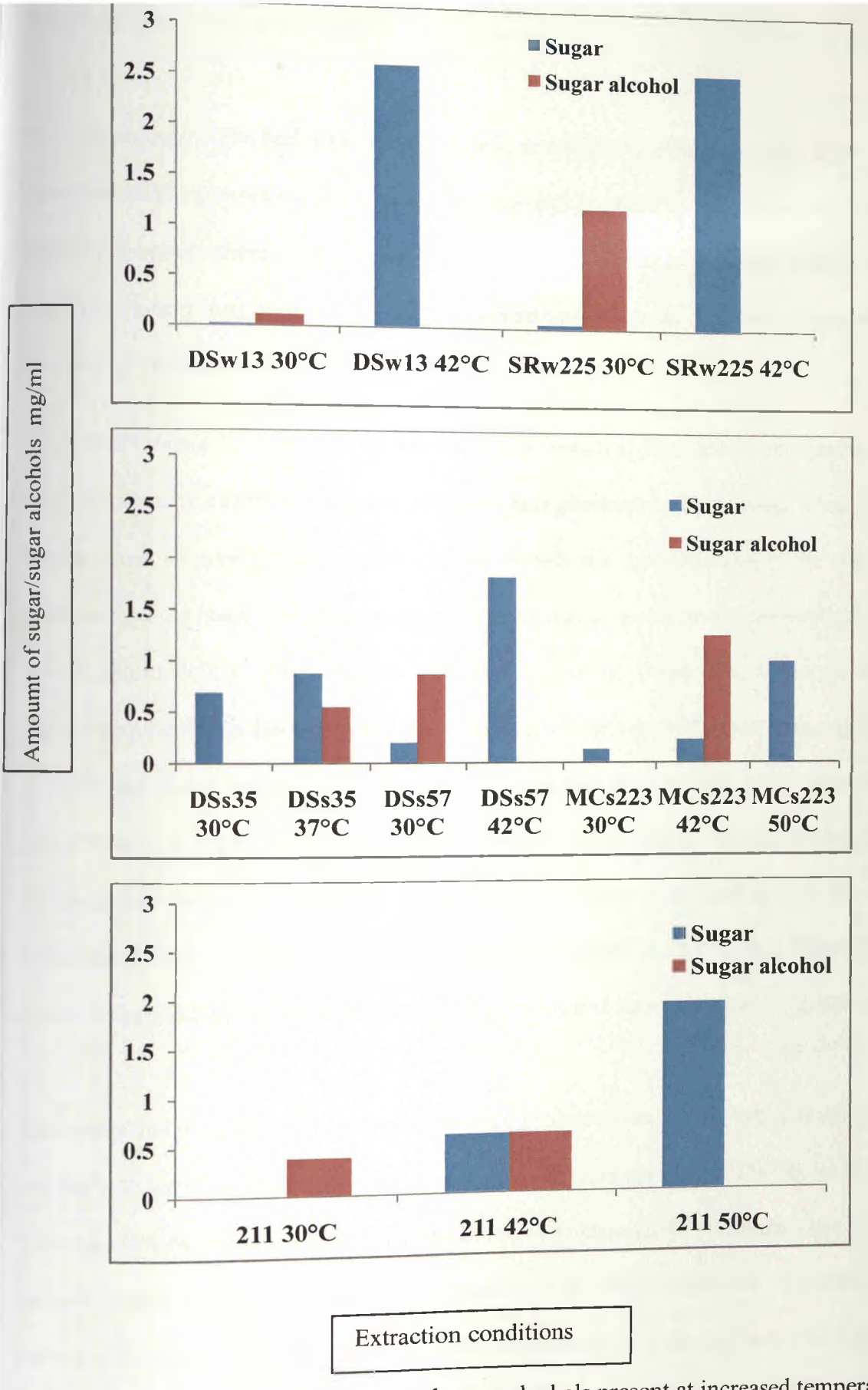


Fig. 5.5b. Amount of sugars and sugar alcohols present at increased temperature

5.4 Discussion

The obligate halophiles had a prolonged lag phase indicative of slow growth of the obligate halophiles as compared to that of facultative halophiles where the lag phase was shorter. At higher temperature these isolates showed a prolonged lag phase and poor growth. Obligate halophiles took a long time of 39-41hrs to germinate where-as facultative halophiles took less time of 26-30hrs to germinate.

It has been shown that inorder to survive under unfavourable conditions, yeasts need to repair the damage caused by salt and to adapt their phenotype. This causes a lag in growth, in some cases because the cells actively arrest growth at a specific phase of the cell cycle. In other cases, a lag phase can arise because the cells cannot generate the resources to continue growth (Smits 2005). The obligate halophiles from the Dead Sea, Mandovi, mangrove showed a similarity in their growth curves, although there was difference with regard to time at which lag phase, log phase and stationary phase was approached; while variations with regard to time at which lag phase, log phase and stationary phase was approached was seen in facultative halophiles as well. There was an increase in dry weight at optimum temperature while increased temperature showed decrease in dry weight. Growth rates of different fungi differed at optimum salt concentration and also at increased temperature.

The organisms produced not one but a mixture of osmolytes. As reported, microorganisms use not just one solute but rather two or more to meet this challenge (Del Moral *et al.* 1994, Ventosa 1998, Wood *et al.* 2001, Roberts 2005). The strategy of accumulating a mixture of polyols is also common in many fungi (Davis *et al.* 2000). Mixtures of osmolytes may reduce the toxicity associated with high concentrations of a single osmolyte and obviate

feedback mechanisms that down regulate metabolic pathways in the presence of high concentrations of product (Davis *et al.* 2000). Such a salinity-dependent switch in osmolyte was observed in archaea and bacteria (Madkour *et al.* 1990, Muller and Saum 2005, Regev *et al.* 1990). Kayingo *et al.* (2001) reported that *Zygosaccharomyces rouxii* and *Pichia sorbitophila* and the less tolerant *Saccharomyes cerevisiae* released glycerol upon hypo-osmotic shock. However, *Z. rouxii* also released arabitol, whereas *P. sorbitophila* released erythritol in addition to arabitol and glycerol.

Glucose, trehalose, erythritol, glycerol, inositol and arabitol were the common osmolytes produced. Other compatible solutes besides polyols are also produced in response to salt stress in microorganisms, such as nitrogen containing compounds like glycine-betaine and free amino acids (Galinski 1995), while mycosporine-like amino acids were suggested as osmoprotectants in halophilic cyanobacteria (Oren 1997).

Petrovic *et al.* (2002) showed that the intracellular glycerol concentration in halophilic *H. werneckii* cells grown at different salinities steadily increases from 0% to 10% NaCl. At higher salinities, the intracellular glycerol concentration remains virtually unchanged. On the other hand, the extracellular glycerol concentrations are seen to be low and independent of salt concentration between 0% and 17% NaCl and start to increase above this salinity. Ravishankar *et al.* (2006) showed that the fungus *Cirrenalia pygmea* synthesized more polyols when the solute concentration in the growth medium was increased. A sudden exposure of the fungus to higher salinities increased the polyol content.

Considerable amount of polyols were produced by the isolates which were more in the stationary phase than in the mid-exponential phase. Actively growing cells at the mid-exponential phase possibly use the osmolytes as the carbon source. Kayingo *et al.* (2004) stated that most yeasts species can utilize glycerol as a sole carbon source or as a osmolyte during osmoregulation and have consequently developed elaborate mechanisms to regulate its flux across cell membranes.

Glucose, trehalose, erythritol and glycerol were the osmolytes that were present in the stationary phase. In some isolates the osmolytes present in the stationary phase were also present in the mid-exponential phase while in some others a particular osmolyte was replaced by another osmolyte in the stationary phase. Additional osmolytes were present in the stationary phase in some isolates. Variation in the osmolytes produced at different growth phases with respect to types and amount was observed among different species of the same genera, as well as the species from different echoniches. More amount of sugars were produced in *C. cladosporioides* as compared to sugar alcohols during mid exponential phase and stationary phase. *P. chrysogenum* produced more sugar alcohols during mid exponential phase and more of sugars during stationary phase whereas *P. corylophilum* produced more of sugar during both phases of growth. *A. versicolor* produced more of polyols during different phases of growth. *A. penicillioides* and *A. terreus var terreus* produced more sugars at different phases of growth. *E. amstelodami* produced polyols at mid-exponential phase and sugars at stationary phase. *H. werneckii* produced sugars at mid-exponential phase and polyols at stationary phase.

H. werneckii produced glucose during the mid-exponential phase, while glucose was the major solute during the stationary phase other than erythritol. Kogej (2006) reported that *H.*

werneckii accumulates other higher polyols besides glycerol in a salt-dependent manner. Polyols such as erythritol, inositol, arabinitol, xylitol and mannitol, were also found to be used for osmoadaptation in fungi (Pfyffer *et al.* 1986, Blomberg & Adler 1992). It is stated that in fungi glycerol and erythritol is linked to osmotic stress and growth at low water activity. Plemenitas *et al.* (2008) reported glycerol as its main compatible solute, since its intracellular amount was the highest of all solutes and it increased with salinity. *H. werneckii* also compensated for the external osmotic pressure caused by increased NaCl concentrations by accumulating various additional substances (erythritol, arabitol, mannitol and mycosporines).

Luxo (1993) showed that mannitol was the main polyol accumulated during the late exponential and stationary phases of growth in media without added NaCl in *Geotrichum penicillatum.* The osmolyte production depends on the amount of stress given to an organism and how an organism combats it.

Glucose, trehalose, erythritol and glycerol were the osmolytes that were present at increased temperature. In some isolates the osmolytes present at increased temperature were also present at 30°C.

Predominance of trehalose was seen in most of the isolates at increased temperature. At increased temperature intracellular trehalose concentration has often been associated with tolerance of heat shock (Hallsworth & Magan 1994). Trehalose and mannitol provide protection against heat, cold and drought, and trehalose is linked to longevity (Dijksterhuis *et al.* 2002). Singer and Lindquist (1998) and Hincha (2004) report the functions of trehalose as stabilizing proteins and membranes. It may function as a radical scavenger (Benaroudj

2001) and act as a compatible solute (Housna 1998). Studies on *Schizosacchaomyces pombe* by (Soto 1999) stated that trehalose renders the cells tolerant to various stresses. Elbein (2003) and Fillinger (2001) showed the importance of trehalose for various stresses, including heat stress, cold stress, oxidative stress, high ethanol concentrations dehydration, osmotic and salt stress, and weak organic acids by and thermo-tolerance in *A. nidulans*. Nesci *et al.* (2004) found that temperature can have an impact on growth rate and on intracellular accumulation of total polyols and sugars. The accumulation of sugar and sugar alcohols in mycelia was dependent on temperature and water stress. Large amounts of trehalose are accumulated in *Saccharomyces cerevisiae* shifted to temperatures exceeding the normal growth range (Attfield 1987, Hottiger *et al.* 1987). A report by Hottiger *et al.* (1987) indicated that trehalose is most important in the induction of thermotolerance in *S. Cerevisiae*, more than heat shock proteins. The induction of trehalose by temperature upshift has been studied in great detail (Hottiger *et al.* 1987, Wiemken 1990, Bell *et al.* 1992, Vuorio *et al.* 1993, Piper 1995, Parrou *et al.* 1997).

Interestingly arabitol and galactose which was present at 30°C in some isolates was not found at increased temperature. *Penicillium* species and *A. penicillioides* produced trehalose at 30°C and also at increased temperature, although the amount of trehalose produced at increased temperature were more. Additional osmolytes were present at 30°C in some isolates.

Variation in the osmolytes produced at 30°C with respect to types and amount was observed among different species of the same genera, as well as the species from different echoniches. At increased temperature trehalose was common with same genera, different genera and different echoniches.

A. penicillioides produced more sugars at 30°C and 37°C as compared to sugar alcohols. *Penicillium* produced more of polyols at 30°C and more of sugars at 37°C. *Eurotium* produced sugars at increased temperature of 50°C. Sugar alcohols were present at 42°C but not at 50°C. *A. terreus var terreus* produced sugar at 30°C and also at increased temperature while it produced sugar alcohol at 42°C. Temperature could have an impact on growth rate and on intracellular accumulation of polyols and sugars.

The study by Nesci *et al.* (2004) showed that modifying water stress and temperature not only affected growth of *A. flavus* and *A. parasiticus* strains but also intracellular accumulation of total polyols and sugars and stated that the increase in temperature, which also represents an environmental stress, may have resulted in the increased total polyols. They report that the temperature can have an impact on growth rate and on intracellular accumulation of total polyols and sugars. At higher temperature the major accumulation of polyols and sugars occurred in the matrically stressed medium (Nesci *et al.* 2004). The accumulation of sugars and sugar alcohols in mycelia was dependent on temperature and water stress. Increase in total polyols at 30°C when compared with 20°C on unammended medium was reporded. Thus an increase in temperature may have resulted in the increased polyols.

This work thus shows the osmolyte pool of the obligate halophile, facultative halophile and the halotolerant, mesophilic fungi. The result obtained in this study provides information underlying the physiological mechanism of osmoadaptation in relation to production osmolytes in the halophilic fungi.

5.5 Conclusion

Prolonged lag phase was seen in all the obligate halophiles and the isolates at an increased temperature. The organisms produced not one but a mixture of osmolytes. Considerable amount of polyols were produced by these organisms. The osmolyte produced by organisms varied considerably in the types and amount. They depend on the amount of stress given to an organism and how an organism combats it. Glucose, trehalose, erythritol, glycerol, arabitol were the osmolytes that were produced. The amount of osmolyte produced in the stationary phase was more as compared to the mid-exponential phase. Predominance of trehalose was seen among isolates from different genera, species and econiches at increased temperature.

Variation in the osmolytes produced at 30°C was observed among different species of the same genera, as well as the species from different echoniches.

SUMMARY

Summary

Halophilic fungi have been isolated from Dead Sea, Mandovi estuary, mangroves and salterns and the salt tolerance of the isolates were studied. Studies on salt tolerance of the cultures revealed that all the cultures tolerated salt at a concentration of 20%-30%. Lag in growth and decrease in sporulation with respect to increasing salt concentration was observed. *A. penicillioides* was the obligate halophile found in the study. Water sample yielded *A. penicillioides*, *P. corylophilum* and *C. cladosporioides* while the sediment sample yielded *A. penicillioides*, with *A. versicolor* in few numbers. *C. cladosporioides* from water sample and *A. versicolor* from sediment sample were exclusively isolated on CzA+10% salt.

Mandovi estuary samples showed obligate halophiles *A. penicillioides* regardless of having low salinity. Most of the isolates from Mandovi estuary tolerated 25% salt or even 30% salt. *C. cladosporioides* was isolated from the Mandovi estuary.

Mangroves showed three obligate halophiles *A. penicillioides*. Obligate halophiles were not isolated from saltern samples. *H. werneckii* was isolated from mangroves and from salterns. *Penicillium* isolates were found in water rather than in sediment. *Cladosporium* was neither isolated from the mangroves nor salterns. The salinity of water was more than the sediment in mangroves.

Study on heat tolerance of the cultures revealed that all the cultures had an optimum temperature of 30°C-37°C and were mesophilic. Most of the isolates had a heat tolerance range within 25°C-42°C. *A. penicillioides* had a heat tolerance range of 25°C-37°C. *C. cladosporioides* and *H. werneckii* had a heat tolerance range of 25°C-30°C. *E. amstelodami*, *A. terreus var tererus* from MRw showed heat tolerance of 25°C-50°C.

With increase in salt, cultural and morphological changes were induced in presence of increasing salt concentration and increased temperature. The isolates showed similar cultural and morphological responses to increased salt; there was a lag in growth, undulated and compact appearance. Distorted and decrease in the number of fruiting bodies was observed with increased concentration of salt. With increase in temperature the isolates showed distinct cultural changes such as compact appearance, lag in growth, decreased sporulation while morphological changes such as, distorted and decrease in the number of fruiting body. *Cladosporium* showed bigger spores with increased concentration of salt and increased temperature. Culturally with increased salt the colony was undulated and there was decrease in the colony diameter. With increase in temperature *Eurotium* showed the presence of cliestothecia as well as the conidial head. These cultural and morphological changes in presence of salt and with varying temperature are survival mechanisms of the isolates.

