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The world of halophilic fungi

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ABSTRACT

Halophilic fungi exist in hypersaline and polyhaline marine environments. Studies have shown their presence in the Dead Sea, Salt Lakes, solar salterns in different continents, as well as in deep-sea, estuary and mangroves. These have been found to belong to *Ascomycota* and *Basidiomycota*. They develop a mechanism of osmo-adaptation to the lower water activity of their environment through internal accumulation of compatible solutes. They hold much biotechnological potential.

Keywords: Extremophiles, halophiles, fungi, osmo-adaptation, compatible solutes, *Aspergillus penicillioides*

INTRODUCTION

The study of microbial life in extreme environments has become one of the leading areas in current scientific research. The mesophiles had dominated centre stage ever since the study of microorganisms began; that organisms survive and actually thrive in environments with extreme physicochemical parameters that are detrimental to the majority of other species, opened the field of microbiology to a whole new world of marvels and exciting discoveries.

The study of organisms under conditions once regarded as hostile to microbial life, has become a focus of increasing scientific attention. The term 'extreme environments' has been used to designate extreme conditions such as that of high or low temperature, high pressure, acidity or alkalinity, very low nutrient content, and low activity of water (a_w) which denotes a low moisture content available to the organism to sustain its biochemical or physiological functions. The low a_w may be brought about either by arid conditions or by low levels of 'available water' caused by the presence of high/ hyperosmotic concentrations of solutes such as sugar or salts. Microorganisms that can grow and thrive in environments which were formerly considered too hostile to support life were termed as extremophiles.

Thus the organisms discovered were able to withstand and live at temperatures of 250 °C of hot springs or - 30 °C of the Antarctic and Arctic, at pressures of 100-300 bar of the deep sea, at acidic conditions of pH 3-4 of sulphurous springs, the Norris Geyser Basin in Yellowstone National Park, Wyoming, or at an alkalinity of pH 9-11 of playa lakes, in concentrations of 60% sugar in food products or of 275‰ salts of the Salt Lake, Utah or 340‰ salt of the Dead Sea. These organisms have been generally termed as extremophiles and more specifically as thermophilic, psychrophilic, barophilic, acidophilic, alkaliphilic, osmophilic or halophilic. Growing either preferentially in such extreme environments, or merely able to exist therein but preferring less extreme conditions, they have been further classified as extremophilic or extremotolerant towards the given environment.

The study of organisms living in such extreme conditions assumed great significance, to understand the diversity of microbiota able to survive in these environments as well as the mechanisms of survival. Such extremophilic microorganisms are structurally adapted at the molecular level to withstand the harsh conditions (Gomes and Steiner, 2004). While initial research on microbiology of extreme environments centred on the prokaryotes, it was believed that eukaryotes were unable to inhabit such ecosystems. The first extremophilic microorganisms to be discovered were the archaea; subsequently eubacteria too were found amongst this exclusive group. It was later conceded that fungi are tolerant to unfavourable extreme conditions, able to survive such untoward situations, and would at the most, be extremotolerant, but are not necessarily extremophilic in nature.

More recently, fungi have also begun to be focussed upon for their capacity to withstand extreme conditions, and in some instances to even have an obligate need for conditions out of the ordinary. Extremophiles are promising models to further our understanding of the functional evolution of stress adaptation and the development of stress-tolerant mechanisms. Their biology widens our views on the diversity of terrestrial life and it has come as a surprise that not only prokaryotes but also eukaryotes have a great capacity to adapt to extreme conditions. Particularly successful examples can be found in the fungal kingdom. Thus these specialized fungi have been discovered in extreme cold, dry, salty, acidic and deep-sea habitats (Gostinčar *et al*., 2010).

HALOPHILES

Halophiles are salt-loving organisms that are found inhabiting hypersaline environments (DasSarma and Arora, 2001). These organisms are found distributed all over the world, in natural hypersaline brines in arid, coastal, and even deep sea locations, as well as in artificial salterns used to mine salts from the sea. Their novel characteristics and capacity for large scale culturing make halophiles potentially valuable for biotechnological applications (DasSarma and Arora, 2001; Margesin and Schinner, 2001).

Hypersaline environments necessarily have a very low a_w brought about by the chemical bonding of water to NaCl. Organisms able to grow under conditions of low water activity below 0.85, imposed by high levels of soluble solids such as salts or sugars, or due to dry conditions, have often been termed as xerotolerant or xerophilic (Andrews and Pitt, 1987; Grant, 2004; Pitt and Hocking, 2009; Tamura *et al.*, 1999). While xerophiles grow in relatively dry conditions, organisms that grow in high osmotic environments of sugar solutions are termed osmophiles (Tucker and Featherstone, 2011) and those that require salt mainly in the form of NaCl, or any other salt along with a small amount of NaCl, are known as halophiles (Kushner, 1978). The microorganisms growing in saline environments are therefore adapted to low a_w levels as well as high levels of ions, and are described as halotolerant or halophilic, rather than merely xerotolerant or xerophilic (Grant, 2004).

Halophiles have been classified according to levels of salt required for optimal growth (Larsen, 1962; Kushner, 1978; DasSarma and Arora, 2001), as shown in **Table 1.**

Organisms inhabiting salt environments may also be categorised (Kushner, 1978) as:

(i) true or obligate halophiles, having an essential requirement of NaCl and a low a_w for growth,

(ii) facultative halophiles, able to grow in low concentrations of salt of <0.1M NaCl, or in environment of high a_w, but growing optimally at higher concentrations of salt,

(iii) halotolerant microorganisms, that do not show an absolute requirement for salt for growth, but grow well up to often very high salt concentrations, and are considered to be extremely halotolerant if the growth range extends above 2.5 M salt.

Furthermore, those that have a capacity to grow at a wide range of salt concentrations have been termed as euryhaline, and those growing over a narrow range of salt concentrations are referred to as stenohaline.

The presence of bacteria in hypersaline environments was first recorded by Pierce in 1914 (Gunde-Cimerman *et al.*, 2000), and for a while it was believed that hypersaline environments were dominated mainly by prokaryotes. The first group of halophiles to be studied were the archaea, with eubacteria being subsequently added to the list (Oren, 1999; 2002a; Gunde-Cimmermn *et al.*, 2009). Among halophilic microorganisms reported were archaeal *Halobacterium* species and a variety of heterotrophic and methanogenic archaea, photosynthetic, lithotrophic, and heterotrophic bacteria, including cyanobacteria such as *Aphanotheca halophytica*. The extremely halophilic archaea, in particular, 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 phototrophic growth, sensory rhodopsins that mediate the phototactic response, and gas vesicles that promote cell flotation (Oren, 2002b).