Isolates were selected based on the salt tolerance and growth characteristics and examined for osmolyte contents as means of osmoregulation. Different organisms produced different amount of osmolytes. Variation in the osmolytes produced at 30°C and at different growth phase was observed between different genera, different species of the same genus, and also different echoniches More than one type of osmolyte was produced with increased salt concentration and high temperature. At high temperature trehalose was the osmolyte produced other than glucose, trehalose, erythritol, glycerol, arabitol. The amount of osmolyte produced in the stationary phase was more as compared to the mid exponential phase. More amounts of sugars were produced than the polyols.

Fungi exhibit a great diversity in morphology and distribution. Some species can survive and in the high salinity and at elevated temperature or in various other adverse environments.

Significance of the work

This work presents the salt tolerance range and the heat tolerance range of the halophilic fungi. As compared to bacteria and archae fungi have been less studied. Although fungi have been studied from Dead Sea, estuaries and saltern, their levels of salt tolerance have not been studied. Obligate halophiles from the genus *Aspergillus* with the obligate requirement for salt have been isolated and studied for the first time. Interestingly obigate halophiles were also isolated from Mandovi estuary having low salinity and many halophiles showed salt tolerance levels till 25% salt. Hypersaline waters which were considered to be a primary ecological niche of *H. werneckii* and reported from a number of salterns have also been found in the mangroves of Goa with low salinity. Effect of heat on halophilic fungi was studied, thus showing heat stress in combination with salt stress. This study along with cultural and morphological changes with increased temperature has revealed that heat affects germination, thus affecting the growth of fungi at higher temperature.

It shows the role of osmolytes: sugars and polyols, as an adaptive mechanism of survival in response to high salt and heat stress. There was a variation in the amount and type of osmolytes produced in relation to different growth phases; trehalose has been shown to be an important osmolyte during heat stress.

Knowledge on the adaptive mechanisms of these halophilic fungi would possibly be helpful to minimize the food spoilage caused by the fungi. Since salt stress is an increasing threat to agriculture in many productive areas of the world the knowledge about halophilic fungi may be beneficial. The compatible solutes of the halophiles find their use in cosmeceuticals and pharmaceuticals industry, protein and cell protectants, enhancing PCR, protein folding.

Osmolytes also have a thermostabilizing role and various applications in biotechnology. This would not be possible without the knowledge of halophiles and their compatible solutes produced by the organism.

APPENDICES

APPENDIX-A

Czapeks Dox Broth (CzB) g/L

Sucrose	-	30.0g
$NaNO_3$	-	3g
K_2HPO_4	-	1g
$MgSO_4.7H_2O$	-	0.5g
KCl	-	0.5g
$FeSO_4$	-	0.01g
D/w	-	1000ml

Czapeks Dox Agar (CzA) g/L

CDB	-	As above
Agar	-	15gm

Caesin Agar g/L

Peptone	-	5g
Beef extract	-	3g
Agar	-	15g
Skimmed Milk	-	100ml
D/w	-	1000ml

Tyrosine agar g/L

Tyrosine	-	5g
Peptone	-	5g
Beef extract	-	3g
Agar	-	15g
D/w	-	1000ml

KmNO4

0.5% $KMnO_4$ in 1N NaOH.

Solution 1	-	1N NaOH
NaOH	-	4g
D/w	-	100ml

Mix 0.5gm $KmNO_4$ in solution 1

Antibiotic streptomycin 0.5g/ml

Stock solution

Streptomycin - 0.05g

D/w-10ml

Add 5 ml of stock streptomycin to 95ml 2% CzA media

EDTA + $CaCl_2$

EDTA - 29.2mg

$CaCl_2$ - 25.6mg

D/w - 100ml

Add 10 ml of stock solution and make up to 1L

Solution boiled and used fresh.

Spore suspension

2% NaCl in 0.5% Tween 80

Sterilized for 20 minutes

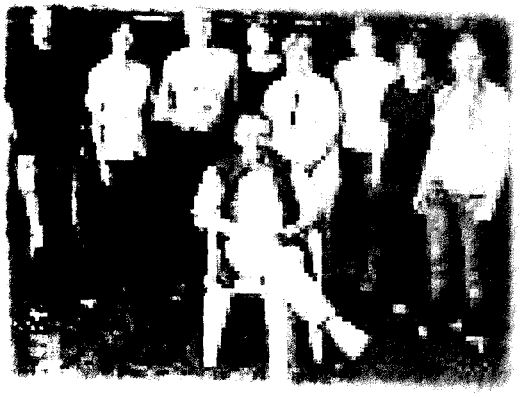
Crude Salt stock solution

Crude salt - 20g

D/w - 100ml

Dissolve and mix with vigorous stirring. Filter the solution using filter paper and use as stock solution for CzA with different salt concentration.

Salt concentration	Stock salt solution (ml)	Vol of D/w (ml)
2%	15	135
5%	38	112
10%	75	75
15%	112	38



BIBLIOGRAPHY

Abadias M, Teixidó N, Usall J, Viñas I and Magan N (2000). Solute stresses affect growth patterns, endogenous water potentials and accumulation of sugars and sugar alcohols in cells of the biocontrol yeast *Candida sake*. J Appl Microbiol, 89:1009–1017.

Adler L, Blomberg A and Nilsson A (1985). Glycerol metabolism and osmoregulation in the salt-tolerant yeast *Debaryomyces hansenii*. J Bacteriol, 162:300–306.

Adler L and Gustafsson L (1980). Polyhydric alcohol production and intracellular amino acid pool in relation to halotolerance of the yeast *Debaryomyces hansenii*. Arch Microbiol, 124:123–130.

Andre L, Nilsson A and Adler L (1988). The role of glycerol in osmotolerance of the yeast *Debaryomyces hansenii*. J Gen Microbiol, 134:669–677.

Andreishcheva EN, Isakova EP, Sidorov NN, Abramova NB, Ushakova NA, Shaposhnikov GL, Soares MIM and Zvyagilskaya RA (1999). Adaptation to salt stress in a salt-tolerant strain of the yeast *Yarrowia lipolytica*. Biochemistry (Moscow), 64:1061–1067.

Ansell R, Granath K, Hohmann S, Thevelein JM and Adler L (1997). The two isoenzymes for yeast NAD+-dependent glycerol 3-phosphate dehydrogenase encoded by GPD1 and GPD2 have distinct roles in osmoadaptation and redox regulation. EMBO J, 16:2179 – 2187.

Attfield PV (1987). Trehalose accumulates in *Saccharomyces cerevisiae* during exposure to agents that induce heat shock response. FEBS Lett, 225:259–263.

Ayerst G (1969). The effects of moisture and temperature on growth and spore germination in some fungi. Journal of Stored Products Research, 5(2):127-141.

Babich H and Stotzky G (1982). Gaseous and heavy metal air pollutants, In:RG Burns, Slater JH (eds) Experimental microbial ecology. Blackwell Scientific Publications, Oxford, pp 631-670.

Babitha S, Ricardo CS and Pandey (2007). Effect of stress on growth, pigment production and morphology of *Monascus* sp. in solid cultures. J Basic Microbiol, 47:118-126.

Bamford S and Heath LAF (2011). The effects of temperature and pH on the vermination of spores of the Chalkbrood fungus, *Ascosphaera apis*. Journal of Apicultural Research, 28:36-40

Bardgett RD and Saggar S (1994). Effects of heavy metal contamination on the short-term decomposition of labeled [C14] glucose in pasture soil. Soil Biol Biochem, 26:727- 733.

Bartnicki-Garcia S, Bracker CE, Gierz G, Lopez-Franco R and Lu H (2000). Mapping the growth of fungal hyphae: orthogonal cell wall expansion during tip growth and the role of turgor. Biophys J, 79:2382-2390.

Beever RE and Laracy EP (1986). Osmotic adjustment in the filamentous fungus *Aspergillus nidulans*. J Bacteriol 168:1358–1365.

Bell W, Klaassen P, Ohnacker M, Boller T, Herweijer M, Schoppink P, van der Zee P and Wiemken A (1992). Characterization of the 56-kDa subunit of yeast trehalose-6-phosphate synthase and cloning of its gene reveal its identity with the product of Cl Fl, a regulator of carbon catabolite inactivation, Eur: J . Biochem, 209:951-959.

Benaroudj N, Lee DH and Goldberg AL (2001). Trehalose accumulation during cellular stress protects cells and cellular proteins from damage by oxygen radicals. J Biol Chem, 276:24261-24267.

Blazquez MA, Stucka R, Feldmann H and Gancedo C (1994). Trehalose-6-P synthase is dispensable for growth on glucose but not for spore germination in *Schizosaccharomyces pombe*. J Bacteriol, 176:3895–3902.

Blomberg A and Adler L (1992). Physiology of osmotolerance in fungi. Adv Microb Physiol, 33:145–212.

Blomberg A (2000). Metabolic surprises in *Saccharomyces cerevisiae* during adaptation to saline conditions: questions, some answers and a model. FEMS Microbiol Lett, 182:1–8.

Bohnert HJ (1995). Adaptation to environmental stresses. Plant Cell, 7:1099-1111.

Borgne SL, Paniagua D and Vazquez-Duhalt R (2008). Biodegradation of organic pollutants by halophilic bacteria and archaea. J Mol Microbiol Biotechnol, 15:74–92.

Borowitzka LJ and Brown AD (1974). The salt relations of marine and halophilic species of the unicellular green alga, *Dunaliella*. The role of glycerol as a compatible solute. Arch Mikrobiol, 96:37-52.

Borse BD (1988). Frequency of Occurrence of Marine fungi from Maharashtra Coast, India. Ind J Mar Sci, 17: 165-167.

Brown AD (1976). Microbial water stress. Bacteriaol Rev,40:803-846.

Brown AD (1978). Compatible solutes and extreme water stress in eukaryotic micro-organisms. Adv Microb Phys, 17:181–242.

Brown AD and Simpson JR (1972). Water relations of sugar-tolerant yeasts: the role of intracellular polyols. J Gen Microbiol, 72:589–591.

Brownell KH and Schneider RW (1985). Roles of matric and osmotic components of water potential and their interaction with temperature in the growth of *Fusarium oxysporum* in synthetic media and soil. Phytopathology 75:53–57.

Buchalo AS, Nevo E, Wasser SP, Oren A and Molitoris HP (1998). Fungal life in the extremely hypersaline water of the Dead Sea: first records. Proc R Soc Lond, 265:1461-1465.

Buchalo AS (2003). Fungi in saline water bodies. In: Nevo E, Oren A, Wasser SP (eds) *Fungal life in the Dead Sea*. Ruggel, Gantner Verlag, pp 41-16.

Butinar L, Sonjak S, Zalar P, Plemenitas A, and Gunde-Cimerman N (2005). Melanized halophilic fungi are eukaryotic members of microbial communities in hypersaline waters of solar salterns. Bot Mar 48:73-79.

Butinar L, Zalar P, Frisvad JC and Gunde-Cimerman N (2005). The genus *Eurotium* - members of indigenous fungal community in hypersaline waters of salterns. FEMS Microbiol Ecol. 51:155-66.

Cantrell SA, Casillas-Martínez L and Molina M (2006). Characterization of fungi from hypersaline environments of solar salterns using morphological and molecular techniques. Mycol Res, 110:962-970.

Casonka LN and Hanson AD (1991). Prokaryotic osmoregulation: genetic and physiology. Anul Rev Microbiol, 45:569–606.

Cayley DS, Lewis BA, Guttman HJ and Record MT (1991). Characterization of the cytoplasm of *Escherichia coli* K-12 as a function of external osmolarity. J Mol Biol, 222:281.

Chen Kuei-Yu and Chen Zuei-Ching (2004). Heat shock proteins of thermophilic and thermotolerant fungi from Taiwan. Bot Bull Acad Sin, 45:247-257.

Clipson N, Jennings D, and Smith J (1989). The response to salinity at the microscopic level of the marine fungus *Dendryphiella salina* as investigated stereologically. New Phytol, 113:121–127.

Cooke JC (1980). Dispersal of microfungi in the Thames River Estuary of Eastern Long Island Sound. Estuarine and Coastal Marine Science, 11:537-545.

Cooney DG and Emerson R (1964). Thermophilic Fungi – an account of their biology, activities and classification. WH Freeman and Co, San Francisco.

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

Cushman JC (2001). Osmoregulation in plants: implications for agriculture. Amer Zool, 41:758-769.

Da Costa MS, Duarte JC and Williams RA, (1989). Microbiology of Extreme Environments and it's Potently for Biotechnology. Elsevier Sci Pub, Ltd England.

D'Amore T, Crumplen R and Stewart GG (1991). The involvement of trehalose in yeast stress tolerance. J In Microbiol, 7:191–196.

Danson MJ and Hough DW (1997). The Structural Basis of Protein Halophilicity. Comparative Biochemistry and Physiology Part A: Physiol, 117:307-312.

DasSarma S and Arora P (2002). Halophiles. In: Encyclopedia of life sciences. Vol 8 London, Nature Publishing Group, pp 458-466.

Das S, Lyla PS and Khan SA (2006). Marine Microbial Diversity And Ecology: Importance And Future Perspectives. Curr Sci, 90:1325-1335.

Davis DJ, Burlak C and Money NP (2000). Osmotic pressure of fungal compatible osmolytes. Mycol Res, 104:800–804.

Deacon JE and Minkley (1974). Desert fishes In GW Brown Jr Ed, Methods in Microbiology Vol 4 pp385-488 Academic press,London.

Del Moral AJ, Severin A, Ramos-Cormenzana, Truper HG and Galinski EA (1994). Compatible solutes in new moderately halophilic isolates. FEMS Microbiol Lett, 122:165–172.

Dennis PP and Shimmin LC (1997). Evolutionary divergence and salinity-mediated selection in halophilic *Archaea*. Microbiol Mol Biol Rev, 61:90–104.

Detkovaa EN and Boltyanskaya YV (2007). Osmoadaptation of haloalkaliphilic bacteria: role of osmoregulators and their possible practical application. Microbiology, 76:511-522

Diano A, Bekker-Jensen S, Dynesen J and Nielsen J (2006). Polyol synthesis in *Aspergillus niger*: influence of oxygen availability, carbon and nitrogen sources on the metabolism. Biotechnol Bioeng, 94:899–908.

Diaz-Munoz G and Montalvo-Rodriguez R (2005). Halophilic black yeasts in the Cabo Rojo solar salterns: its first record for this extreme environment in P.R. Caribb J Sci, 41: 360–365.

Dijksterhuis J, van Driel KGA, Sanders MG, Molenaar D, Houbraken JAMP, Samson R A and Kets EPW (2002). Trehalose degradation and glucose efflux precede cell ejection during germination of heat-resistant ascospores of *Talaromyces macrosporus*. Arch Microbiol, 178:1–7.

Domsch KH, Gams W, and Anderson TH (1980). Compandium of Soil Fungi. Vol 1 and 2. Academic Press, London.

D'Souza J, Araujo A, Karande A and Freitas YM (1979). Studies on fungi from coastal waters of Bombay and Goa. Ind J Mar Sci, 8: 98-102.

D'Souza-Ault MR, Smith LT and Smith GM (1993). Roles of N-acetylglutaminylglutamine amide and glycine betaine in adaptation of *Pseudomonas aeruginosa* to osmotic stress. Appl Environ Microbiol, 59:473-478.

Elbein AD, Pan YT, Pastuszak I and Carroll D (2003). New insights on trehalose: a multifunctional molecule. Glycobiology, 13:17r–27r, ISSN 0959-6658.

El Meleigy MA, Hoseiny EN, Ahmed SA and Al Hoseiny AM (2010). Isolation, Identification, Morphogenesis and ultrastructure of obligate halophilic fungi. Journal of applied sciences in environmental sanitation, 5:201-212.

Falkenberg P, Yaguchi M, Roy C, Zucker M and Matheson AT (1986). The primary structure of the ribosomal A-protein (L12) from the moderate halophile NRCC 41227. Biochem Cell Biol, 64:675–680.

Fell JW and IM Master (1980). The association and potential role of fungi in mangrove detrital systems. Bot Mar, 23:257–263.

Fillinger S, Chaveroche MK, van Dijck P, de Vries R, Ruijter G and Thevelein J (2001) Trehalose is required for the acquisition of tolerance to a variety of stresses in the filamentous fungus *Aspergillus nidulans*. Microbiol, 147:1851–1862.

Fougere F and Streeter LR (1991). Effects of salt stress on amino acids, organic bacteroids and cytosol of alfalfa. Pl Physiol, 96:1228–36.

Franco A, Soto T, Vicente-Soler J, Valero Guillen P, Cansado J and Gacto M (2000). Characterization of tpp1+ as encoding a main trehalose-6P phosphatase in the fission yeast Schizosaccharomyces pombe. J Bacteriol, 182:5880–5884.

Galinski EA (1995). Osmoadaptation in Bacteria. Adv Microb Physiol, 37:273-328.

Galinski EA (1993). Compatible solutes of halophilic eubacteria: molecular principles, water-solute interactions, stress protection. Experientia, 49:487-496.

Galinski EA and Herzog RM (1990). The role of trehalose as a substitute for nitrogen-containing compatible solutes (*Ectothiorhodospira halochloris*). Arch Microbiol, 153:607-613.

Galinski EA and Oren A (1991). Isolation and structure determination of a novel compatible solute from the moderately halophilic purple sulfur bacterium *Ectothiorhodospira marismortui*. Eur J Biochem, 198:593-598.

Galinski EA, Pfeiffer HP and Trüper HG (1985). 1,4,5,6-Tetrahydro-2-methyl-4-pyrimidine carboxylic acid: A novel cyclic amino acid from halophilic phototrophic bacteria of the genus *Ectothiorhodospira*. *Eur* J Biochem 149:135-139.

Galinski EA and Truper HG (1982). Betaine, a compatible solute in the extremely halophilic phototropic bacterium *Ectothiorhodospira halochloris*. FEMS Microbiol Lett, 13:357-360.

Galinski EA and Trüper HG (1994). Microbial behaviour in salt-stressed ecosystems. FEMS Microbiol Rev, 15:95-108.

Grant WD, Gemmell RT and McGenity TJ (1998). Halophiles, in Hirokoshi K and Grant WD (eds) Extremophiles: Microbial life in extreme environments. Wiley-Liss, New York Inc. pp 93-132.

Grishkan I, Nevo E and Wasser SP (2003). Soil micromycete diversity in the hypersaline Dead Sea coastal area, israel. Mycol Prog 2:19–28.

Gunde-Cimerman N, Lorena B, Sonjak S, Turk M, Uršič V, Zalar P and Plemenitaš A (2005). Halotolerant and halophilic fungi from coastal environments in the Arctics. In: Gunde-Cimerman N, Oren A, Plemenitas A (eds) Adaptation to Life at High Salt Concentrations in Archaea, Bacteria, and Eukarya. Netherlands, Springer, pp 397-423.

Gunde-Cimerman N, Ramos J and Plemenitas A (2009). Halotolerant and halophilic fungi. Mycol Res, 113:1231-1241.

Gunde-Cimerman N, Zalar P, de Hoog GS and Plemenitas A (2000). Hypersaline water in salterns–natural ecological niches for halophilic black yeasts. FEMS Microb Ecol, 32:235 – 240.

Gunde-Cimerman N, Zalar P, Petrovic U, Turk M, Kogej T, de Hoog GS and Plemenitas A (2004). Fungi in the salterns. In:Ventosa A (ed) Halophilic microorganisms. Springer-Verlag, Heidelberg, pp. 103-113.

Guiraud P, Steinman R, Seigle-Murandi F and Sage L (1995). Mycoflora of soil around the Dead Sea. Systematic and Applied Microbiology 18:318-322.

Han KH and Prade RA (2002). Osmotic stress-coupled maintenance of polar growth in *Aspergillus nidulans*. Mol Microbiol, 43:1065 –1078.

Hallsworth JE and Magan N (1994). Effect of carbohydrate type and concentration on polyhydroxy alcohol and trehalose content of conidia of three entomopathogenic fungi. Lett Appl Microbiol, 18:8–11.

Han KH, Seo JA and Yu JH (2004). Regulators of G-protein signalling in Aspergillus nidulans: RgsA down regulates stress response and stimulates asexual sporulation through attenuation of GanB (Gα) signaling. Mol Microbiol, 53:529 –540.

Hincha DK and Hagemann M (2004). Stabilization of model membranes during drying by compatible solutes involved in the stress tolerance of plants and microorganisms. Biochem J, 383:277-283.

Hochachka PW and Somero GN (2002). Biochemical adaptation, mechanism and process in physiological evolution. Oxford University Press, UK.

Hohmann S (2002). Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev, 66:300-372.

Hottiger T, Boller T and Wiemken A (1987). Rapid changes of heat and desiccation tolerance with changes of trehalose content in *Saccharomyces cerevisiae* cells subjected to temperature shifts. FEBS Lett, 220:113–115.

Hottiger T, De Virgilio C, Hall M N, Boller T and Wiemken A (1994). The role of trehalose synthesis for the acquisition of thermotolerance in yeast. I. Genetic evidence that trehalose is a thermoprotectant. II. Physiological concentrations of trehalose increase the thermal stability of proteins in vitro. Eur J Biochem, 219:187–193.

Hounsa CG, Brandt, Thevelein J, Hohmann S and Prior BA (1998). Role of trehalose in survival of *Saccharomyces cerevisiae* under osmotic stress. Microbiol, 144:671-680.

Hyde KD and Jones EBG (1988). Marine Mangrove fungi. Mar Ecol, (P.S.Z.N.I) 9:15-33

Jaenicke R and Böhm G (1998). The stability of proteins in extreme environments. Curr Opin Struct Biol, 8:738-748.

Javor BJ (1989). Hypersaline Environments: Microbiology and Biogeochemistry, SpringerVerlag, Berlin.

Jennings DH (1995). The physiology of fungal nutrition. Cambridge, UK: Cambridge University Press.

Jennings DH and Lysek G (1996). Fungal Biology: Understanding the Fungal Lifestyle, Bios Scientific, Oxford.

Jones EBG and Alias SA (1997). Biodiversity of mangrove fungi. In Hyde KD (ed) Biodiversity of tropical microfungi. Hong kong University Press, Hong kong, pp71-92.

Juniper S and Abbott LK (2006). Soil salinity delays germination and limits growth of hyphae from propagules of arbuscular mycorrhizal fungi. Mycorrhiza.16:371-379.

Kane J and Summerbell RC (1987) Sodium Chloride as aid in identification of *Phaeoannellomyces werneckii* and other medically important dematiaceous fungi. J Clin Microbiol, 25:944-946.

Kathiresan.K (2000). A review of studies on Pichavaram mangrove, southeast India. Hydrobiologia, 430:185-205.

Kayingo G, Kilian S G and Prior B A (2001). Conservation and release of osmolytes by yeasts during hypo-osmotic stress. Arch Microbiol, 177:29–35.

Kayingo G, Sirotkin V, Hohmann S and Prior BA (2004). Accumulation and release of the osmolyte glycerol is independent of the putative MIP channel Spac 977.17p in *Schizosaccharomyces pombe*. Anton Van Leeuwenhoek, 85:85–92.

Kemph B and Bremer E (1998). Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. Arch Microbiol, 170:319-330.

Kemph B and Bremer E (1995). Opu A, an osmotically regulated binding protein-dependent transport system for the osmoprotectant glycine betaine in Bacillus subtilis. J Biol Chem, 270:16701-16713.

Kessarkar PM, Rao VP, Shynu R, Mehra P and Viegas BE (2008). The Nature and Distribution of Particulate Matter in the Mandovi Estuary, Central West Coast of India. Estuaries and coasts, 33:39-44.

Kim SY, Lee KH, Kim JH and Oh DK (1997). Erythritol production by controlling osmotic pressure in *Trigonopsis variabilis*. Biotechnol Lett, 19:727–729.

Kis-Papo T, Grishkan I, Gunde-Cimerman N, Oren A, Wasser SP and Nevo E (2003a). Spatiotemporal patterns of filamentous fungi in the hypersaline Dead Sea. In: Nevo E, Oren A, Wasser SP (eds) Fungal Life in the Dead Sea, Gantner Verlag, Ruggel, pp 271-292.

Kis-Papo T, Oren A, Wasser SP and Nevo E (2003b). Physiological adaptation of filamentous fungi to the Dead Sea: survival of spores and vegetative cells. In: Nevo E, Oren A, Wasser SP (eds) Fungal life in the Dead Sea. Ruggel, Gantner Verlag, pp 293-313.

Kis-Papo T, Grishkan I, Gunde-Cimerman N, Oren A, Wasser SP and Nevo E (2003c). Spatiotemporal diversity of filamentous fungi in the hypersaline Dead Sea. In: Nevo E, Oren A, Wasser SP (eds) Fungal life in the Dead Sea. Ruggel, Gantner Verlag, pp 271-292.

Kis-Papo T, Grishkan I, Oren A, Wasser SP and Nevo E (2001). Spatiotemporal diversity of filamentous fungi hypersaline Dead Sea. Mycol Res, 105:749-756.

Kogej T, Ramos J, Plemenita A and Gunde-Cimerman N (2005). The Halophilic Fungus *Hortaea werneckii* and the Halotolerant Fungus *Aureobasidium pullulans* Maintain Low Intracellular Cation Concentrations in Hypersaline Environments. Appl Environ Microbiol, 71:6600–6605.

Kogej T, Gostinčar C, Volkmann M, Gorbushina AA and Gunde-Cimerman N (2006). Mycosporines in extremophilic fungi novel complementary osmolytes? Environ Chem, 3:105–110.

Kogej T, Stein M, Volkmann M , Gorbushina A., Galinski EA and Nina Gunde-Cimerman (2007) Osmotic adaptation of the halophilic fungus *Hortaea werneckii*: role of osmolytes and melanization. Microbiol, 153:4261-4273.

Kuei-Yu Chen and Zuei-Ching Chen (2004). Heat shock proteins of thermophilic and thermotolerant fungi from Taiwan. Bot Bull Acad Sin, 45: 247-257.

Kuhlmann AU and Bremer E (2002). Osmotically regulated synthesis of the compatible solute ectoine in *Bacillus pasteurii* and related *Bacillus* spp. Appl Environ Microbiol. 68:772-783.

Kultz D and Chakravarty D (2001). Hyperosmolality in the form of elevated NaCl but not urea causes DNA damage in murine kidney cells. Proc. Natl. Acad. Sci. USA 98, 1999-2004.

Kuncic MK, Kogej T, Drobne D, and Gunde-Cimerman N (2010). Morphological Response of the Halophilic Fungal Genus Wallemia to High Salinity. Appl Environ Microbiol. 76:329–337.

Kunte HJ, Crane RA, Culham DE, Richmond D and Wood JM (1999). Protein ProQ influences osmotic activation of compatible solute transporter ProP in *Escherichia coli* K-12. J Bacteriol, 181:1537-1543.

Kunte JH, Truper HG and Stan-Lotter S (2002) Halophilic microorganisms. In: Horneck G. Baumstark C (eds) Astrobiology: the quest for the conditions of life. Springer, Berlin. pp.185-199.

Kushner DJ (1978). Life in high salt and solute concentrations. In *Microbial Life in Extreme Environments* Edited by: Kushner DJ. London: Academic Press, pp. 317-368.

Lacey J and Magan N (1991). Fungi in cereal grains: Their occurrence and water and temperature relationships. In: Cereal grain - Mycotoxins, fungi and quality in drying and

storage. Developments in Food Science 26 (Chelkowski J, ed). Elsevier, Amsterdam-London-NewYork-Tokyo, pp.77-118.

Lahav R, Fareleira P, Nejidat A and Abeliovich A (2002). The identification and characterization of osmotolerant yeast isolates from chemical wastewater evaporation ponds. Microb Ecol, 43:388-396.

Lamosa P, Martins LO, Da Costa M S, and Santo H (1998). Effects of temperature, salinity, and medium composition on compatible solute accumulation by *Thermococcus* spp. Applied and environmental microbiology 64:3591–3598.

Lamosa P, Turner DL, Ventura R, Maycock C and Santos H (2003). Protein stabilization by compatible solutes. Effect of diglycerol phosphate on the dynamics of *Desulfovibrio gigas*rubredoxins studied by NMR. Eur J Biochem, 270:4606–4614.

Lanyi J (1974). Salt-dependent properties of proteins from extremely halophilic bacteria. Bacteriol Rev, 38:272-290.

Larsson C and Gustavsson L (1993). The role of physiological state in osmotolerance of the salt-tolerant yeast *Debaryomyces hansenii.* Can J Microbiol, 39: 603 – 609.

Larsson C, Morales C, Gustafsson L and Adler L (1990). Osmoregulation of the salt-tolerant yeast *Debaryomyces hansenii* grown in a chemostat at different salinities. J Bacteriol. 172:1769-1774.

Leong WF, Tan TK and Jones EBG (1991). Fungal colonization of submerged *Bruguiera cylindrica* and *Rhizophora apiculata* Wood. Bot Mar, 34:69–76.

Lewis LG, Learmonth RP and Watson K (1995). Induction of heat, freezing and salt tolerance by heat and salt shock in *Saccharomyces cerevisiae*. Microbiol, 141: 687-694.

Litchfield CD (1998). Survival Strategies for Microorganisms. Hypersaline Environments and Their Relevance to Life on Early Mars, Meteoritics and Planetary Sci. 33:813-819

Luxo C, Nobre MF and Da Costa MS (1993). Intracellular polyol accumulation by yeast like fungi of the genera *Geotrichum* and *Endomyces* in response to water stress (NaCl). Cand J Microbiol, 39:868-873.

Mackey MA, Norton RS and Borowitzka LJ (1984). Organic osmoregulatory solutes in cyanobacteria. J Gen Microbiol, 130: 2177–2191.

MacMillan SV, Alexander DA,Culhqm DE, Kunte HJ, Marshall EV, Rochon D, and Wood JM (1999). The ion coupling and organic substrate specificities of osmoregulatory transporter ProP in *Escherichia coli*. Biochim Biophys Acta, 1420:30-44.

Madern D, Ebel C and Zaccai G (2000). Halophilic adaptation of enzymes. Extremophiles, 4:91-98.

Madkour MA, Smith LT and Smith GM (1990). Preferential osmolyte accumulation: a mechanism of osmotic stress adaptation in diazotrophic bacteria. Appl Environ Microbiol, 56:2876-2881.

Magan N (1988). Effects of water potential and temperature on spore germination and germ-tube growth in vitro and on straw leaf sheaths. Trans Br Mycol Soc, 90:97–107.

Magan N, Challen MP and Elliot TJ (1995). Osmotic, matric and temperature effects on in vitro growth of isolates of *Agaricus bisporus* and *A. bitorquis*. In: Elliot TJ, ed. Science and technology of edible mushrooms. Rotterdam: Balkemaar. pp. 773–780.

Mager WH and de Kruijff A1J (1995). Stress-induced transcriptional activation. Microbiol Rev, 59:506-531.

Makut M.D and Godiya EM (2010). A survey of cellulolytic mesophilic fungi in the soil environment of Keffi Metropolis, Nasarawa State, Nigeria African Journal of Microbiology Research 4:2191-2195.

Marbaniang T and Nazareth S (2006). Isolation of Halotolerant *Penicillium* species from mangroves and salterns and their resistance to heavy metals. Curr Sci, 92:895-896

Martins LO, Empadinhas N, Marugg JD, Miguel C, Ferreira C, da Costa MS and Santos H (1999). Biosynthesis of mannosylglycerate in the thermophilic bacterium *Rhodothermus marinus*. Biochemical and genetic characterization of a mannosylglycerate synthase. J Biol Chem, 274:35407-35414.

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

Maria GL and Sridhar KR (2002). Richness and diversity of filamentous fungi on woody litter of mangroves along the west coast of India. Curr Sci, 83: 25

Mbata TI (2008) Isolation of fungi in hyper saline Dead Sea water. Sudanese J Public Health, 3:170-172.

Mermelstein LD and JG Zeikus (1998). Anaerobic nonmethanogenic extremophiles, p. 255-284. *In* K. Horikoshi, and Grant WD (ed.), Extremophiles, Microbial life in extreme environments. Wiley-Liss, New York.

Miekle AJ, Chudek JA, Reed RH and Gadd GM (1991). Natural abundance 13C-nuclear magnetic resonance analysis of acyclic polyol and trehalose accumulation by several yeast species in response to salt stress. FEMS Microbiol Lett, 82:163–168.

Momani K, El-Hasan T, Auaydeh S and Al-Nawayseh K (2009). Heavy Metals Distribution in the Dead Sea Black Mud, Jordan JJEES Jordan Journal of Earth and Environmental Sciences, 2:50-59.

Moore DJ, Reed RH, and Stewart WDP (1987). A glycine betaine transport system in *Aphanothece halophytica* and other glycine betaine synthesising cyanobacteria. Arch Microbiol, 147:399-405.