The single-celled green alga *Dunaliella salina* was the first eukaryotic halophilic organism recorded (Ramos-Cormenzana, 1991). Fungi that could survive in low a_w were only known as contaminants in food that had been preserved using high concentrations of salt or sugar. Crystalline sodium chloride is generally considered to be hostile to most forms of life, and has been used for centuries as a food preservative. However, in reality, food preserved with high concentrations of salt form an environment for halophilic and halotolerant microorganisms.

It had been assumed that fungi do not populate hypersaline environments and only a few reports described their isolation from moderately saline environments such as salt marshes, saline soil and sea water (Gunde-Cimerman *et al.*, 2000). These isolates were seen as ubiquitous fungi not specifically adapted to saline environments, or as halotolerant. Findings proved that fungi do indeed exist and are in fact an integral part of halophilic communities in hypersaline waters, as found in the Dead Sea (Buchalo *et al.*, 1998), and as active inhabitants of solar salterns, which were considered as a new group of eukaryotic halophiles (Gunde-Cimerman *et al.*, 2000). The photosynthetic and heterotrophic eukaryotes were examples of well-adapted and widely distributed extremely halophilic microorganisms.

Since then, many new species and indeed species only previously known as food contaminants, have been discovered in hypersaline environments around the world (Gunde-Cimerman *et al.*, 2009). These are now recognized as an integral part of indigenous microbial communities. These microorganisms can adapt to extreme concentrations of NaCl, and often, also to high concentrations of other ions, to high ultraviolet radiation and, in some cases, to extremes of pH (Gunde-Cimerman *et al.*, 2000). Though the occurrence of fungi in hypersaline environments was formerly thought to

Table 1: Classification of halophiles

HalophilicNature	**NaCl required for optimal growth as defined by**		
	Larsen (1962)	**Kushner (1978)**	**Das Sarma and Arora(2001)**
Nonhalophile	<2.0 %	0.2M / [1.17%]	<0.2 M
Slight Halophile	2 – 5 %	0.2M – 0.5M [1.17% – 2.925%]	0.2 – 0.85 M [1.17% – 5%]
Moderate Halophile	5 – 20 %	0.5M – 2.5M [2.925% – 14.625%]	0.85 – 3.4 M [5% – 20%]
Borderline Extreme Halophile	____	2.5M – 4.0M [14.625% – 23.4%]	___
Extreme Halophile	20 – 30 %	2.5M – 5.2 M [23.4% – 30.42%]	> 3.4 – 5.1 M [20% – 30%]

be due to a random event caused by airborne inoculum and that 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 (Gunde-Cimerman *et al.*, 2000; Cantrell *et al.*, 2006). Halophilic fungi, therefore, have been recognized as a novel category of extremophilic eukaryotes that play an important role in saline and hypersaline ecosystems (Gunde-Cimerman *et al.*, 2005b). Thus, halophiles have been found in each of the three domains: Archaea, Bacteria and Eukarya.

HALOPHILIC FUNGI

The halophilic fungi have been the best studied fungal extremophiles. Various econiches world-wide have been studied for the presence of halophilic fungi, in particular, the hypersaline environments, such as the Dead Sea, the Salt Lakes, solar salterns, salt marshes, and other marine environments such as mangroves and estuary.

1) Hypersaline environments

Hypersaline environments contain salt concentrations in excess of sea water and can be perceived as an extreme condition where the salinity is beyond the normal acceptable range (Das *et al.*, 2006; DasSarma and Arora, 2001). Reports of fungi from extremely hypersaline environments were very few before 2000. However, Javor (1989) expressed that the lack of such a record may reflect not the inability of fungi to colonize these extreme environments, but rather the low quantum of effort on research in this field.

Hypersaline conditions drastically decrease the biodiversity of fungal life by exerting a strong selective pressure, resulting in the development of halophilic and halotolerant microbes (Das *et al.,* 2006). These extreme habitats, in more recent years, have attracted attention for a greater understanding of the microorganisms therein and their relationship with the environment (Grishkan *et al.*, 2003). Isolation and identification of marine fungi have been frequently hindered due to the low competitive ability of these fungi and their slow growth, difficulties in obtaining sporulating structures and in morphological identification (Gunde-Cimerman and Plemenitaš, 2006).

Hypersaline environments may be thallasohaline or athallasohaline. Thallasohaline econiches, where the relative proportion of constituent salts is the same as that of sea water and is obtained by the evaporation of sea water, are those of solar salterns and salt marshes. Athallasohaline environments denote those wherein the composition of constituent salts is different from that of sea water, as obtained in the Dead Sea and the Great Salt Lake.

1.1) The Dead Sea

The Dead Sea has an extremely hypersaline environment, with a unique ionic character and extreme high salinity of about 340gL^{-1} total dissolved salts comprising 41‰ magnesium, 39‰ sodium, 17‰ calcium, 7‰ potassium, and 212‰ chloride and 5‰ bromide as the major ions in the water, which is slightly acidic (Buchalo *et al.*, 1998) and has a water activity of 0.699 (Krumgalz & Millero, 1982), and minerals like oxides of silicon, calcium, aluminium, magnesium, iron, sodium, potassium, titanium, sulphur and phosphorous, chloride and bromide are present in the sediment (Ma'or *et al.*, 2006). This distinctive ionic composition, with its high concentration of the divalent cations magnesium and calcium, is highly inhibitory even to those microorganisms best adapted to life at high salt concentrations, the halophilic and halotolerant microorganisms. The precipitation of halite from the water column to the lake bottom, has caused an additional increase in this already extremely high ratio of divalent to monovalent cations of water (Molitoris *et al.*, 2000; Buchalo, 2003; Kis-Papo *et al.,* 2003a; Mbata, 2008), which has been suggested as a plausible reason for the paucity in the diversity of the Dead Sea biota.

The existence of an indigenous microflora in the Dead Sea was first reported by Wilkansky (1936). Until recently prokaryotic microorganisms were considered to be the only decomposers in the Dead Sea (Oren, 1988). However, it was found that fungi, long neglected as a component of the food web in the Dead Sea and in other hypersaline environments as well, may also play a role (Buchalo *et al.,* 1998). Most species identified were common soil fungi, not well adapted to life at high salt concentrations (Molitoris *et al.* 2000).