Moubasher AH, Abdel-Hafez S, Bagy MM and Abdel-Sater MA (1990). Halophilic and halotolerant fungi in cultivated desert and salt march soil, from Egypt. Acta Mycological. 26:56–81.

Muller V and Saum SH (2005). The chloride regulon of *Halobacillus halophilus*: a novel regulatory network for salt perception and signal transduction in bacteria, p. 303-310. In N. Gunde-Cimerman, A. Oren, and A. Plemenitas (ed.), Adaptation to life at high salt concentrations in Archaea, Bacteria, and Eukarya, Vol 9. Springer, Dordrecht, The Netherlands.

Nass R and Rao R (1998). Novel localization of a Na^+/H^+ exchanger in a late endosomal compartment of yeast. Implications for vacuole biogenesis. J Biol Chem, 273:21054-21060.

Nazareth S, Gonsalves V and Nayak S. A first record of obligate halophilic aspergilli from the Dead Sea Ind J microbiol DOI: 10.1007/s12088-011-0225-z.

Nesci A, Etcheverry M and Magan N (2004). Osmotic and matric potential effects on growth, sugar alcohol and sugar accumulation by Aspergillus section Flavi strains from Argentina. J Appl Microbiol, 96:965–972.

Neves ML, Oliveria RP. and Lucas CM (1997). Metabolic flux response to salt-induced stress in the halotolerant yeast *Debaryomyces hansenii*. Microbiol, 143:1133–1139.

Newell S (1996). Established and potential impacts of eukaryotic mycelia decomposers in marine/terrestrial ecotones. Journal Experimental of Marine Biology and Ecology 200:187-206.

Nikolaou E, Agrafioti I, Stumpf M, Quinn J, Stansfield I and Brown AJP (2009). Phylogenetic diversity of stress signalling pathways in fungi. BMC Evolutionary Biology. 9:44 62.

Nobre MF and da Costa MS (1985). The accumulation of polyols by the yeast *Debaryomyces hansenii* in response to water stress. Can J Microbiol, 31:1061–1064.

Nyyssola A, Kerovuo J, Kaukinen P, von Weymarn N and Reinikaiuem T (2000). Extreme halophiles synthesize betaine from glycine by methylation. J Biol Chem, 275:22196-22201.

Obuekwe CO, Badrudeen AM, Al-Saleh E and Mulder JL (2005). Growth and hydrocarbon degradation by three desert fungi under conditions of simultaneous temperature and salt stress. Int Biodet Biodegrad, 56:197–205.

Oren A (1997). Mycosporine-like amino acids as osmotic solutes in a community of halophilic cyanobacteria. Geomicrobiol J, 14:231–240.

Oren A (1999). Microbial studies in the Dead Sea: Future challenges towards the understanding of life at the limit of salt concentration. Hydrobiologia, 405:1-9.

Oren A (1999). Bioenergetic Aspects of Halophilism Microbiol Mol Biol Rev, 63:334–348.

Oren A (2002) Diversity of halophilic microorganisms: environments, phylogeny, physiology, and applications. J Ind Microbiol Biotechnol, 28:56-63.

Oren A (2003) Physical and chemical limnology of the Dead Sea. In: Nevo E, Oren A, Wasser SP (eds) Fungal life in the Dead Sea. Ruggel, Gantner Verlag, pp 45-67.

Oren A (2010). Industrial and environmental applications of halophilic microorganisms. Environ Technol. 31(8-9):825-834.

Oren A and Gurevich P (1993). The fatty acid synthetase of *Haloanaerobium praevalens* is not inhibited by salt. FEMS Microbiol. Lett, 108:287–290.

Parrou L, Teste MA and Frangois L (1997). Effect of various types of stress on the metabolism of reserve carbohydrates in *Saccharomyces cerevisiae* : genetic evidence for a stress-induced recycling of glycogen and trehalose. Microbiol, 143:1891-1900.

Pedros-Alio C (2004). Trophic ecology of salterns. In: Ventosa A (ed) Halophilic Microorganisms. Springer-Verlag Heidelberg, pp 33-48.

Pedros-Alio C, Calderon-Paz JI, MacLean ML, Medina G, Marrase C, Gasol JM and Guixa-Boixereu N (2000). The microbial food web along salinity gradients. FEMS Microbiol Ecol, 32:143-155.

Peter H, Weil B, Burkovski A, Kramer R and Morbach S (1998). Corynebacterium glutamicum is equipped with four secondary carriers for compatible solutes: identification, sequencing and characterization of the proline/ectoine uptake system, ProP and the ectoine/proline/glycine betaine carrier, EctP, J Bacteriol, 180:6005-6012.

Petrovic U, Gunde-Cimerman N and Plemenitas A (2002). Cellular responses to environmental salinity in the halophilic black yeast *Hortaea werneckii*. Mol Microbiol, 45: 665–672.

Pflüger K, Baumann S, Gottschalk G, Lin W, Santos H and Müller V (2003). Lysine-2,3-aminomutase and β-lysine acetyltransferase genes of methanogenic archaea are salt induced and are essential for the biosynthesis of Nε-acetyl-β-lysine and growth at high salinity. Appl Environ Microbiol, 69:6047-6055.

Pfyffer G E, Pfyffer B U and Rast DM (1986). The polyol pattern, chemotaxonomy, and phylogeny of the fungi. Sydowia, 39:160–201.

Pfyffer GE and Rast DM (1988). The polyol pattern of fungi as influenced by the carbohydrate nutrient source. New Phytol, 109:321–326.

Piper PW (1993). Molecular events associated of heat tolerance by the yeast *Saccharomyces cerevisiae*. FEMS Microbiol Rev, 11:339–356.

Plemenitas A and Gunde-Cimerman N (2005). Cellular reponses in the halophilic black yeast *Hortaea weneckii* to high environmental salinity. In: Adaptation to Life at High Salt Concentrations in *Archea, Bacteria and Eukarya* (Gunde-Cimerman N, Oren A, Plemenitas A, eds). Springer, the Netherlands, pp 455 –470.

Plemenitas A, Vaupotic T, Lenassi M, Kogej T and Gunde-cimerman N (2008). Adaptation of extremely halotolerant black yeast *Hortaea werneckii* to increased osmolarity: a molecular perspective at a glance. Stud Mycol, 61:67-75.

Pul U, Wurm R and Wagner R (2007). The role of LRP and H-NS in transcription regulation: involvement of synergism, allostery and macromolecular crowding. J Mol Biol, 366:900–915.

Qasim SZ and Gupta Sen R (1981) Environmental characteristics of the Mandovi-Zuari estuarine system in Goa Estuarine, Coastal and Shelf Science, 13:557-578.

Rai JN and Chowdhery HJ (1978). Microfungi from mangrove swamps of West Bengal, India. Geophytology, 8:103-110.

Ramos J (2005). Introducing *Debaryomyces hansenii*, a salt-loving yeast. In: *Adaptation* to Life at High Salt Concentrations in Archea, Bacteria and Eukarya (Gunde-Cimerman N, Oren A, Plemenitas A, eds). Springer, the Netherlands, pp 441–451.

Ramirez ML and Chulze SN (2004) Impact of osmotic and matric water stress on germination, growth, mycelia water potentials and endogenous accumulation of sugars and sugar alcohols in *Fusarium graminearum* Mycologia, 96:470–478.

Raper KB and Fennell DI (1965):The genus *Aspergillus*. Baltimare U.S.A, Williams & Wilkins.

Ravishankar JP, Suryanarayanan TS and Muruganandam (2006). Strategies for osmoregulation in the marine fungus *Cirrenalia pygmea* Khol (Hyhomycetes). Indian J Mar Sci, 34:351-358.

Ray B (1986). Impact of bacterial injury and repair in food microbiology: its past, present and future. J Food Prot, 49:651–655.

Redkar RJ, Lemke PA and Singh NK (1996). Altered gene expression in *Aspergillus nidulans* in response to salt stress. Mycologia, 88:256-263.

Regev R, Peri I, Gilboa H and Avi-Dor Y (1990). ^{13}C NMR study of the interrelation between synthesis and uptake of compatible solutes in two moderately halophilic eubacteria. Bacterium Ba1 and *Vibrio costicola*. Arch Biochem Biophys, 278:106-112.

Roberts MF (2004). Osmoadaptation and osmoregulation in archaea: update 2004. Front Biosci, 9:1999-2019.

Roberts MF (2005). Organic compatible solutes of halotolerant and halophilic microorganisms. Saline Syst, 1:1-30.

Roberts MF, Lai MC and Gunsalus RP (1992). Biosynthetic pathway of the osmolytes Nε-acetyl-β-lysine, β-glutamine, and betaine in *Methanohalophilus* strain FDF1 suggested by nuclear magnetic resonance analyses. J Bacteriol, 174:6688-6693.

Robertson DE, Noll D and Roberts M (1992). Free amino acid dynamics in marine methanogens. β-Amino acids as compatible solutes. J Biol Chem, 267:14893-14901.

Robertson DE, Roberts MF, Belay N, Stetter KO and Boone DR (1990). Occurrence of β-glutamate, a novel osmolyte, in marine methanogenic bacteria. Appl Environ Microbiol, 56:1504–1508.

Roeßler M, and Müller V (1998). Quantitative and physiological analyses of chloride dependence of growth of *Halobacillus halophilus*. Appl Environ Microbiol, 64:3813-3817.

Rosasa AL and Casadevall A (1997). Melanization affects susceptibility of Cryptococcus neoformans to heat and cold. FEMS Microbiol Lett, 153:265 – 272.

Rubenhagen R, Ronsch H, Jung H, Kramer R and Morbach S (2000). Osmosensor and osmoregulator properties of the betaine carrier BetP from the *Corynebacterium glutamicum* in proteoliposomes. J Biol Chem, 275:735-741.

Russell NJ, Evans RI, terSteeg PF, Hellemons J, Verheul A and Abee T (1995). Membranes as a target for stress adaption. Int J Food Microbiol, 28:255–261.

Salar RK and Aneja KR (2006). Thermophilous fungi from temperate soils of northern India. J Agr Technol, 2(1): 49-58.

Sarma VV and Vittal BPR (2001). Biodiversity of mangrove fungi on selected mangrove plants in the Godavari and Krishna deltas,east coast of India. Fungal Divers, 6:113-129.

Saum SH, Sydow JF, Palm P, Pfeiffer F, Oesterhelt D, and Müller V (2006). Biochemical and molecular characterization of the biosynthesis of glutamine and glutamate, two major compatible solutes in the moderately halophilic bacterium *Halobacillus halophilus*. J Bacteriol, 188:6808-6815.

Schlesinger MJ (1990). Heat shock proteins. J Biol Chem, 265: 12111 – 12114.

Schliller C, Brewster L, Alexander M R, Gustin M C and Ruis H (1994). The HOG pathway controls osmotic regulation of transcription via stress response element (STRE) of the Saccharornyces cerevisiae CTTl gene. EMBO J, 13: 4382-4389.

Shin CS, Kim HJ, Kim MJ and Ju JY (1998). Morphological change and enhanced pigment production of Monascus when co-cultured with Saccharomyces cerevisiae or Aspergillus oryzae. Biotechnol Bioeng, 59:576–581.

Shivanand P and Jayaraman G (2009). Production of extracellular protease from halotolerant bacterium, *Bacillus aquimaris* strain VITP4 isolated from Kumta coast. Process Biochem.44:1088–1094.

Shivanand P and Mugeraya G (2011). Halophilic bacteria and their compatible solutes osmoregulation and potential applications Curr Sci, 100(10)1516-1518.

Silva Z, Borges N, Martins LO, Wait R, da Costa M S and Santos H (1999). Combined effect of the growth temperature and salinity of the medium on the accumulation of

compatible solutes by *Rhodothermus marinus* and *Rhodothermus obamensis*. Extremophiles, 3:163-172.

Silva-Graça M, and Lucas C (2003). Physiological studies on long-term adaptation to salt stress in the extremely halotolerant yeast *Candida versatilis* CBS 4019 (syn. *C. halophila*). FEMS Yeast Res, 3:247-260.

Smith LT and Smith GM (1989). An osmoregulated dipeptide in stressed *Rhizobium meliloti*. Bacteriol, 171:4714-4717.

Smits GJ and Brul S (2005). Stress tolerance in fungi-to kill a spoilage yeast. Current opinion in Biotechnology 16:225-230.

Singer MA and Lindquist S (1998). Multiple effects of trehalose on protein folding in vitro and in vivo. Mol Cell, 1:639-648.

Soto T, Fernandez J, Vicente-Soler J, Cansado J and Gacto M (1999). Accumulation of trehalose by overexpression of tps1, coding for trehalose-6-phosphate synthase, causes increased resistance to multiple stresses in the fission yeast Schizosaccharomyces pombe. Appl Environ Microbiol, 65:2020-2024.

Suh JH and Shin CS (2000). Physiological analysis on novel coculture of *Monascus* sp. J101 with Saccharomyces cerevisiae. FEMS Microbiol Lett, 190:241–245.

Tansey MR and Brock TD (1978) Microbial life at high temperatures: ecological aspects. In: Microbial Life in Extreme Environments (ed. D. Kushner). Academic Press, London: pp. 159-216.

Tresner HD and Hayes JA (1971). Sodium chloride tolerance of terrestrial fungi. Appl Microbiol, 22:210–213.

Tsui, C.K.M. and Hyde K.D (2004). Biodiversity of fungi on submerged wood in a stream and estuary in the Tai Ho Bay, Hong Kong. Fungal Divers 15:205-220.

Tunbald-Johansson I and Adler L (1987). Effects of sodium chloride concentration on phospholipid fatty acid composition of yeasts differing in osmotolerance. FEMS Microbiol Lett, 43:275-278.

Van Alstyne KL and Houser LT (2003). Dimethylsulfide release during macroinvertebrate grazing and its role as an activated chemical defense. Mar Ecol Prog Ser, 250:175-181.
Van Eck J H, Prior BA and Brandt E V (1989). Accumulation of polyhydroxy alcohols by *Hansenula anomala* in response to water stress. J Gen Microbiol, 135:3505–3513.

Van Laere AJ (1989). Trehalose: reserve and/or stress metabolite? FEMS Microbiol Rev, 63:201-210.

Ventosa A, Nicto JJ and Oren A (1998), Biology of moderately halophilic aerobic bacteria. Microbiol. Mol Biol Rev, 62: 504–544.

Vigants A, Kruce R, Bekers M and Zikmanis P (1998). Response of Zymomonas mobilis levansucrase activity to sodium chloride addition. Biotechnol Lett, 20:1017 – 1019.

Vijaykrishna D, Jeewon R, Hyde KD (2006). Molecular taxonomy,origins and evolution of freshwater ascomycetes. Fungal Divers 23:351-390.

Vittal PR and Sarma V (2006). Diversity and ecology of fungi on mangroves of Bay of Bengal region - An overview. Ind J Mar Sci 35:308-317.

Voegele RT, Hahn M, Lohaus G, Link T, Heiser I and Mendgen K (2005). Possible roles for mannitol and mannitol dehydrogenase in the biotrophic plant pathogen *Uromyces fabae*. Plant Physiol, 137:190–198.

Vossenberg JLCM, Driessen AJM, Grant D and Konings WD (1999) Lipid membranes from halophilic and alkali-halophilic Archae have a low H^+ and Na+ permeability at high salt concentrations. Extremophiles, 3:253-257

Vuorio OE, Kalkkinen N and Londesborough J (1993). Cloning of two related genes encoding the 56-kDa and 123-kDa subunits of trehalose synthase from the yeast *Saccharomyces cerevisiae*. Eur J Biochem, 216:849-861.

Wasser SP, Grishkan I, Kis-Papo T, Buchalo AS, Paul AV, Gunde-Cimerman N, Zalar P and Nevo E (2003) Species diversity of the Dead Sea fungi. In: Nevo E, Oren A, Wasser SP (eds) Fungal life in the Dead Sea. Ruggel, Gantner Verlag, pp 203-270.

Watanabe Y and Takakuwa M (1984). Effects of sodium chloride on lipid composition of *Saccharomyces rouxii*. Agricultural and Biological Chemistry, 48:2415-2422.

Wegmann K (1986). Osmoregulation in eukaryotic algae. FEMS Microbiol Rev, 39:37–43.

WelchWJ and Brown CR (1996). Influence of molecular and chemical chaperones on protein folding. Cell Stress Chaperones, 1:109-115.

Wiemken A (1990). Trehalose in yeast, stress protectant rather than reserve carbohydrate. AntonieVan Leeuwenhoek, 58:209-217.

Witteveen CFB and Visser J (1995). Polyol pools in *Aspergillus niger*. FEMS Microbiol. Lett. 134:57-62.

Wolfe GV (2000). The chemical defense ecology of marine unicellular plankton: contraints, mechanisms, and impacts. Biol Bull, 198:225-244.

Wood JM, Bremer E, Csonka LN, Kramer R, Poolman B van der Heide T and Smith LT 2001. Osmosensing and osmoregulatory compatible solute accumulation by bacteria. Comp Biochem Physiol, 130:437–460.

Yancey P H (2001). Protein, osmolytes and water stress. Am, Zool, 41:699-709.

Yancey PH, Clark ME, Hand SC, Bowlus RD and Somero GN (1982). Living with water stress: Evolution of osmolyte systems. Sci, 217:1214-1222.

Zalar P, De Hoog GS and Gunde-Cimerman N (1999). Ecology of halotolerant dothideaceous *black yeasts*. Stud. Mycol. 43:38-*48*.

Zalar P, Kocuvan MA, Plemenitas A and Gunde-Cimerman N (2005). Halophilic black yeast colonize wood immersed in hypersaline water. Botanica Marina, 48:323–326.

PUBLICATIONS

Journals

- Nazareth S, Gonsalves V, Nayak S. A first record of obligate halophilic aspergilli from the Dead Sea Ind J microbiol DOI: 10.1007/s12088-011-0225-z.
- Nayak S, Gonsalves V, Nazareth S. Isolation and salt tolerance of halophilic fungi from mangroves and solar salterns in Goa – India. (Galley proof-in print).

Papers presented

- Nayak S, Gonsalves V and Nazareth S (2009). Fungal diversity and salt tolerance of fungi from the Dead Sea and Sediment. 35th Annual Meeting of the Mycological Society, University of Madras, Guindy Campus, Chennai.
- Gonsalves V, Nayak S and Nazareth S (2009). Diversity of fungi in salterns and mangroves situated at two different sites in Goa. 36th Annual Meeting of the Mycological Society, Department of Botany, Goa University.
- Nayak S, Gonsalves V and Nazareth S (2009). Halophilic fungal isolates from the Mandovi estuary in Goa. Association of Microbiologists in India National Chemical Laboratory, Pune.
- Nazareth S, Gonsalves V, Nayak S (2011). Diversity of halophilic filamentous fungi and black yeasts in Goa. National symposium ICAR Goa.
- Nazareth S, Nayak S, Gonsalves V (2011). Halophilic filamentous fungi isolated from marine econiches of Goa–India. Asian mycological congress Icheon Korea.
- Gonsalves V, Nayak S and Nazareth S (2012). Halophilic fungi from Dead Sea and their production of osmolytes. 38th Annual Meeting of the Mycological Society of India, Department of Botany, Amaravati.

Nazareth S, Nayak S, Gonsalves V. Kavaka. A first record for the occurrence of halophilic black yeast Hortaea werneckii from brackish waters of mangroves of Goa-India (accepted).

Indian J Microbiol
DOI 10.1007/s12088-011-0225-z

ORIGINAL ARTICLE

A First Record of Obligate Halophilic Aspergilli from the Dead Sea

Sarita Nazareth · Valerie Gonsalves · Shweta Nayak

Received: 7 June 2011 / Accepted: 22 August 2011

Abstract The isolation of obligate halophilic aspergilli from the Dead Sea and the range of salt tolerance of halophilic fungi isolated, are reported here for the first time. The mycobiota of the Dead Sea isolated in this study, was dominated by *Aspergillus* and *Penicillium* species; *Cladosporium* were found in lesser numbers. All three genera were obtained from the water sample; however, *Aspergillus* was the only genus obtained from the sediment. There was significant difference in growth of each isolate at different salt concentrations and intraspecies analysis revealed dissimilarity in response of strains to different salt concentrations in the growth medium The isolates were euryhaline, with halotolerance up to 20–25% solar salt, *Aspergillus* and *Penicillium* species showing a higher level of halotolerance, as compared to that of *Cladosporium*. Halophilic fungi were found in greater numbers in the sediment sample as compared to that in the water sample. *Penicillium* and *Cladosporium* species were exclusively facultative halophiles, while some species of *Aspergillus* were facultative halophiles. All the obligate halophiles isolated, belonged to the genus *Aspergillus* and were identified as *A. penicillioides* and *A unguis*, the latter being a first record of the species from the Dead Sea.

Keywords Dead Sea · Obligate halophile · *Aspergillus* · *Penicillium* · *Cladosporium*

S. Nazareth (✉) · V. Gonsalves · S. Nayak
Department of Microbiology, Goa University, Taleigao Plateau, Panaji, Goa 403206, India
e-mail: saritanazareth@yahoo.com

Introduction

The hypersaline environment contains salt concentrations in excess of sea water [1]. Hypersaline conditions drastically decrease the biodiversity by exerting a strong selective pressure, resulting in the development of halophilic and halotolerant microbes [2], the study of which has drawn much interest [3]. Fungi are known to adapt to certain extreme environments, but isolation and identification of fungi from these habitats have been frequently hindered due to their slow growth and low ability to compete [4].

The Dead Sea, an extremely hypersaline environment, has high concentrations of about 340‰ total dissolved salts, with levels of divalent ions like magnesium and calcium being higher than that of monovalent ions. This unique ionic composition makes it hostile to microorganisms and could be responsible for the paucity in the diversity of the Dead Sea biota [5, 6]. Microbes that inhabit high-salt environments are adapted to high levels of ions, as well as to low a_w, and are described as halophilic or halotolerant, while the term xerophilic or xerotolerant is restricted to describing those organisms growing at a_w values imposed by *other than* inorganic ions [7].

A number of filamentous fungi have been isolated from the Dead Sea [3, 5, 6]; however, only the species of *Gymnascella marismortui* has been reported as a true halophile, having an absolute requirement of salt for growth [5, 8].

The present study was aimed at investigating the existence of obligate halophilic fungi in the Dead Sea, and records the isolation of obligate halophilic aspergilli for the first time. It also illustrates the range of salt tolerance of halophilic fungi, differentiating these as obligate or facultative halophiles, an aspect which has been insufficiently studied hitherto.

Published online: 10 September 2011

Materials and Methods

Sample Collection

Dead Sea water (DSw) and sediment (DSs) samples were aseptically collected at Kalya beach (31°45′50.62″N and 35°30′13.10″E) at about 0.5 m depth and 3–4 m from the shore.

Salinity and pH Measurement

Salinity was measured with an Atago refractometer; pH was recorded by Cyberscan pH meter. DSs sample was suspended in deionised water (1:5 w/v), centrifuged at 3000 rpm, 10 min (Remi centrifuge), to remove particulate matter, before analysis.

Isolation and Identification of Fungi

DSw and a suspension of DSs were each plated on 10 plates of Czapek-Dox Agar + 20% solar salt (20%S-CzA) and on CzA + 10% solar salt (10%S-CzA) plate, then incubated at 30°C for 30 days.

All isolates obtained from the water sample were picked; those obtained from sediment, being in greater numbers, were selected based on apparent dissimilarity in cultural characteristics. Purified isolates were identified on basis of colony and micromorphology characteristics with reference to identification keys [9–11].

The identification of selected obligate halophilic isolates was confirmed by ITS rDNA sequence analysis (Merck-GeNei Services), using consensus primers for 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA gene fragment, using PCR conditions of one cycle of denaturation at 94°C (5 min), followed by 35 cycles of denaturation at 94°C (1 min), annealing at 55°C (45 s) and extension at 72°C (1.30 min), then maintained at 72°C (10 min) Sequence data was analyzed to find the closest homologs; GenBank accession numbers were obtained. Sequence similarities were obtained using NCBI BLAST. Alignment and phylogenetic tree were constructed in Clustal X version 2 and the NJ distance method.

Determination of Halotolerance

Spore suspensions of isolates were prepared in 2% saline containing Tween 80 (0.05% final concentration), and spot-inoculated on CzA containing solar salt (0–30%). These were subcultured by inoculating 10^3 spores in triplicate on plates containing corresponding salt concentrations. Growth was recorded in terms of colony diameter after 7 days incubation, or after 15 days for those showing delayed growth.

Statistical Analysis

The correlation of viable counts with salinity of the sample, or, with salinity of isolation media was performed by the Pearson correlation test. Fungal diversity of water or of sediment, each on 10%S-CzA and on 20%S-CzA, was analyzed by Shannon–Weaver index (H). Statistically significant difference ($P < 0.05$) in the effect of different salt concentrations on growth of all isolates, as well as in growth within the species, was analysed by two-way ANOVA.

Results

Salinity and pH

The DSw sample had a salinity of 370‰ and pH of 5.65; DSs showed 450‰ salinity, pH 5.3.

Density and Diversity of Fungal Isolates

The fungal count of DSw was 0.2 cfu ml^{-1} on 20% S-CzA and 0.1 cfu ml^{-1} on 10% S-CzA; DSs yielded 91.1 cfu g^{-1} on 20% S-CzA and 8.9 cfu g^{-1} on 10% S-CzA. The density of fungal isolates showed a positive correlation with salinity of the sample ($r = 1$) and with salt concentration used in the isolation medium ($r = 1$).

Twenty-five isolates were obtained from DSw: twenty-one were isolated on 20% S-CzA, of which eight were identified as *Aspergillus*, twelve as *Penicillium* and one with sterile mycelia; four isolates were obtained on 10% S-CzA, identified as *Cladosporium*. DSs was dominated exclusively by aspergilli; thirty from the total of 414 obtained on 20% S-CzA and all four obtained on 10% S-CzA were picked for further study. The identification of the isolates is shown in Table 1. On 20% S-CzA, DSw showed seven species ($H = 0.72$), DSs yielded two species ($H = 0.15$); on 10% S-CzA, DSw and DSs each showed only one species ($H = 0$).

Halotolerance

Figure 1 shows the halotolerance of an individual isolate, of isolates within a species with statistically significant difference, or of an isolate as indicative of a group having similarity. The halophilic nature of each isolate is indicated in Table 1.

Isolates from Water Sample

Five of the eight aspergilli—DSw1, DSw5, DSw6, DSw17 and DSw22, did not grow in absence of salt and were

Table 1 Fungi from Dead Sea water and sediment samples isolated on Czapek-Dox Agar supplemented with 10 or 20% solar salt

Isolate	Sample	Solar salt added to CzA (%)	Isolate number	Halophilic nature
A. penicillioides	Water	20	DSw1, DSw5, DSw6, DSw17, DSw22	Obligate
A. unguis	Water	20	DSw12	Obligate
A. restrictus	Water	20	DSw14	Facultative
A. flavus var columnaris	Water	20	DSw9	Facultative
P. corylophilum	Water	20	DSw4, DSw8, DSw10, DSw11, DSw13, DSw15, DSw18, DSw19, DSw20.	Facultative
P. steckii	Water	20	DSw7, DSw21	Facultative
P. waksmanii	Water	20	DSw16	Facultative
C. cladosporioides	Water	10	DSw23, DSw24, DSw25, DSw26	Facultative
Sterile mycelia	Water	20	DSw2	Obligate
A. penicillioides	Sediment	20	DSs28, DSs29, DSs30, DSs31, DSs32, DSs33, DSs34, DSs35, DSs36, DSs37, DSs38, DSs39, DSs40, DSs41, DSs42, DSs43, DSs44, DSs45, DSs46, DSs49, DSs53, DSs54, DSs56	Obligate
			DSs47, DSs48, DSs51, DSs55	Facultative
A. versicolor	Sediment	20	DSs50, DSs52, DSs57	Facultative
		10	DSs58, DSs61	Facultative
			DSs59, DSs60,	Halotolerant

obligate halophiles, showing identical cultural and morphological characteristics; the ITS sequence analysis of one from this group, DSw22, confirmed it to be *A. penicillioides*. Similarly, DSw12, also an obligate halophile and DSw14 which showed delayed growth in absence of added salt, were identified by ITS sequence analysis as *A. unguis* and *A. restrictus*, respectively. The GenBank accession numbers were HQ702381 for DSw12, HQ702382 for DSw14, HQ702383 for DSw22. The phylogenetic relationship of these isolates is shown in Fig. 2. The other isolates grew in absence of added salt, but showed enhanced growth at increased levels of salt and were termed as facultative halophiles; these were identified as *A. flavus* var. *columnaris*, *Penicillium* species: *P. corylophilum*, *P. steckii*, *P. waksmanii* and *Cladosporium cladosporioides*. The sterile fungus was an obligate halophile.

The six obligate halophilic aspergilli: five *A. penicillioides* and *A. unguis* grew in presence of 2 to 20–25% salt, with maximal growth at 10 and 10–15% salt, respectively; the facultative halophiles *A. restrictus* and *A. flavus* var. *columnaris*, grew optimally at 5% salt, with tolerance of 20% salt during growth.

The obligate halophilic sterile fungus grew in presence of 2–15% salt and maximally at 10% salt.

The facultative halophilic penicilli cultures all required 2–5% salt for optimal growth. *P. corylophilum* had maximum salt tolerance of 15% (two isolates), 20% (six isolates), or 25% (one isolate); tolerance levels of *P. steckii* and *P. waksmanii* were 20–25% salt. The *Cladosporium* isolates were also facultative halophiles, which grew optimally with 5% salt and showed tolerance of 15–20% salt.

Significant difference ($P < 0.05$) was obtained in growth of each isolate at different salt concentrations, as well as in growth amongst the strains of *A. penicillioides*, *C. cladosporioides* and *P. corylophilum*; however, the strains of *P. steckii* were similar.

Isolates from Sediment Sample

Twenty-three of the isolates from DSs isolated on 20% S-CzA, did not grow in absence of salt and were obligate halophiles, identified as *A. penicillioides*; DSs35 and DSs40, one each from amongst those showing delayed growth at salt concentrations of 2 and 5% salt, respectively, were confirmed by gene sequence analysis as *A. penicillioides;* the GenBank accession numbers were HQ702384 for DSw35 and HQ702385 for DSw40. Figure 2 indicates the phylogenetic relationship of these isolates. Four other isolates which showed delayed growth in absence of salt were also identified as *A. penicillioides* and three facultative halophiles were identified as *A. versicolor*. The four isolates obtained on 10%S-CzA plates were identified as *A. versicolor*.