The first filamentous halophilic fungi from the Dead Sea to be recorded were the halophilic ascomycetous fungus *Gymnascella marismortui* from surface water samples at Ein-Zukim and the halotolerant *Penicillium westlingii* and *Ulocladium chlamydosporum* from deep waters at Ein Gedi (Buchalo *et al.,* 1998). A number of filamentous fungi have then been isolated from the Dead Sea, from water samples at Ein Bokek and Ein Gedi (Buchalo, 2003; Grishkan *et al.,* 2003; Kis-Papo *et al.,* 2003b; Mbata, 2008, Wasser *et al.,* 2003), as well as water and sediment samples at Kalya Beach (Nazareth *et al.,* 2012b). These included the species of *Aspergillus fumigatus*, *A. sydowii*, *A. terreus*, *A. versicolor*, *A. niger*, *A. penicillioides, A. restrictus*, *Aureobasidium pullulans*, *Chaetomium globosum*, *Eurotium herbariorum*, *Gymnascella marismortui*, *Hortaea werneckii, Penicillium aurantiogriseum, P. corylophilum, P. steckii,* the dematiaceous fungi *Alternaria alternata, Cladosporium cladosporioides* and *C. sphaerospermum*; *Aspergillus unguis* was reported for the first time by Nazareth *et al.* (2012b). Species of *Candida: C. glabrata, C. tropicalis, C. parapsilosis* and *C. krusei*, were also isolated; among these, only *C. parapsilosis* was known previously as a food-borne halotolerant yeast, while the others were not known for their halotolerance (Butinar *et al.,* 2005a).

Nazareth *et al.* (2012b) have shown that amongst the halophilic genera obtained, the genera *Penicillium* and

Cladosporium were found exclusively in the water, while the sediment yielded only *Aspergillus* species, with *A. penicillioides* predominant and only a few isolates of *A. versicolor*. 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 halophilic as the genera of *Penicillium* and *Aspergillus*. The high density of *A. penicillioides* in the sediment sample has been attributed to the low a_w caused by the higher matrix potential and salinity higher than that of the water, which could be the result of precipitation of halites from the water column due to evaporation (Oren, 2003). The lower diversity seen in the sediment is possibly because only those able to withstand these conditions can thrive (Kunte *et al.,* 2002); likewise, the hypersaline environment would be expected to support to a greater extent, the survival and growth of halophilic fungi and/or the presence of greater numbers of these groups. The high population density seen in the sediment could be due to the capacity of these select microorganisms to balance the osmotic pressure of the environment and resist denaturation by salt (DasSarma and Arora, 2001).

Kis-Papo *et al.* (2003a) have shown that spores of the isolates obtained from the terrestrial shore of the Dead Sea generally proved less tolerant to suspension in water of the Dead Sea than spores of the species isolated from the water column, thus indicating that fungi in the Dead Sea have adapted to a halotolerant and/or halophilic existence.

The spatial and temporal diversity in the fungal community of the Dead Sea was also examined at eight near-shore localities and at different stations offshore over a 1-year period, as well as, depth profiles were sampled at a deep station of 304 m in the centre of the sea (Kis-Papo *et al*., 2003b). The 476 isolates obtained belonged to 38 species from 19 genera: one from *Oomycota*, two from *Zygomycota*, thirteen from *Ascomycota*, and three mitosporic fungi. Approximately 43% of the isolates belonged to the genera *Aspergillus* and *Eurotium*. Most of the species appeared only in winter and fungal diversity increased near the outlets of less saline springs near the shore. The species *Aspergillus terreus, A. sydowii, A. versicolor, Eurotium herbariorum, Penicillium westlingii, Cladosporium cladosporioides* and *C. sphaerospermum* were isolated consistently and probably form a stable core of the community. The observations suggest that a remarkably diverse fungal diversity may be found in the hypersaline Dead Sea waters.

1.2) Salt Lakes

The Great Salt Lake, Utah, is second in salinity only to the warmer Dead Sea and was once considered equally, as being devoid of life. The North arm of Great Salt Lake is saturated with dissolved salts that are close in relative proportion to sea water, with a salinity of 290-360‰, and slightly alkaline. Yet it is now known that hypersaline bodies of water that exceed the modest 3.5 % salt of earth's oceans are populated with rich communities of 'halophiles' or salt-lovers. These microbes are in all three Domains of life, Archaea, Bacteria, and Eukarya (Baxter *et al*., 2005); however, eukaryotes are in small numbers (DasSarma and Arora, 2001). The isolation of a halophilic filamentous fungus, *Cladosporium glycolicum*, a saprophytic hyphomycetes, of the anamorphic *Mycosphaerellaceae*, on a submerged piece of pine wood in the Great Salt Lake, Utah (Cronin and Post, 1977) was the first report of halophilic fungi from this hypersaline lake; non-filamentous 'fungi' from the genus *Thraustochytrium* were also subsequently found (Amon 1978; Brown 1990). Halophilic fungi have likewise been isolated from the Enriquillo Salt Lake, Dominican Republic, as recorded by Gunde-Cimerman *et al.* (2005a). The Mono Lake, California, with a salinity of 69-78‰, is alkaline and is lacking in magnesium and calcium divalent cations which have a low solubility at alkaline pH (DasSarma and Arora, 2001). Although ubiquitous fungi were isolated from Mono Lake, the isolation procedures chosen were not suitable to obtain the obligate halophilic fungi which could be present (Steiman *et al*., 2004).

1.3) Solar Salterns

Hypersaline waters of solar salterns, termed as thalassohaline (Oren 2002a), have salt concentrations greater than 35‰ of sea water, estimated at about 300‰ just before the time of halite precipitation, the pH being mildly alkaline (Nayak *et al*., 2012). Salinity of these salterns steadily increases to saturation point; variations in pH, temperature, light intensity, oxygen and nutrient concentrations, permit the growth of different microbial communities (Gunde-Cimerman *et al*., 2000; Pedros-Alio, 2004).

In 2000, the first reports appeared showing that fungi do exist in solar salterns (Gunde-Cimerman *et al.*, 2000). These were melanized fungi, isolated from the Adriatic seasonal solar salterns, at the border between Slovenia and Croatia, and represented by black, yeast-like hyphomycetes: *Hortaea werneckii*, *Phaeotheca triangularis*, *Trimmatostroma salinum*, *Aureobasidium pullulans*, together with phylogenetically closely related *Cladosporium* species. These and other non-melanized filamentous fungi: *Aspergillus, Eurotium, Penicillium* and *Wallemia* showed that there exists a considerable diversity of halophilic and halotolerant fungi, and it became increasingly clear that this indigenous halophilic mycobiota are adapted to long-term survival and vegetative growth. Melanized fungi have been isolated from hypersaline waters on different continents, indicating that they are present globally (Butinar *et al.*, 2005c; Zalar *et al.*, 2005b).