The twenty-three obligate halophilic *A. penicillioides* were grouped on the basis of the minimum requirement of 2 or 5% salt for growth, fifteen and eight isolates, respectively, with some of these showing delayed growth at the minimum salt requirement. Two isolates with slow growth at 2% salt, grew optimally with 5% salt, while the other

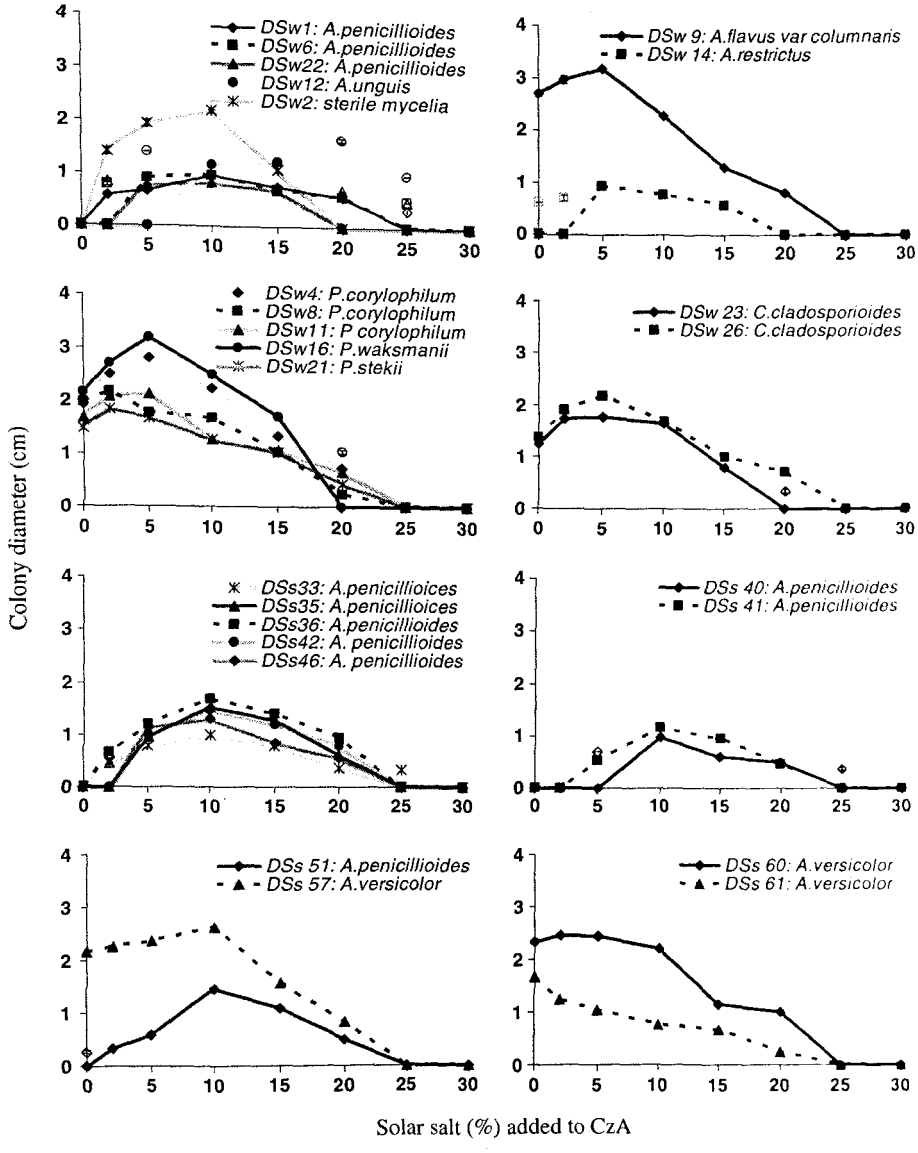


Fig. 1 Salt tolerance curves of the isolates from Dead Sea water and sediment samples as recorded after 7 days incubation; unconnected *open symbols* indicate delayed growth at respective salt concentrations, after 15 days incubation

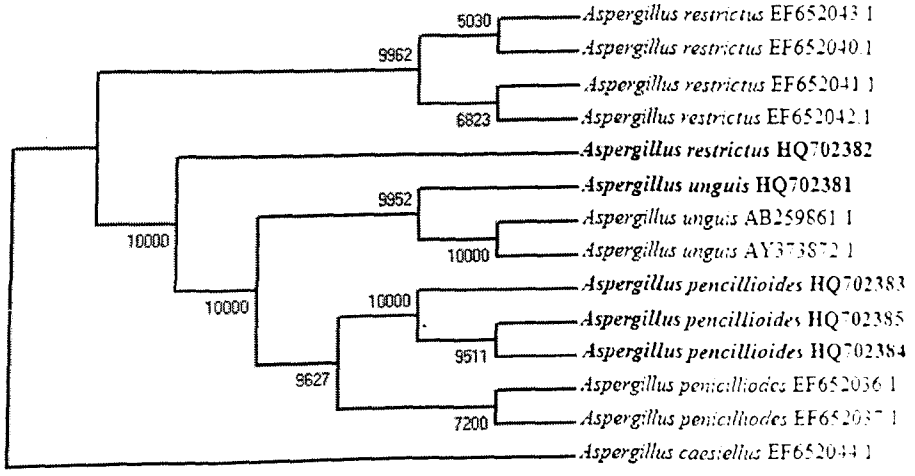


Fig. 2 Phylogenetic tree obtained from the alignment of ITS region of rDNA of *Aspergillus* species; isolates obtained from the present study are indicated in *bold*, along with the GenBank accession numbers

isolates grew optimally with 10% salt; all tolerated 20% salt concentration, with some of these growing even at 25% salt within 15 days.

Three of the facultative halophilic *A. penicillioides* grew at 20% salt, with optimum growth of one at 5% and two at 10% salt; the fourth isolate grew at 25% salt within 15 days, and optimally at 10% salt.

The facultative *A. versicolor*, three isolated on 20% S-CzA and one on 10% S-CzA.all grew at a range of 0–20% salt with maximal growth obtained in presence of 2, 5 or 10% salt. Two other isolates picked from 10% S-CzA, were halotolerant, with optimal growth in absence of added salt, and decreased growth in presence of increased levels of salt.

Significant difference ($P < 0.05$) was obtained for the growth of isolates at different salt concentrations. Analysis of growth of *A. penicillioides* at different salt concentration revealed that those strains that required a minimum of 2% salt for growth were dissimilar, while those that required a minimum of 5% salt for growth, were similar. There was also similarity among strains of the facultative halophilic *A. penicillioides* and among that of *A. versicolor*, all isolated on 20% S-CzA; however, there was dissimilarity between strains of *A. versicolor* isolated on 10% S-CzA.

Discussion

The Dead Sea is a hypersaline environment with a unique ecology, its salinity being higher than the recorded 275‰ of the Great Salt Lake, Utah [1, 8]; the study of its microbiota has attracted much interest in recent decades. The acidic pH of the Dead Sea, as recorded herein and confirmed in earlier reports, is unlike other neutral or alkaline hypersaline lakes [12] and would be conducive to fungal life.

The fungi obtained in the present work could be native as well as of terrestrial source, brought by the influx of water from the Jordan River, or through water movement at the shore line, as well as by airborne contamination and human activity. Microbes may be present in the dormant state, able to return to the physiologically active state under favourable conditions [13]; mycelial forms may develop only during the rare episodes wherein the salinity of the upper water layers is reduced as a result of inflow of freshwater. The high concentration of salt used for fungal isolation in this study, and the avoidance of a rich/more easily assimilable carbon medium, would not only have aided the selection of halophiles, but also served to inhibit the growth of any faster-growing terrestrial fungi.

Aspergillus and *Penicillium* were the principal genera obtained with the methodology used in this study. Diversity studies have revealed that at low water potential the active mycota is dominated by species of *Aspergillus* and *Penicillium*, which are thus numerically the most common taxa [2], as observed in studies on Mono Lake [14], salterns of Slovenia [15], Arctic [16] and Cabo Rojo, [17], and our own studies on local salterns (unpublished data). Amongst the species obtained in this study, the isolation of *A. unguis* is a first report, while *A. penicillioides*, *A. restrictus*, *A. versicolor*, *C. cladosporioides*, *P. steckii* and *P. corylophilum* have been recorded in earlier work on the Dead Sea mycobiota [2, 3, 18, 19], *A. versicolor* and *C. cladosporioides* having been reported as indigenous species [18]. The isolation of these fungi in the summer months corroborates the report of Kis-Papo et al. [18].

It was observed that while almost all of the fungi from different genera were halophilic in nature, obligate halophiles were found only in the genus *Aspergillus*, namely *A. penicillioides* and *A. unguis*, which could be indigenous fungi; these species add to the recorded strains of the obligate halophilic *Gymnascella marismortui* [5]. *A. versicolor* and *A. flavus var columnaris*, *Penicillium* and *Cladosporium* species were facultative halophiles. Although some of these species have been reported earlier, their halophilic nature as obligate or facultative halophiles, or halotolerant, had not been defined.

It was interesting to note that the facultative halophiles *Penicillium* and *Cladosporium* were found exclusively in the water, while the sediment yielded only *Aspergillus* species, mainly the obligate halophilic *A. penicillioides* and, in lesser numbers, the facultative halophiles *A. penicillioides* and *A. versicolor*. The facultative halophilic *A. flavus var. columnaris* and *A. restrictus*, were isolated only from the water, while *A. versicolor* was obtained only from the sediment. Notably, *Cladosporium* species were isolated only on medium containing 10% salt but not in presence of 20% salt, indicating that the genus as a whole is not as halotolerant as the genera of *Aspergillus* and *Penicillium*. Furthermore, *A. penicillioides* was present in high density and formed the principal fungi in the sediment sample, where, the a_w would be low due to the higher matrix potential and salinity [13].

The precipitation of halites from the water column due to evaporation [12] could be responsible for the higher salinity of the sediment as compared to that of the water; this could be a reason for obligate halophiles to survive more readily and in greater numbers, in the sediment, while the facultative halophiles were found more in the water column. Only those organisms able to withstand these conditions can thrive [13], which could explain the lower diversity obtained in the sediment.

The obligate halophiles isolated were observed to grow slower, yielding smaller colonies than the facultative halophiles and halotolerant fungi. Marine fungi are noted to grow very slowly on artificial media as compared to

terrestrial fungi [20]; the reduced growth rate of salt adapted cultures may be related to the increase in energy demands under stress, wherein cells may utilize energy for compartmentalization or exclusion of Na^+ ion, direct synthesis of compatible osmolytes and synthesis of proteins conferring salt tolerance [21].

The isolates were obtained in greater numbers on isolation medium containing 20% salt, as compared to that obtained on medium with 10% salt, and showed an extreme halotolerance of 20–25% salt; however, no extreme halophile was obtained, the isolates being mainly moderate halophiles. The isolates were euryhaline in nature, growing over a wide range of salt concentrations.

In the present study, it was observed that while almost all of the fungi from different genera were halophilic in nature, obligate halophiles were found only in the genus *Aspergillus*: *A. penicillioides* and *A. unguis,* which could be indigenous mycobiota; these species add to the known strains of *Gymnascella marismortui.* [5]. *A. versicolor* and *A. flavus* var. *columnaris*, *Penicillium* and *Cladosporium* species were facultative halophiles. Although some of these species have been reported earlier, their halophilic nature as obligate or facultative halophiles, or halotolerant, had not been defined.

This is a first description of aspergilli species as obligate halophiles and those of the genera *Penicillium* and *Cladosporium* as facultative halophiles, establishing all the isolates as euryhaline in nature; it is also a first report of obligate halophilic *A. penicillioides* and *A. unguis* from the Dead Sea.

References

1. DasSarma S, Arora P (2002) Halophiles. In: Encyclopedia of life sciences, vol 8. Nature Publishing Group, London, pp 458–466
2. Das S, Lyla PS, Khan SA (2006) Marine microbial diversity and ecology: importance and future perspectives. Curr Sci 90:1325–1335
3. Grishkan I, Nevo E, Wasser SP (2003) Soil micromycete diversity in the hypersaline Dead Sea coastal area, Israel. Mycol Prog 2:19–28
4. Gunde-Cimerman N, Plemenitas A (2006) Ecology and molecular adaptation of the halophilic black yeast *Hortaea werneckii.* Rev Environ Sci Biotechnol 5:323–331
5. Buchalo AS, Nevo E, Wasser SP, Oren A, Molitoris HP (1998) Fungal life in the extremely hypersaline water of the Dead Sea: first records. Proc R Soc Lond 265:1461–1465
6. Kis-Papo T, Oren A, Wasser SP, Nevo E (2003) Survival of filamentous fungi in hypersaline Dead Sea water. Microbial Ecol 45:183–190
7. Grant WD (2004) Life at low water activity. Phil Trans R Soc Lond B 359:1249–1267
8. Kushner DJ (1978) Life in high salt and solute concentrations. In: Kushner DJ (ed) Microbial life in extreme environments. Academic Press, London, pp 317–368
9. Domsch KH, Gams W, Anderson TH (1980) Compendium of soil fungi, vol 1. IHW-Verlag, Eching
10. Ellis MB (1971) Dematiaceous hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, England
11. Raper KB, Fennell DI (1965) The genus *Aspergillus*. Williams and Wilkins Co, Baltimore
12. Oren A (2003) Physical and chemical limnology of the Dead Sea. In: Nevo E, Oren A, Wasser SP (eds) Fungal life in the Dead Sea. Gantner Verlag, Ruggel, pp 45–67
13. Kunte JH, Truper HG, Stan-Lotter S (2002) Halophilic microorganisms. In: Horneck G, Baumstark C (eds) Astrobiology: the quest for the conditions of life. Springer, Berlin, pp 185–199
14. Steiman R, Ford L, Ducros V, Lafond JL, Guiraud P (2004) First survey of fungi in hypersaline soil and water of Mono Lake area (California). Antonie van Leeuwenhoek 85:69–83
15. Gunde-Cimerman N, Cerovac S, Zalar P (2001) Biodiversity of filamentous fungi in the saltpans Secovlje. Acta Biol Slov 44:25–30
16. Gunde-Cimerman N, Lorena B, Sonjak S, Turk M, Uršič V, Zalar P, Plemenitaš A (2005) Halotolerant and halophilic fungi from coastal environments in the Arctics. In: Gunde-Cimerman N, Oren A, Plemenitaš A (eds) Adaptation to life at high salt concentrations in Archaea bacteria and Eukarya. Springer, The Netherlands, pp 397–423
17. Cantrell SA, Casillas-Martinez L, Molina M (2006) Characterization of fungi from hypersaline environments of solar salterns using morphological and molecular techniques. Mycol Res 110:962–970
18. Kis-Papo T, Grishkan I, Gunde-Cimerman N, Oren A, Wasser SP, Nevo E (2003) Spatiotemporal patterns of filamentous fungi in the hypersaline Dead Sea. In: Nevo E, Oren A, Wasser SP (eds) Fungal life in the Dead Sea. Gantner Verlag, Ruggel, pp 271–292
19. Wasser SP, Grishkan I, Kis-Papo T, Buchalo AS, Paul AV, Gunde-Cimerman N, Zalar P, Nevo E (2003) Species diversity of the Dead Sea fungi. In: Nevo E, Oren A, Wasser SP (eds) Fungal life in the Dead Sea. Gantner Verlag, Ruggel, pp 203–270
20. Ravishankar JP, Suryanarayanan TS, Muruganandam (2006) Strategies for osmoregulation in the marine fungus *Cirrenalia pygmea* Khol. (Hyhomycetes). Indian J Mar Sci 34:351–358
21. Redkar RJ, Lemke PA, Singh NK (1996) Altered gene expression in *Aspergillus nidulans* in response to salt stress. Mycologia 88:256–263

Indian Journal of Geo-Marine Sciences
Vol. 41(1), February 2012, pp.

Isolation and salt tolerance of halophilic fungi from mangroves and solar salterns in Goa - India

Shweta S Nayak, Valerie Gonsalves & Sarita W Nazareth*
Department of Microbiology, Goa University, Taleigao Plateau, Goa 403 206, India
[E.Mail: saritanazareth@yahoo.com]

Received 30 May 2011 ; revised 16 September 2011

High salt tolerant fungal genera were isolated from mangroves and solar salterns of Goa. These belonged mainly to the genus *Aspergillus*, some *Penicillium* and a few *Eurotium* and *Hortaea*. Most of the isolates were facultative halophiles, euryhaline in nature, not having an absolute requirement of added salt for growth. They were able to grow even at high salt concentrations and showing optimal growth only in presence of added salt in the medium. Only one species, *A. penicillioides*, had an absolute requirement of added salt in the medium for growth and were termed as obligate halophiles.

[Key words: Mangroves; salterns; fungi; euryhaline; obligate halophile; *A. penicillioides.*]

Introduction

The salinity of the brackish water of mangroves, situated at the intertidal zone, is less than that of sea water. Hypersaline waters of solar saltens, termed as thalassohaline[1], have salt concentrations greater than 3.5%, that of sea water. Salinity of these saltens steadily increase to saturation point; variations in pH, temperature, light intensity, oxygen and nutrient concentrations permit the growth of different microbial communities[2,3].

Halophiles include mostly prokaryotic and eukaryotic microorganisms with the capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salt. Fungi living in different saline environments are generally adapted to extreme conditions of low a_w, temperature, pH and salinity[4]. Mangicolous filamentous fungi have been isolated from different parts along the Indian West Coast[5-9] and East Coast[10-15], and from around the globe[15-18]. However, there is no record of these fungi characterised in terms of halophily, other than that of *Penicillium* by Marbaniang and Nazareth[9] and the studies on the osmoregulation of the hyphomycetes *Cirrenalia pygmea* Kohl[19]. The study on the microbiota of salterns in India, has focussed mainly on bacteria[20-25] with reports on haloarchaea being exclusively from Goa, on the West Coast of the Indian peninsula. Investigations on the mycobiota from salterns of Slovenia-Adriatic[2,26,27] and Cabo Rojo, Puerto Rico[28,29] and from saline soils, Soos, Czech Republic[30] have revealed the presence of halophilic black yeasts and halotolerant dematiaceous and other filamentous fungi.

The present study describes the isolation of high salt-tolerant fungi from two different econiches - the brackish environment of the mangroves and the hypersaline system of solar salterns in Goa. It also demonstrates the halophilic nature of these isolates, together with their range of salt tolerance.