Subsequently, other salterns were studied, seasonal and those that operated throughout the year in other geographic locations around the world, including those along the Red Sea coast in Eilat, Israel, along the Mediterranean coast in Spain, France and Portugal, the Atlantic coast in Namibia, the coasts of the Dominican Republic and Puerto Rico (Butinar *et al.*, 2005c; Cantrell *et al.*, 2006; Diaz-Munoz and Montalvo-Rodriguez, 2005; Gunde-Cimerman *et al.,* 2005a) and at Goa on the Western coast of the Indian peninsula (Nayak *et al.,* 2012).

The halophilic or halotolerant mycobiota discovered in hypersaline waters around the world comprise the meristematic melanized yeast-like fungi and different related species of the genus *Cladosporium* (Gunde-Cimerman *et al.,* 2000; Butinar *et al.,* 2005b), non-melanized yeasts (Butinar *et al.,* 2005a), filamentous genera *Wallemia, Scopulariopsis* and *Alternaria* (Gunde-Cimerman *et al.,* 2005a; Zalar *et al.,* 2005a) and different species of the genera *Aspergillus* and *Penicillium*, with their teleomorphic stages *Eurotium, Emericella* and *Petromyces* (Butinar *et al.,* 2005c; Cantrell *et al.*, 2006; Nayak *et al.,* 2012). *Aspergillus* and *Penicillium* have been shown to be the principal genera obtained (Nayak *et al.,* 2012). Diversity studies have revealed that at low water potential the active mycota is dominated by the species of *Aspergillus* and *Penicillium* and is thus numerically the most common taxa (Buchalo, 2003).

Melanized fungi are stated to have a selective advantage over the other mycoflora in saline environments (Gunde-Cimerman *et al.,* 2000), representing 85-100% of the total isolated mycobiota from highly saline waters, and partially replaced by non-melanized fungi at lower salinities, being detected only occasionally with NaCl concentrations below 5% (Butinar *et al*., 2005b). However, findings of Nayak *et al.* (2012) suggested a greater number of non-melanised filamentous fungi as compared to melanized fungi from amongst the isolates from salterns as well as that from the hypersaline waters of the Dead Sea (Nayak *et al.,* 2012; Nazareth *et al*., 2012b).

Gunde-Cimerman *et al.* (2009) classified the halophilic or halotolerant fungi isolated. Within *Ascomycota*, the main orders were *Capnodiales, Dothideales* and *Eurotiales*. Both *Capnodiales* and *Dothideales* were xerotolerant in nature, containing a large number of extremotolerant species that can grow not only in salterns (Butinar *et al.,* 2005b), but also as epilithic or cryptoendolithic species at high or low temperatures and in Arctic glacier ice (Gunde-Cimerman *et al.,* 2003). They have been found only in natural environments, not even in salted food products. The dominant halophilic species were represented by *Hortaea werneckii, Phaeotheca triangularis, Trimmatostroma salinum* and the halotolerant *Aureobasidium pullulans.*

Another important group of extremophilic fungi within *Capnodiales* were members of the genus *Cladosporium* (section *Cladosporium*). These fungi have a wide distribution as universal decomposers of dead plant material (David, 1997). Some species were consistently isolated from salterns and salt lakes worldwide as the most common and frequent fungal taxa (Gunde-Cimerman *et al.,* 2000). Taxonomic analyses revealed a complex of eight new species with either narrow or wide ecological distributions (Gunde-Cimerman *et al.,* 2009).

Among the *Eurotiales*, xerotolerance and halotolerance were recurrent phenomena. These species were located in remote clades within the order, and thus it is likely that halotolerance is the main plesiomorphic trait shared by the entire order. The most important group of halotolerant species was represented by *Aspergillus niger, A. sydowii, Eurotium amstelodami* and *Penicillium chrysogenum*, which have been recovered from brine, baits and biofilms. *Aspergillus flavus, A. tubingensis, A. versicolor, Emericella* species, *Eurotium herbariorum, Penicillium citrinum* and *P. steckii* are species that are detected at relatively high frequencies of occurrence, but at lower mean counts. *P. chrysogenum* and *P. brevicompactum*, appear pan-globally. Statistical analyses have indicated that the most consistent group of multisite species is represented by *Aspergillus niger, Eurotium amstelodami* and *Penicillium chrysogenum*. Yeasts that have been isolated from hypersaline waters belong to the genera *Candida, Debaryomyces, Metschnikowia* and *Pichia.*

The division *Basidiomycota* contains three orders with halophilic and halotolerant representatives. *Trichonosporales* (Scorzetti *et al.* 2002), containing *Trichosporon mucoides*, and *Sporidiales*, comprising *Rhodosporidium sphaerocarpum, R. babjevae* and *Rhodotorula laryngis* (Butinar *et al.* 2005a), have been frequently isolated. The order *Wallemiales* had the single genus *Wallemia*, (Zalar *et al.*, 2005a). Taxonomic analyses of isolates from sweet, salty and dried food and from hypersaline evaporation ponds in the Mediterranean, Namibia, and the Dead Sea (Wasser *et al.,* 2003) have shown this genus to comprise three species: *W. sebi, W. muriae* and *W. ichthyophaga* (Zalar *et al.,* 2005a). The entire genus *Wallemia* and /or the order *Wallemiales*, was either xerophilic/halophilic or xerotolerant.

Although halophilic fungi require sodium chloride for optimal growth, they do not need salt for viability, and, contrary to many halophilic prokaryotes, are able to grow and adjust to the whole salinity range, from freshwater to solutions saturated with NaCl. They can adapt to this broad ecological amplitude by surviving periods of extreme environmental stress in a resting state, but when conditions change, they can use the available water immediately and respond with increased metabolic activity, growth and propagation. This adaptive halophilic behaviour, named poikilophilic halophily, enables continuous colonization of the hypersaline environments (Gunde-Cimerman *et al.*, 2005b).

1.4) Saline soils, salt marshes, desert soils

Halophilic fungi have also been isolated from saline soils of Soos, Czech Republic (Hujslova *et al.*, 2010), identified as belonging mainly to *Penicillium* and to the genera *Mucor, Fusarium* and *Paecilomyces*, as well as from saline soils, salt marshes and desert soils (Grishkan *et al*., 2003; Guiraud *et al*., 1995; Moubasher *et al*., 1990; Abdel-Hafez, 1981).