Materials and Methods

Collection of Samples

Samples of water (w) and sediment (s) were collected during the pre-monsoon summer season from Mangroves (M) and solar Salterns (S) at two sites in Goa: at Ribander (R), lying alongside the Mandovi estuary, Goa, leading into the Arabian Sea, and one interiorly at Santa Cruz (C), along the offshoot of the estuary. Water from mangroves are channeled into the salterns. Sampling of the mangrove water was done from five different sites about 15 cm apart, at the intertidal zone during low tide, at a distance of 1m from the shore and a depth of 0.5m, and pooled together. Sediment samples were also collected from the same points. Brine and sediment samples from salterns were obtained from five different saltpans at each site and pooled. The temperature of the mangroves and salterns was

*Corresponding author

recorded in the morning (07.00 hr) and at mid-day (12.00 hr). Physicochemical conditions of pH and salinity of the samples were determined using a Cyberscan pH meter and Atago Handheld refractometer; for sediment samples, 1 g was shaken in 5 ml deionised water and the supernatent used for pH and salinity measurement.

Samples were plated on Czapek Dox agar (CzA) ammended with 20% solar salt (20%S-CzA) and containing streptomycin, 0.5g/l. The plates were incubated at 30°C for a total of three weeks. All the black yeasts were picked, while the other isolates were picked up based on apparent dissimilarity of cultural characteristics and purified. The isolates were identified on the basis of colony and morphological characteristics: nature of growth, spore colour, pigmentation, the fruiting body and arrangement of spores with reference to identification keys[31,32] and http: www.doctorfungus.org/thefungi/hortaea.php. The identification of the obligate halophilic species and the black yeast was confirmed by DNA sequence analysis using consensus primers for 18S rRNA (partial sequence), ITS1, 5.8S rRNA, ITS2 (complete sequence); and 28S rRNA (partial sequence) gene fragment and the sequence data was analysed for closest homologues (Merck, Bangalore GeNei Services); the GenBank accession number was obtained.

Halotolerance was studied as described earlier[9]. Spore suspensions of the isolates were prepared in 2% saline containing Tween 80 at a final concentration of 0.05% and spot-inoculated on plates of CzA amended with different concentrations from 0 to 30% solar salt, and incubated at 30°C. These were then subcultured in triplicate, with 10^3 spores inoculum onto the respective medium. Growth was measured in terms of colony diameter after 7d incubation, or after 15d incubation for those showing delayed growth. Those isolates which did not grow on CzA without added salt, were also grown on Malt Extract Agar (MEA), Potato Dextrose Agar (PDA) and Sabouraud Agar (SA), each with and without solar salt, to confirm the obligate requirement of salt for growth.

Results

The temperatures recorded at the mangroves, Ribander (MR) and Santa Cruz (MC) varied from 23°C in the morning to 32°C-33°C at noon; that at the salterns, Ribander (SR) and Santa Cruz (SC) was 39°C in the morning and 41°C at noon. The pH of the mangrove water (w) and sediment (s) samples was close to neutral: MRw and MRs at 6.65 and 7.03 respectively, MCw and MCs at 7.03 and 7.16 respectively, the pH of the sediment samples being marginally higher than that of the water samples. Saltern waters were slightly alkaline, while the sediment was a little acidic: SRw at pH 8.06 and SRs at 6.00, SCw at 7.62 and SCs at 6.15. The salinity of the mangrove waters and sediment at Ribander was 32‰ and 15‰ respectively, while salinity at Santa Cruz was 25‰ and 10‰ respectively. Salinity of the salterns waters and sediment was 295‰ and 125 ‰ respectively at Ribander, and 230‰ and 140‰ respectively at Santa Cruz.

Fungal isolates

Total number of highly salt-tolerant isolates obtained in the 100 mL water samples or of 4 g of sediment samples from each site, were 17 from MRw, 112 from SRw, 30 from SRs, 4 from MCw, 7 from MCs and 15 from SCw. The selected isolates obtained from MRw were identified as *Aspergillus* species: *A. penicillioides, A. ochraceus, Eurotium* species: *E. amstelodami, E. repens,* and *Penicillium* species: *P. asymmetrica sec fasiculata, P. corylophilum; A. tamarii and Hortaea werneckii* were isolated from MCw and *A. flavipes, A. terreus var terreus, A. versicolor, E. amstelodami, E. repens* from MCs. The isolates obtained from brine of SR were identified as *A. versicolor, A. wentii, P. asymmetrica sec fasiculata, P. chrysogenum, P. corylophilum, P. griseofulvum* and those from SRs were, *A. candidus, A. flavus, A. sydowii, A. versicolor, A. wentii, E. amstelodami and H. werneckii.* SC brine samples yielded isolates of *A. penicillioides, A. versicolor, P. asymmetrica sec fasiculata and H. werneckii;* no isolate was obtained from SCs.

Halotolerance

Salt tolerance curves of the isolates were grouped according to the genus and then by the species, placing in priority, those having an absolute requirement of added salt for growth (Fig. 1). The salt tolerance range and concentrations for optimal growth of the isolates, together with their halophilic nature, are summarized in Table 1. *A. penicillioides* MRw201, MRw202, MRw203, MRw204, MRw207 and MRw208, required a minimum of 2% added salt for growth on CzA and could grow in presence of up to 20% salt, with MRw201 and MRw207 growing at salt concentrations of 2-25%, or even 30% in 15 days;

SCw255 required a minimum of 5% added salt for growth and was able to grow at 20% salt, or at 25% salt in 15 days. All showed optimal growth at 10% salt concentration. The essential requirement of salt for growth of these isolates was further confirmed by inoculating the isolates on MEA, PDA and SA without salt and with 10% solar salt; no growth was seen on media without addition of solar salt, however, growth was obtained on all media with 10% salt, comparable to that obtained on CzA+10% salt (Fig. 2). The identification of MRw202, *A. penicillioides,* was confirmed by ITS sequence analysis, GenBank Accession No. HQ891824.

The rest of the cultures could grow in the absence of added salt, most of these growing at 0-20% added salt within 7d, with optimal growth at salt concentrations of 2 /5 /10% as shown below viz. *A. sydowii* SRs240, *A. terreus var terreus* MCs223, *A. versicolor* MCs224, SRw235 and SRs239, *A. wentii* SRs247, *P. asymmetrica sec fasiculata,* MRw206, SRw226 and SCw256 and *P. corylophilum* SRw233, optimum growth at 2% salt; *A. flavipes* MCs219, optimum at 2-5% salt; *A. ochraceus* MRw213, *A. sydowii* SRs 243, *A. versicolor* MCs220, MCs222, SRw229, SRw230, SRw231, SRs237, SRs242, SCw252, SCw253 and SCw254, *A. wentii* SRw234, *E. amstelodami* MRw211, *P. asymmetrica sec fasiculata,* SRw232 and SCw257, *P. chrysogenum* SRw225 and SRw227, *P. corylophilum* MRw205 and *P. griseofulvum* SRw228, all growing optimally at 5% salt, *A. flavus* SRs244, *A. versicolor* SRw236, *E. amstelodami* MRw209, *E. repens* MRw210, MRw212 and MCs218 with optimal growth at 10% salt. *A. tamarii* MCw216 could grow at 0-15% salt, and optimally at 10% salt.

Some isolates that could grow in the absence of added salt, were able to grow in presence of 25% salt in 15d, namely, *A. candidus* SRs248, *A. versicolor* SRs241, SRs246, *H. werneckii* MCw217 and *E. amstelodami* SRs245, all with optimal growth at 5% salt, *H. werneckii* SRs238 and *A. versicolor* SRs246, at 5-10% and at 10% salt respectively. Other isolates grew at the range of 0-25% salt in 7d, as in *H. werneckii* MCw215, SCw250, SCw251 with optimal growth at 5% and MCw214 at 10% salt, and *E. amstelodami* MCs221 at 10% salt. *A. wentii* SRs249 grew even in the presence of 30% salt in 15 days, and optimally at 2% salt.

Discussion

The present study revealed the presence of diverse genera of halophilic filamentous fungi and black yeasts in mangroves and solar salterns in Goa, India. Although previous reports have indicated the presence of halophilic fungal species in salterns, these were mainly of the dematiaceous fungi and black yeasts and other halotolerant species[2,26-29] and there is no record of these from mangroves; the only report is of slight halophilic *Penicillium* from mangroves and salterns of Goa, India, with maximum tolerance around 17.5% solar salt and optimal growth at 2% salt[9]. A range of fungi are known to occur in the mangrove ecosystem, although these differ as to their location and some fungi occur more frequently than others, with many factors such as salinity, temperature, availability and diversity of substrata effecting species occurance[33]. This was also seen in the present work, particularly with respect to the isolation of the halophilic *A. penicillioides* mainly from mangrove waters, and only a single isolate from saltern brine.

Cantrell *et al.*[29] report that most of the fungi that have been isolated from mangroves, saline soils, marine sediments, sea water, salt marshes and sand dunes belong to the imperfect stage of the Ascomycota. Fungi from mangroves in different parts of India, isolated on laboratory media, were mainly of the Fungi imperfectii[6,8,10,12], which was also seen in the present study; the reported species of *A. candidus, A. flavipes, A. flavus, A. ochraceus, A. sydowii, A. tamarii, A. terreus, A. versicolor, A. wentii, E. amstelodami* and *P. chrysogenum* were common to the isolates identified in this work. However, numbers of halophilic fungi isolated in the present work were seen to be lower as compared to that isolated on media without high salt concentrations. Though the occurrence of fungi in hypersaline environments was formerly thought to be due to a random event caused by airborne inoculum and the fungi had no specific ecological function, it was later seen, through studies on growth on laboratory media, that some species were capable of growing and reproducing in hypersaline environment[26,29].

There was a predominance of *Aspergillus* species, with *Penicillium* also in fairly high proportion while the telemorphic form *Eurotium*, as well as the black yeast *Hortaea werneckii*, were obtained in fewer numbers, amongst the present isolates. The predominance of *Aspergillus* and *Penicillium*, corroborates the report of Buchalo[34] that at low water

potential the active mycota is dominated by species of these genera and is thus numerically the most common taxa. Cantrell et al.[29] and Gunde-Cimerman *et al.*[2] reported the presence of *Cladosporium cladosporioides* in salterns. In keeping with this finding, *Cladosporium* and *Alternaria* have also been isolated from these econiches, on isolation media with comparatively lower salt concentrations, and were found to be halotolerant[35,36], however, the medium incorporating high solar salt concentration used in the present study did not favour the growth of these dematiaceous fungi. From amongst the diverse isolates obtained from the hypersaline waters of salterns of Slovenia[2,26,27] and of Cabo Rojo[28,29], the species *A. candidus*, *A. flavus*, *A. penicillioides*, *Eurotium*, *P. chrysogenum* and *H. werneckii* were amongst those also found in the present study.

Melanized fungi are stated to have a selective advantage over the other mycoflora in saline environments[2], representing 85-100% of the total isolated mycobiota from highly saline waters, and partially replaced by non-melanized fungi at lowered salinities, being detected only occasionally with NaCl concentrations below 5%[27]. However, our findings showed that the non-melanised filamentous fungi were in greater numbers than the melanized fungi from amongst the isolates from salterns. This was also seen amongst the fungi isolated from the hypersaline waters of the Dead Sea[37].

The mycobiota obtained from mangroves and salterns in this study were classified as obligate and as facultative halophiles or as halotolerant[34,37,38]. The isolates obtained were slight to moderate halophiles, requiring 2% or 5-10% salt respectively for optimal growth, as defined by Kushner[38], mainly of euryhaline nature, able to grow at a wide range of salt. Most were facultative halophiles, growing even in the absence of added salt; isolates which had an absolute requirement for salt added to the medium were termed as obligate halophiles, their obligate halophilic nature being confirmed by their essential requirement of salt for growth on different media.

The salt tolerance profiles of *H. werneckii* at 0-25%, with optimal growth at 5-10% salt, corroborated earlier findings wherein isolates from salterns have been shown to grow in presence of a wide range of salt concentrations, from absence of salt, up to near saturation of 32%, with a broad growth optimum of 6-14%[2], or from 0-25% salt and optimally at 10%[28]. Their presence in the nonhypersaline waters of mangroves is also shown in this study. Further, their isolation from salterns in India, Asia, adds to their presence on yet another Continent and confirms the observation of Butinar et al.[27] that they are present globally in hypersaline waters of man-made salterns.

Hortaea werneckii has been termed as halophilic[2,27], or as halotolerant[39]. Based on their ability to grow at near 0% salt, yet with the requirement of salt for optimal growth, they were categorised in this study, as facultative halophiles.

Significantly, *A. penicillioides* was the only obligate halophilic species obtained in the present work. It is particularly noteworthy that all but one of the obligate halophiles were isolated from mangroves and only one from the salterns, contrary to the expectation that the saltern brine, with a low a_w, lower than that of the mangroves, would yield halophilic species. The other isolates obtained from the salterns could also include terrestrial/aerial fungi that adapted to the hypersaline conditions and were therefore not true halophiles. It appears therefore that a hypersaline condition is not necessarily an indication of the existence of a high number of obligate halophilic fungi, more than that from low saline environments.

The obligate halophiles isolated were observed to grow slower, yielding smaller colonies than the facultative halophiles. Ravishankar[19] noted that marine fungi grow very slowly on artificial media as compared to terrestrial fungi. As indicated by Redkar et al.[40] the reduced growth rate of salt adapted cultures may be related to the increase in energy demands under stress, wherein cells may utilize energy for compartmentalization or exclusion of Na^+ ion, direct synthesis of compatible osmolytes and synthesis of proteins conferring salt tolerance.

The present work is a first report on the isolation of halophilic filamentous fungi and black yeasts, which were classified as moderate halophiles, with high salt tolerance levels, from amongst isolates from mangroves and solar salterns in India and from mangroves across the globe. The isolation of the obligate halophile *A. penicillioides* from these econiches is also recorded herein, for the first time.

Acknowledgements

Authors are grateful to Mofeeda Gazem for help in the identification of the fungi.

References

1 Oren A, Diversity of halophilic microorganisms: environments, phylogeny, physiology, and applications, *J. Ind Microbiol Biotechnol*, 28 (2002) 56-63.

2 Gunde-Cimerman N, Zalar P, De Hoog G S & Plemenitas A, Hypersaline waters i n salterns: natural ecological niches for halophilic black yeasts, *FEMS Microbiol. Ecol.*, 32 (2000) 235-240.

3 Pedros-Alio C, Trophic ecology of salterns, in: *Halophilic Microorganisms*, edited by A.Ventosa, (Springer-Verlag, Heidelberg) 2004, pp. 33-48.

4 Griffith D H, Fungal Physiology, (John Wiley & Sons, New York) 1994.

5 Raghukumar S, Marine lignicolous fungi from Maharashtra India, *Kavaka*, 1 (1973) 73-85.

6 D'Souza J, Araujo A, Karande A & Freitas Y M, Studies on fungi from coastal waters of Bombay and Goa, *Ind J Mar Sci*, 8 (1979) 98-102.

7 Borse B D, Frequency of occurrence of marine fungi from Maharashtra Coast, India, *Ind J Mar Sci,* 17 (1988) 165-167.

8 Maria G L & Sridhar K R, Richness and diversity of filamentous fungi on woody litter of mangroves along the West Coast of India, *Curr Sci,* 83 (2002) 25.

9 Marbaniang T & Nazareth S, Isolation of halotolerant *Penicillium* species from mangroves and salterns and their resistance to heavy metals, *Curr Sci*, 92 (2006) 895-896.

10 Rai J N & Chowdhery H J, Microfungi from mangrove swamps of West Bengal, India, *Geophytol.*, 8 (1978) 103-110.

11 Kathiresan K, A review of studies on Pichavaram mangrove, Southeast India, *Hydrobiologia*, 430 (2000) 185-205.

12 Vittal P R & Sarma V, Diversity and ecology of fungi on mangroves of Bay of Bengal region - An overview, *Ind J Mar Sci*, 35 (2006) 308-317.

13 Nambiar G R & Ravieendran K, Diversity of mangrove fungi of North Malabar, Kerala (India), *Indian Forester,* 134 (2008) 1658-1662.

14 Rani C & Paneerselvam A, Fungal diversity in the sediments of Point Calimere East Coast of India, *J Pure Appl Microbiol.*, 4 (2010) 199-206.

15 Sahoo K & Dhal N K, Potential microbial diversity in mangrove ecosystem: A review, *Ind J Mar Sci,* 38 (2009) 249-256.

16 Hyde K D, A comparison of the intertidal mycota of five mangrove tree species, *Asi. Mar. Biol.,* 7 (1990) 93-107.

17 Zhong-shan C, Jia-Hui P, Wen-cheng T, Qi-jin C & Yong-cheng L, Biodiversity and biotechnological potential of mangrove-associated fungi, *J Forestry Res,* 20 (2009) 63-72.

18 Alias S A, Zainuddin N & Jones E B G, Biodiversity of Marine fungi in Malaysian Mangroves, *Bot. Mar.*, 53 (2010) 545-554.

19 Ravishankar J P, Suryanarayanan T S & Muruganandam, Strategies for osmoregulation in the marine fungus *Cirrenalia pygmea* Khol. (Hyhomycetes), *Ind J Mar Sci*, 34 (2006) 351-358.

20 Khandavilli S, Sequeira F & Furtado I, Metal tolerance of extremely halophilic bacteria isolated from estuaries of Goa, India, *Ecol Env & Cons,* 5 (1999) 149-152.

21 Raghavan T M & Furtado I, Occurrence of extremely halophilic Archaea in sediments from the continental shelf of west coast of India, *Curr Sci*, 86 (2004) 1065-1067.

22 Dave S & Desai H, Microbial diversity of marine salterns near Bhavnagar, Gujarat, India, *Curr Sci*, 90 (2006) 497-500.

23 Braganca J M & Furtado I, Isolation and characterization of Haloarchaea from low- salinity coastal sediments and waters of Goa, *Curr Sci,* 99 (2009) 1182-1184.

24 Khan A S, Sheik Ali M & Ahmed I J, Halophilic (aerobic) bacterial growth rate of mangrove ecosystem, *J Environ Biol,* 30 (2009) 705-707.

25 Manikandan M, Kannan V R & Pasic L, Diversity of microorganisms in solar salterns of Tamil Nadu, India, *World J Microbiol Biotechnol*, 25 (2009) 1007-1017.

26 Gunde-Cimerman N, Zalar P, Petrovic U, Turk M, Kogej T, de Hoog G S & Plemenitas A, Fungi in the Salterns, in: *Halophilic microorganisms*, edited by A. Ventosa, (Springer-Verlag, Heidelberg) 2004, pp. 103-113.

27 Butinar L, Sonjak S, Zalar P, Plemenitas A & Gunde-Cimerman N, Melanized halophilic fungi are eukaryotic members of microbial communities in hypersaline waters of solar salterns, *Bot Mar*, 48 (2005) 73-79.

28 Diaz-Munoz G & Montalvo-Rodriguez R, Halophilic black yeasts *Hortaea wernekii* in the Cabo Rojo Solar Salterns: its first record for this extreme environment in Puerto Rico, *Caribb J Sci,* 41 (2005) 360-365.

29 Cantrell S A, Casillas-Martínez L & Molina M, Characterization of fungi from hypersaline environments of solar salterns using morphological and molecular techniques, *Mycol Res,* 110 (2006) 962-970.

30 Hujslova M, Kubatova A, Chudickova M & Kolarik M, Diversity of fungal communities in saline and acidic soils in the Soos National Natural Reserve, Czech Republic, *Mycol. Progress,* 9 (2010) 1-15.

31 Raper K B & Fennell D I, The genus *Aspergillus*, (Williams & Wilkins, Baltimore U.S.A) 1965.

32 Domsch K H, Gams W & Anderson T H, Compendium Of Soil Fungi,vol 1, (IHW-Verlag, Eching) 1980.

33 Jones E B G & Alias S A, Biodiversity of mangrove fungi, in: *Biodiversity of tropical microfungi*, edited by K.D. Hyde, (Hong Kong University Press, Hong Kong) 1997, pp. 71-92.

34 Buchalo A S, Fungi in saline water bodies, in: *Fungal life in the Dead Sea*, edited by E. Nevo, A. Oren, S.P. Wasser, (Ruggell, GantnerVerlag) 2003, pp. 141-161.

35 Nazareth S & Marbaniang T, unpublished data.

36 Gonsalves V & Nazareth S, unpublished data.

37 Nazareth S, Gonsalves V & Nayak S, A first record of obligate halophilic aspergilli from the Dead Sea, *Ind J Microbiol,* DOI: 10.1007/s12088-011-0225-z.

38 Kushner D J, *Microbial Life in Extreme Environments*, (University of Ottawa Academic press London New York San Francisco) 1978.

39 Plemenitas A, Vaupotic T, Lenassi M, Kojej T & Gunde-Cimerman N, Adaptation of extremely halotolerant black yeast *Hortaea werneckii* to increased osmolarity: A molecular perspective at a glance, *Stud Mycol*, 61 (2008) 67-75.

40 Redkar R J, Lemke P A & Singh N K, Altered gene expression in *Aspergillus nidulans* in response to salt stress, *Mycologia*, 88 (1996) 256-263.

Table 1—Obligate and Facultative Halophiles

Isolate		% Salt level supporting growth*		Halophile
Species	Number	Range	Optimal	
A. penicilloides	MRw201, MRw207	2 -25 (30)	10	Obligate
	MRw202, MRw203, MRw204, MRw208	2-20	10	Obligate
	SCw255	5-20 (25)	10	Obligate
A. candidus	SRs248	0-20 (25)	5	Facultative
A. flavipes	MCs219	0-20	2-5	Facultative
A. flavus	SRs244	0-20	10	Facultative
A. ochraceus	MRw213	0-20	5	Facultative
A. sydowii	SRs240	0-20	2	Facultative
	SRs243	0-20	5	Facultative
A. tamarii	MCw216	0-15	10	Facultative
A. terreus var terreus	MCs223	0-20	2	Facultative
A. versicolor	MCs220, MCs222,	0-20	10	Facultative
	MCs 224	0-20	2	Facultative
	SRw229, SRw230, SRw231, SCw254	0-20	5	Facultative
	SRw235, SRs239	0-20	2	Facultative
	SRw236	0-20	10	Facultative
	SRs237, SRs242	0-20	5	Facultative
	SRs241	0-20 (25)	5	Facultative
	SRs246	0-20 (25)	10	Facultative
	SCw252, SCw253	0-20	5	Facultative
A. wentii	SRw234	0-20	5	Facultative
	SRs247	0-20	2	Facultative
	SRs249	0-25 (30)	2	Facultative
E. amstelodami	MRw211	0-20	5	Facultative
	MCs221	0-25	10	Facultative
	SRs245	0-20 (25)	5	Facultative
E. repens	MRw209, MRw210, MRw212, MCs218	0-20	10	Facultative
P. asymmetrica sec fasiculata	MRw206, SRw226, SCw256	0-20	2	Facultative
	SRw232, SCw257	0-20	5	Facultative
P. chrysogenum	SRw225, SRw227	0-20	5	Facultative
P. corylophilum	MRw205	0-20	5	Facultative
	SRw233	0-20	2	Facultative
P. griseofulvum	SRw228	0-20	5	Facultative
H. werneckii	MCw214	0-25	10	Facultative
	MCw215	0-25	5	Facultative
	MCw217	0-20 (25)	5	Facultative
	SRs238	0-20 (25)	5-10	Facultative
	SCw250, SCw251	0-25	5	Facultative

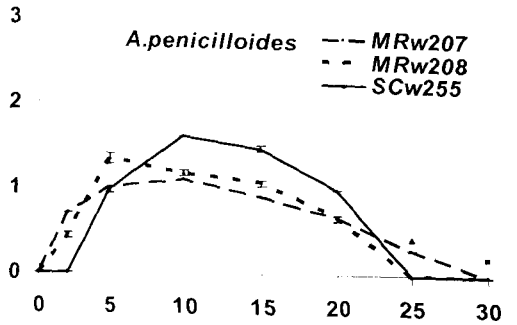
*Salt tolerance of isolates recorded after 7d incubation; data in brackets indicate delayed growth at the respective salt concentration, recorded after 15d incubation

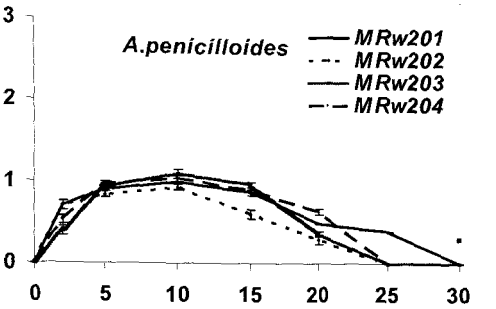
Legends to figures:

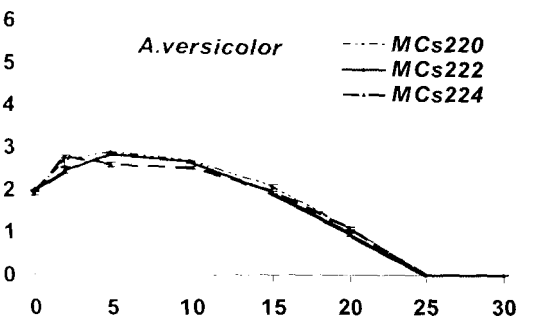
Fig. 1—Salt tolerance of isolates recorded after 7d incubation; unconnected points indicate delayed growth at the respective salt concentration, recorded after 15d incubation.

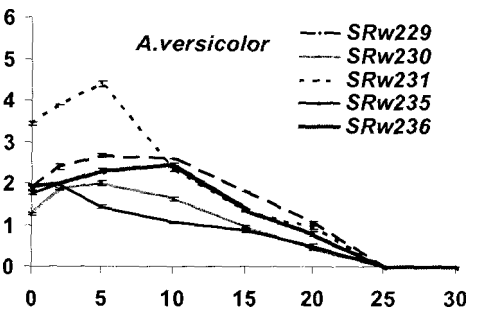
Fig. 1—(cont.). Salt tolerance of isolates recorded after 7d incubation; unconnected points indicate delayed growth at the respective salt concentration, recorded after 15d incubation.

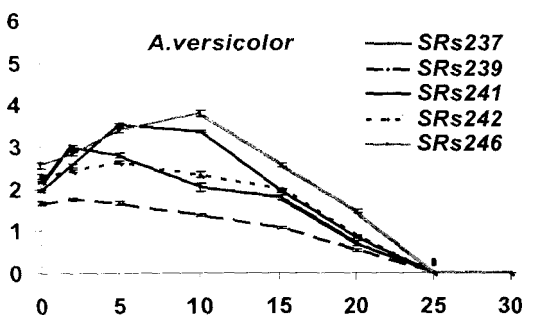
Fig. 2—Growth of *Aspergillus penicillioides* isolates in terms of colony diameter recorded after 7d incubation on CzA, S-CzA, MEA, S-MEA, PDA, S-PDA, SA and S-SA, where S represents 10% solar salt added to the medium.

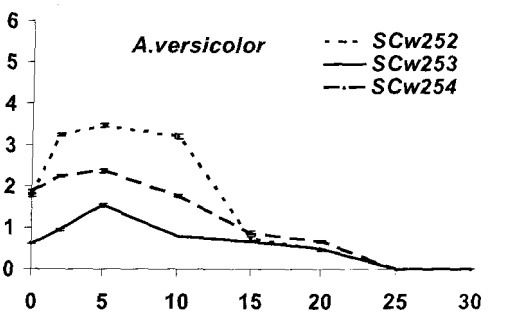


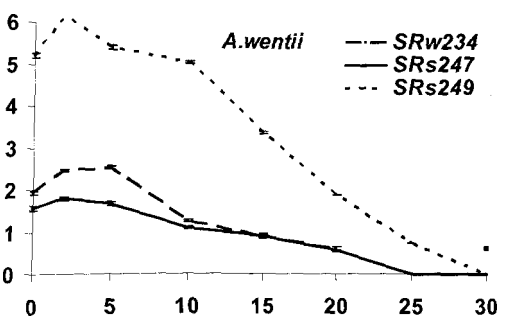






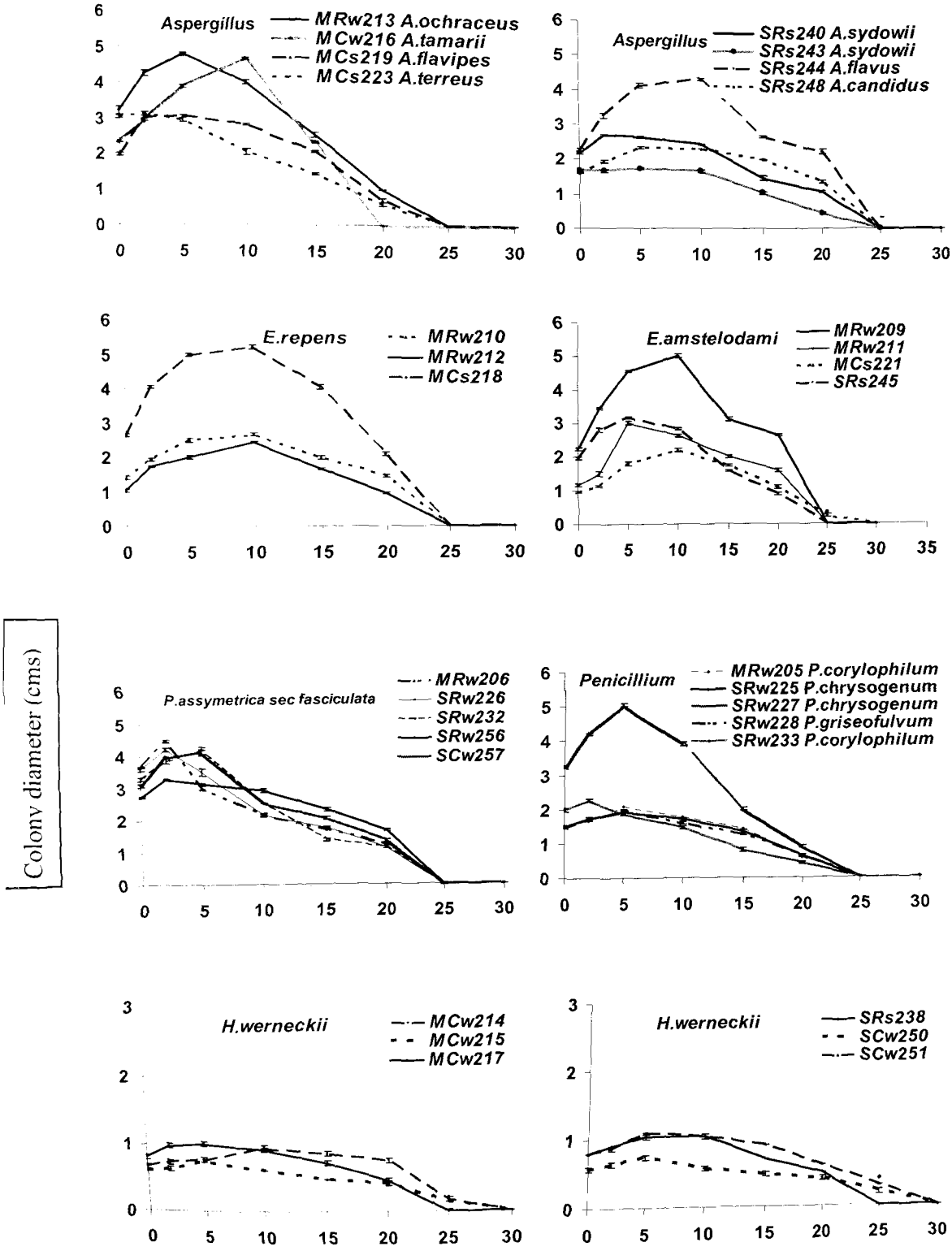






Colony diameter (cms)

Crude salt added (%)


Aspergillus
MRw213 A.ochraceus
MCw216 A.tamarii
MCs219 A.flavipes
MCs223 A.terreus
Aspergillus
SRs240 A.sydowii
SRs243 A.sydowii
SRs244 A.flavus
SRs248 A.candidus
E.repens
MRw210
MRw212
MCs218
E.amstelodami
MRw209
MRw211
MCs221
SRs245
P.assymetrica sec fasciculata
MRw206
SRw226
SRw232
SRw256
SCw257
Penicillium
MRw205 P.corylophilum
SRw225 P.chrysogenum
SRw227 P.chrysogenum
SRw228 P.griseofulvum
SRw233 P.corylophilum
H.werneckii
MCw214
MCw215
MCw217
H.werneckii
SRs238
SCw250
SCw251
Colony diameter (cms)
Crude salt added (%)