2) Polyhaline environments

The study on isolation of halophilic fungi has focussed only on hypersaline econiches which are expected to support the growth of such organisms. However, it has been shown that the existence of halophilic fungi is not restricted to hypersaline environments, and do in fact occur in euryhaline and polyhaline waters of mangroves and of estuary (Nazareth *et al*., 2011; Nayak *et al*., 2012; Gonsalves *et al.,* 2012), which have salinities equal to, or less than that of seawater at 35‰. It may be said that lack of reports of halophilic fungi from such econiches is not a reflection of a meagre presence of this extremophilic group, but rather a consequence of the dearth of work done in these areas.

2.1) Estuary

An estuary is under the influence of marine conditions such as tides, waves, influx of saline water, as well as the flow of fresh water and sediment of the river (Manoharachary *et al.*, 2005). Although reports exist on the isolation of fungi from the estuarine environment, as noted by Gonsalves *et al*. (2012), they do not record halophilic species. Nonetheless, Gonsalves *et al*. (2012) have shown the presence of halophilic fungi from an estuary, through studies carried out on the Mandovi Estuary of Goa, India (**Fig. 1**).

Samples of surface and bottom water and of sediment were collected during the pre-monsoon summer season from ten stations, beginning at the mouth and moving hinterland between 73º46.65´ to 74º2.5´. The salinity of the water at the mouth of the estuary was 37‰, close to that of sea water, with a gradual decrease in salinity to 30-31‰ at Station 5, about the mid-point of the sampling section, a sudden fall to 24-25‰ at the next two stations, and a further sequential decrease to 10-16‰ till the last station interior. The salinity of the surface water was comparable to that of the bottom at all stations, while the salinity of the sediment was 25‰ at the mouth, but showed very low salinity thereafter, becoming nil at the last station. *Aspergillus* and *Penicillium* were the dominant halophilic genera isolated, with *Eurotium* and *Cladosporium* in lesser numbers. These were represented by *Aspergillus flavus, A. fumigatus, A. nidulans, A. penicillioides, A. sydowii, A. versicolor, Penicillium asymetrica* subsec *fasciculata, P. canescens, P. chrysogenum, P. corylophilum, P. steckii, Cladosporium carpophilum, C. cladosporioides, Eurotium amstelodami* and *E. repens*

Fig.1. Map indicating the Mandovi Estuary. (Excerpt from Gonsalves *et al.*, 2012)

2.2) Mangroves

Mangicolous filamentous fungi have been isolated from around the globe, as summarised briefly by Nayak *et al*., (2012). However, these were not characterised in terms of halophily, except for the studies on the osmoregulation of the *Hyphomycetes Cirrenalia pygmea* (Ravishankar *et al*., 2006), and on some isolates from mangroves of Goa, India, as reported by Marbaniang and Nazareth (2007) and by Nazareth *et al.* (2012a), all of which were both mildly halophilic, based on the isolation media used. However, halophilic fungi from the mangroves along the Mandovi Estuary of Goa, situated on the West Coast of the Indian Peninsula, were isolated on 20% NaCl medium (Nazareth *et al.,* 2011; Nayak *et al*., 2012). These mangroves, situated at the intertidal zone, had a salinity of 32 ‰ in the water and 15 ‰ in the sediment, the pH being close to neutral or mildly acidic. The isolates belonged mainly to *Deuteromycota*, with a predominance of *Aspergillus* and *Penicillium* species that were identified as *Aspergillus penicillioides, A. flavipes, A. ochraceus, A. tamarii, A. terreus* var. *terreus* and *A. versicolor, Penicillium asymmetrica* sec. *fasciculata* and *P. corylophilum*, the black yeast *Hortaea werneckii* and *Ascomycetes* species, *Eurotium amstelodami, E. repens.* Cantrell *et al.* (2006) have also reported 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*. Although much work had been done on halophilic fungi from hypersaline environments, there was little or no record of these from mangroves.

HALOPHILY IN FUNGI

Fungi from hypersaline environments had been termed as halophiles because of their growth on high-salt isolation media, but were not examined for their obligate requirement of salt for growth and if these were indeed true halophiles, or whether they had an ability to grow even with minimal salt, and hence could not actually be termed as true halophiles.

Strains of only one species *Gymnascella marismortui* had been reported as obligate halophile (Buchalo, 2003), although its absolute requirement for sodium chloride for its growth was not indicated. According to the definition of Gunde-Cimerman *et al.*, (2004; 2005a), halophilic fungi refers to those species which are isolated regularly with high frequency on selective saline media from environments at salinities above 10%, and are able to grow *in vitro* on media with at least 17% NaCl. Buchalo *et al.* (1998) have described an isolate as obligate halophile, because of its optimal growth in the presence of 0.5 – 2.0 M or 3 – 12% NaCl. However, Nazareth *et al* (2012b), in keeping with the definitions of Kushner (1978), have defined an obligate or true halophile as one having an essential requirement of NaCl for their growth, and a facultative halophile as one that requires high salinity of 0.5 M and above for optimal growth, but can grow even in the absence of NaCl. They have demonstrated the salt tolerance curves of isolates obtained from the Dead Sea, and an estuary, mangroves and solar salterns in Goa, India, obtaining the minimum and maximum salt tolerance levels, together with the salt concentration required for optimal growth, thus describing them as obligate or as facultative halophiles, and classified them as slight or moderate halophiles, on basis of the NaCl concentration required for optimal growth.

Thus the fungi from the Dead Sea as shown by Nazareth *et al.* (2012b), were found to be euryhaline in nature, with very few showing a stenohaline character. Although fungi were found in greater numbers when isolated in presence of 20% rather than 10% salt, no extreme halophile was isolated; however they showed an extreme halotolerance of 20–25% crude salt. Many of the isolates were obligate halophiles, most belonging to *Aspergillus penicillioides*, with an isolate of *A. unguis* and an unidentified non-sporing or sterile fungus, a first report of obligate halophilic aspergilli from the Dead Sea by Nazareth *et al.*, (2012b), while other isolates were facultatively halophilic. *Aspergillus* was the only genus that had true halophiles, while facultative halophiles were found in the genera *Aspergillus, Penicillium* and *Cladosporium*. The obligate halophiles could be classified as moderate halophiles, while the facultative halophiles showed slight to moderate halophily. The fungi obtained from the water were both obligate as well as facultative halophiles, while almost all from the sediment were obligate halophiles. This could be due to the higher salinity of the sediment as compared to that of water.

The obligate halophiles isolated were observed to grow slower, yielding smaller colonies than the facultative halophiles and halotolerant fungi. Ravishankar *et al.* (2006) noted that marine fungi grow very slowly on artificial media as compared to terrestrial fungi. As indicated by Redkar *et al.* (1996), 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. Thus, to counter such physiological conditions, cells may, as an adaptive feature, be subjected to reduction in volume and growth rate.

Similar to the fungi from the Dead Sea, the mycobiota obtained from the estuary, mangroves and salterns were classified as obligate and as facultative halophiles or as halotolerant. The presence of the obligate halophile *Aspergillus penicillioides* in the polyhaline environment of the estuary and from mangroves was reported for the first time by Gonsalves *et al.* (2012) and Nayak *et al.* (2012). The isolates of *A. penicillioides* showed moderate halophily. While the genus *Aspergillus* had both obligate and facultative halophiles, species of *Penicillium*, *Cladosporium* and *Eurotium* were exclusively facultative halophiles, showing slight to moderate halophily, mainly euryhaline in nature (Gonsalves *et al.*, 2012; Nayak *et al.*, 2012).

It was interesting to note from the studies on the estuary that the obligate halophilic *Aspergillus penicillioides* were found in greater numbers at the stations hinterland, although the salinity was lower, as compared to that at the mouth, where the salinity was nearly equal to that of sea water. Borut and Johnson (1962) have demonstrated the isolation of marine fungi from fresh and brackish sediments of an estuary, purported to be due to the irregular pattern of water currents giving rise to changes in environmental factors as well as determining the direction and extent of spore transport.

Furthermore, the presence of obligate halophiles upstream could be due to their ability to synthesize or take up compatible solutes from the estuarine environment, which is abundantly lined with mangroves. Tolerance of salinity has been viewed as a partial function of nutrient level (Borut and Johnson, 1962). It has been reported that fungi associated with standing litter of macrophytes could adapt to daily fluctuations of water availability through adjustment of their intracellular solute concentrations by degradation of organic matter (Kuehn *et al.*, 1998). It is known that in estuarine ecosystems, the detritus and marsh vegetation constitute a major part of the organic content (Manoharachary *et al.*, 2005). The mangroves bordering the estuary, are more abundant upstream, where there is also a decrease in the cross-sectional area, and would serve as a nutrient source for the obligate halophiles. Results have in deed shown the presence of the obligate halophile *A. penicillioides* in the mangroves bordering the Mandovi estuary (Nayak *et al.*, 2012). Tidal movement which extends to a distance of about 50 km (Sundar and Shetye, 2005), a point just beyond the last station sampled, would aid the availability of the nutrient, the receding tide bringing particulate material from upstream of the estuary, and the rising tides carrying it back, thereby forming a nutrient reservoir (Verma and Agarwal, 2007). Interestingly, no halophile was obtained from the samples from the last station hinterland where there were no mangroves.

The work demonstrated the occurrence of obligate moderately halophilic aspergilli in estuarine environment having a salinity lower than that of the sea, thus indicating that hypersaline environments are not the sole econiches for true halophiles. It is particularly noteworthy that obligate halophiles were obtained from the estuary and mangroves, and only one from the salterns, contrary to the expectation that the saltern brine, with a much lower a_w than that of the estuary and mangroves, would yield halophilic species. The isolates obtained from the salterns could also have included 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, by means of their absolute requirement for salt, are truly of marine origin. They can be termed as specialists, with their growth optimum shifted towards extreme values and have a narrow ecological amplitude (Gostinčar *et al.,* 2010). The facultative halophiles could be from terrestrial or fresh water environment, which have adapted so as to grow and sporulate in marine environment.

1) The true halophile *Aspergillus penicillioides*

Aspergillus penicillioides is strictly asexual (Tamura *et al*. 1999). Its growth is favoured by low a_w, and can grow even at an a_w of 0.68, which is inhibitory for most fungi (Tamura *et al*., 1999; Pitt and Hocking, 2009). *A. penicillioides* has been found in diverse habitats of low a_w, such as the Dead Sea, solar salterns, mangroves, estuary (Wasser *et al*., 2003; Butinar *et al*., 2011; Nazareth *et al.,* 2012b; Nayak *et al*., 2012; Gonsalves *et al*., 2012), as well as foods such as grains, dried fruit, baked goods, salted fish, spices, as well as on binocular lenses and human skin (Andrew and Pitt, 1987; Tamura *et al*., 1999; Pitt and Hocking, 2009). Although *A. penicillioides* has been described as an osmophilic fungus (Wasser *et al*., 2003), its capacity to grow in an environment in which the lowering of a_w is contributed by sodium chloride ions, establishes these isolates as halophilic (Grant, 2004), and consequently as true halophiles.

Nazareth and Gonsalves (2013) have demonstrated the absolute requirement of high NaCl and a low a_w for the growth of *A. penicillioides*. The isolates obtained were unable to grow in absence of salt in either a defined mineral salts – sucrose medium such as Czapek Dox Agar, or nutrient rich agar media such as Sabouraud Dextrose, Potato Dextrose or Malt Extract. Micromorphological examination of the isolates of *A. penicillioides* grown on these media in the absence of salt, showed that in one isolate from the Dead Sea, the conidia either appeared swollen and distorted without germination, in another from the Mandovi estuary, Goa, they germinated and then got distorted, while in yet another obtained from mangroves, the conidia germinated and formed distorted mycelia with very little cytoplasm, and lysis at some portions with oozing of the cytoplasm. However, when the same media were supplemented with 10% solar salt concentration which supported maximal growth, the mycelia and vesicles appeared normal. It was seen, therefore, that a low a_w was required not only for the growth of the halophile, but also for conidial germination and germ tube elongation and branching.

A. penicillioides was the only obligate halophile common to the different saline habitats such as the hypersaline thalassohaline Dead Sea and athalassohaline salterns, the polyhaline estuary of Mandovi and brackish water of mangroves, all being moderate halophiles (Nazareth and Gonsalves, 2013). This similarity in the obligate moderate halophily was seen between the isolates from different econiches as well as distant geographical coordinates. This finding implies the ubiquitous distribution of this true halophile in varied environments. Furthermore, these isolates have not only been shown to essentially require a low a_w for their growth, but that they can grow well when this low water activity is met by sodium chloride, rendering these isolates as true halophiles.

2) The black yeast *Hortaea werneckii*

Black yeasts, also termed as black fungi, dematiaceous fungi, microcolonial fungi or meristematic fungi are a diverse group of slow-growing microfungi which mostly reproduce asexually, the fungi imperfecti (Sterflinger, 2006).

Hortaea wernekii is a meristematic fungus. It has been found in tropical and sub-tropical climates, and was amongst the first mycobiota isolated from hypersaline environments of salterns, which have been considered as its primary ecological niche (Gunde-Cimerman *et al*., 2000), existing at 0.5-5.2M NaCl of the salterns (Plemenitaš *et al*., 2008). It has been isolated from a number of salterns in Slovenia-Adriatic (Gunde-Cimerman *et al*., 2000; Butinar *et al*., 2005b; Kogej *et al*., 2005; Gunde-Cimerman and Plemenitaš, 2006), in Cabo-Rojo, Puerto Rico (Diaz-Munoz and Montalvo-Rodriguez, 2005; Cantrell *et al*., 2006), their halophilic behaviour and complex polymorphic life cycle ensuring their dominance over other fungi (Gunde-Cimerman and Plemenitaš, 2006). Their isolation from salterns in Goa, India (Nayak *et al*., 2012), adds to their presence on yet another Continent and confirms the observation of Butinar *et al.* (2005b) that they are present globally in hypersaline waters of man-made salterns.

However, it has not been found in all hypersaline waters. Although isolated from eutrophic thalassohaline waters of the solar salterns, they have not been found in oligotrophic salterns of Eilat, at Red Sea, Israel, nor in athalassohaline waters of Salt Lake, Utah, or that of Dead Sea (Gunde-Cimerman and Plemenitaš, 2006; Nazareth *et al*., 2012b).

Hortaea has furthermore been found in other diverse saline habitats such as sea-water, beach-soil, marine fish and salt-preserved foods, arid inorganic and organic surfaces (Gunde-Cimerman and Plemenitaš, 2006; Plemenitaš *et al*., 2008). Its presence in the non-hypersaline econiche of mangroves in Goa was shown for the first time by Nazareth *et al*., *(*2011). The presence of *Hortaea* in soil and decaying vegetation is known [http://www.doctorfungus.org/thefungi/hortaea.php]. The mangrove ecosystem, which has a salinity of 2.5-3.0% or 0.5M, is rich in detritus, that would help to sustain *H. werneckii* outside of hypersaline environments.

The isolation of *H. werneckii* poses difficulties due to its slow-growing nature and its low competitive ability, amidst its co-existence with a diverse group of microorganisms (Gunde-Cimerman *et al*., 2000). It was, however, successfully isolated from mangroves using a CzA+salt isolation medium (Nazareth *et al*., 2011), in preference to a rich, more easily assimilated carbon medium with salt, which would have encouraged the growth of fast growing halotolerant fungi, that could have outgrown the black yeast.

The slow growth of isolates and a wide salt tolerance range of 0-25% added salt, with optimal growth at 5-10%, corroborates earlier findings (Gunde-Cimerman *et al*., 2000; Kogej *et al*., 2005; Diaz-Munoz and Montalvo-Rodriguez, 2005). *H. werneckii* has been termed as halophilic because of its ability to grow at near saturation salt concentrations of 32%, with a broad growth optimum of 6-14% salt (Gunde-Cimerman *et al*., 2000). On the other hand, because of its ability to grow even in absence of salt, it has also been categorized as halotolerant (Plemenitaš *et al*., 2008). To emphasize this unusually wide adaptability and to distinguish *H. werneckii* from other halotolerant fungi, which have lower maximum salinity limits, it has also been described as extremely halotolerant (Gostinčar *et al*. 2011). However, Nazareth *et al*., *(*2011) have characterized them as facultative halophiles, on the basis that the isolates showed no obligate requirement for salt, yet showed halophilic nature, a moderate halophile with optimal growth in presence of 5-10% salt, growing at concentrations of even 25% salt.

H. werneckii has also been isolated from deep-sea hydrothermal ecosystems (Le Calvez *et al*., 2009) and from deep-sea sediments (Singh *et al*., 2012). It can be quite certain, therefore, that hypersaline environments are not the only econiches for the black yeast, and it may be present in econiches yet to be discovered.

H. werneckii has served for much study on molecular mechanisms involved in salt tolerance (Turk and Plemenitaš 2002; Turk *et al.,* 2003; Lenassi *et al.,* 2007; Gostinčar *et al.,* 2009; Lenassi *et al.,* 2013). Morphological plasticity, incrustation of the cell wall with melanins and presence of other protective substances like mycosporines (Gorbushina *et al.,* 2008) represent passive physiological adaptations which enable black fungi to be highly resistant against environmental stresses (Gostinčar *et al.,* 2010). These and other studies have contributed much to the understanding of halophily in this black yeast.

OSMOADAPTATION IN HALOPHILIC FUNGI

Halophilic organisms must adapt to a lower water potential of the surrounding saline environment as compared to the cell cytoplasm, so as to prevent loss of cytoplasmic water and consequent dehydration and cell shrinkage. Thus in order to cope with this challenge, the cell must develop a means of osmoregulation in order to decrease the chemical potential of its free water and maintain osmotic equilibrium, as well as to resist the denaturing effects of salts. Two basic mechanisms of osmotic adaptation have evolved in such cells**:** a 'salt-in-cytoplasm mechanism' and an 'organic osmolyte mechanism' (DasSarma and Arora, 2001; Kunte *et al*., 2002). Depending on the mechanism of adaptation to the low a_w, organisms are divided into two broad categories: osmoregulators and osmoconformers, respectively (Yancey, 2005).

1) Salt-in-cytoplasm mechanism

The salt-in-cytoplasm mechanism is considered a typical archaeal strategy of osmo-adaptation (DasSarma and Arora, 2001; Kunte *et al.,* 2002; Margesin and Schinner 2001; Roberts, 2005). The intracellular salt concentration of archaea is extremely high and it is generally assumed that organic compatible solutes are not accumulated in these extreme halophiles, and sodium ions are accumulated internally, with a high ratio of cytoplasmic potassium to sodium. Proteins of halophilic anaerobic bacteria and Archaea contain an excess of negative amino acids glutamate and aspartate on their surface, which is neutralized by the binding of significant amounts of hydrated ions like potassium and sodium cations, that maintain a hydration layer thus reducing their surface hydrophobicity and decreasing their tendency to aggregate at high salt concentrations. In low saline environments, the excess of negatively charged ions will destabilize the molecules structure due to repulsion, when the shielding cations are removed. Thus organisms employing this mechanism display a relatively narrow adaptation and their growth is restricted to high saline environments (Kunte *et al.,* 2002).

2) Organic osmolyte mechanism

The organic osmolyte mechanism is widespread among bacteria and eukarya and also present in some methanogenic Archaea. It involves an accumulation of osmolytes, also termed as compatible solutes, so named as these do not interfere with the metabolic processes of the organism, by which the cells maintain their turgor pressure. The osmolytes include polyols such as glycerol, sorbitol, mannitol and arabitol, sugars and sugar derivatives such as sucrose, trehalose, glucosylglycerol; amino acids such as glutamate, proline and derivatives and quaternary amines such as glycine betaine either by synthesis or by uptake from their surrounding environment (DasSarma and Arora, 2001; Margesin and Schinner 2001; Kunte *et al*., 2002; Oren 2002a; Roberts, 2005). Some of these are shown in **Fig. 2**.

Fungi may have more than one type of compatible solutes (Yancey, 2005) which do not compete with each other (Marcelo *et al*., 2007) and may in fact act synergistically

(Davis *et al.*, 2000; Bugni and Ireland, 2004). Organisms employing this mechanism are more flexible, showing a higher degree of adaptability than those employing the salt-in-cytoplasm mechanism, since the intracellular concentrations of organic osmolytes can be up- or down-regulated according to the salinity of the medium to prevent osmotic shrinkage or swelling; thus even though they display wide salt tolerance, they can also grow in low salt environments (Lamosa *et al* 1998; Kunte *et al* 2002; Yancey 2005).

Various polar uncharged solutes have been found in different fungi in response to osmotic stress of NaCl in the medium which include either/or a combination of glycerol, erythritol, arabitol, ribitol and mannitol, as well as glucose (Beever and Laracy, 1986; Kelavkar and Chhatpar, 1993; El-Kady *et al.*, 1994; 1995; Nesci *et al.*, 2004; Ramirez *et al.*, 2004; Roberts, 2005; Bois *et al.*, 2006; Kogej *et al.*, 2007).

The role of melanization has also been implicated in adaptation by *Trimmatostroma* and *Hortaea werneckii* to hypersalinity (Kogej *et al.* 2006a; 2007). Ultrastructural studies showed a distinct layer of melanin in the outer parts of the cell wall of *H. werneckii* at optimal salinity (0.86 M NaCl), whereas cell-wall melanization diminished at higher salinities (Kogej *et al.* 2007). Mycosporines were also found to be accumulated in this black yeast under hypersaline conditions (Kogej *et al.*, 2006b).

CONCLUSION

Halotolerant or halophilic microorganisms offer a wide potential for applications in various fields of biotechnology (Gomes and Steiner, 2004; Margesin and Schinner, 2001). They have been found to produce bioactive metabolites, induced by low a_w (Sepcic *et al.* 2011). They play an important role in food industry 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. Biopolymers, such as biosurfactants and exopolysaccharides, are of interest for microbially enhanced oil recovery. Enzymes, such as new isomerases and hydrolases, that are active and stable at high salt

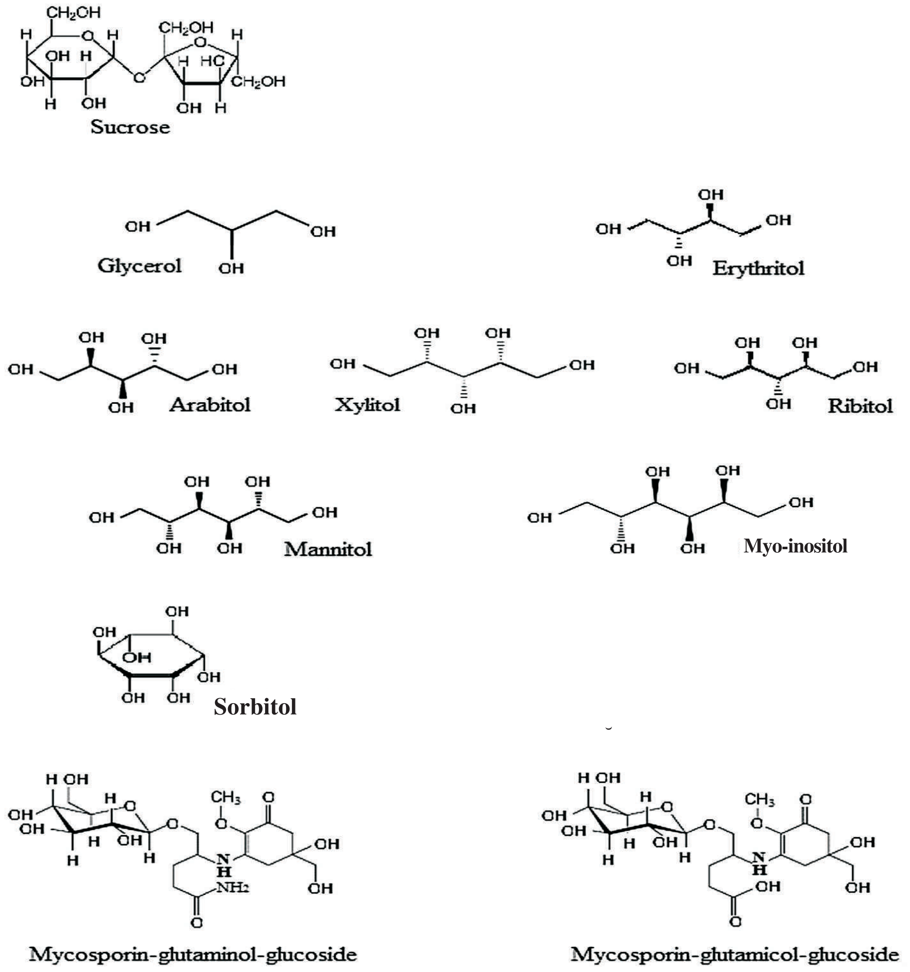

Fig. 2 Structures of compatible solutes accumulated in fungi.

contents offer significant prospects. Compatible solutes are useful as stabilizers of biomolecules and whole cells, salt antagonists, or stress-protective agents (Yancey, 2005). The world of halophilic fungi is yet new, unrecognized to a great extent, and offers great scope for unravelling the unknown and exploiting their benefits.

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