Indian Journal of Marine Sciences
Vol. 35(4), December 2006, pp. 351-358

Strategies for osmoregulation in the marine fungus *Cirrenalia pygmea* Kohl. (Hyphomycetes)

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Received 10 July 2006; revised 18 September 2006

The marine fungus *Cirrenalia pygmea* during growth at increased salinities responded by increasing its intramyceliar polyol content. A similar response was noticed when the fungus was exposed to sudden hyper-osmotic shock; this response was irrespective of the osmotic substance (ionic or nonionic) present in the growth medium. A hypo-osmotic shock treatment led to a decrease in the polyol content. Increasing salinity increased the activity of polyol enzymes such as polyol dehydrogenase and mannitol dehydrogenase. These observations suggested that polyols contribute to osmotic regulation in this marine fungus. The fungus accumulated a mixture of polyols, when the mycelium aged. The sodium content of the mycelium increased with salinity, although the potassium content did not. Higher salinity also led to an increase in the amino acid pool size. Proline and Dragendorff - positive compounds were absent from the mycelium growing in higher salinities. The level of mycelial sugars decreased with increase in salinity whereas that of glycogen and sterols increased with salinity. The fatty acid profile of the fungus was also influenced by salinity. Higher salinities brought about a decrease in the extent of unsaturation of fatty acids. The melanin content of hyphae also increased with external salinity. Based on these results, a synthetic approach is attempted here to explain the various strategies evolved by this manglicolous fungus to survive in a vagarious environment.

[**Key words:** Fungus, osmoregulation, *Cirrenalia pygmea*]

Introduction

Investigations on marine fungi have been mainly concerned with their isolation, cultivation, morphology, taxonomy and systematics[1]. When compared with terrestrial fungi, the growth of many marine fungi on artificial medium is very slow. Furthermore, their capacity to grow and sporulate in culture declines on repeated subculturing[2]. Such recalcitrance has greatly hampered any experimental work with marine fungi. However, marine fungi have been screened for enzymes that can be used for bioremediation of colored pollutants[1,3].

Physiological studies on osmoregulation of marine fungi have essentially centered around the marine hyphomycete *Dendryphiella salina* and the yeast *Debaryomyces hansenii*[4]. Indeed, our understanding of osmotic adjustment in filamentous fungi derives from investigations on a remarkably small number of species. David Jennings and his associates at the University of Liverpool have made seminal contributions to the field through their studies on the marine hyphomycete *Dendryphiella salina* isolated from decaying marine and estuarine plants[4]. It was therefore of interest to investigate the physiology of osmoadaptation in a filamentous marine fungus.

The fungal species *Cirrenalia pygmea* J. Kohlmeyer (Hyphomycetes) is a marine (manglicolous) and melanised hyphomycete, frequently growing on the prop roots of mangrove trees. The fungus occurs throughout the tidal range and is often exposed to sudden changes in salinity due to submergence in seawater and exposure to sunlight[5]. We chose this fungus for the study since we found that it exhibited consistent behaviour in culture despite frequent subculturing.

Materials and Methods

A single spore isolate of fungus *Cirrenalia pygmea* obtained from the prop root of mangrove *Rhizophora mucronata* Poiret (Rhizophoraceae) was used. The fungus was grown in Petri dishes on cellophane discs overlying 2% malt agar medium (containing 0.1% Difco yeast extract) and made up with undiluted, filtered and aged seawater (salinity 34.5‰) or

seawater diluted with deionised water to give a salinity of 20.7 or 6.9 ‰.

Osmotic shock was provided by transferring the fungal colony growing on cellophane overlying nutrient agar medium of a known salinity to a fresh medium of a different solute concentration[6]. This fresh medium was made up with deionised water, seawater, sucrose or NaCl. The concentration of NaCl and sucrose was iso-osmotic with the salinity of the seawater used. The hypo osmotic stress was provided by transferring a 7-day old colony grown on medium with seawater of salinity 20.70 ‰ or 34.5 ‰ to a medium with seawater salinity of 13.80‰ or 6.90 ‰. The hyper osmotic shock was provided by transferring a 7-day old colony growing on malt agar medium made up with distilled water to malt agar medium containing seawater (20.70 ‰), sucrose or NaCl.

Amount of polyols were estimated[7] by spectrophotometry and polyols were extracted from a 7-day old mycelium using the method of Hocking & Norton[8]. The absorbance was measured at 412 nm in a Hitachi 220A double beam spectrophotometer. Standard calibration curve for polyol was made using glycerol. Polyol content was expressed as mg g^{-1} dry weight.

For qualitative estimation of polyols by gas chromatography, the polyols were extracted from the mycelium of a 7-day, 15-day or 30-day old mycelium using the methods of Wethered *et al*.[9] and Pfyffer & Rast[10]. Pure samples of Polyols (Sigma) were used as standards. Solvents of HPLC grade were employed. Standards were prepared and separation of acetylated polyols was carried out in a Shimadzu GC-15A equipped with FID, IBM PC/AT and Oracle 2 integrator. Identification of polyols was done by comparison with the peaks obtained for standard samples. The retention time and peak areas of polyols were used for calculating their amount.

Estimation of glycogen was carried out following the method of Morales *et al.*[11] using glucose as standard. Glycogen was expressed as mg g^{-1} dry weight. Estimation of total sugars was carried out following the method of Roe[12] and expressed as mg g^{-1} dry weight of the mycelium. Trehalose was detected by thin layer chromatography following the method of Stahl[13].

Estimation of monovalent cations was carried out following the method of Galpin *et al*[14]. The mycelium grown at various salinities was processed and analysed for sodium and potassium in an inductively coupled plasma atomic emission spectrometer – 3410 ICP equipped with a mini torch. The sodium and potassium content was expressed as mmol mg^{-1} dry weight.

The free amino acid pool of the fungus was analysed by HPLC after derivatisation with pthaldialdehyde following the method of Rajendra[15]. Estimation of fatty acids by gas chromatography was carried out by following the method of Niller & Berger[16]. Extraction, purification and estimation of melanin was done following the method of Gadd[17]. Melanin extracted and processed from the fungus was used as standard.

Results

The fungus synthesized more polyols when the solute concentration in the growth medium was increased (Fig.1). A sudden exposure of the fungus to higher salinities increased the polyol content within 4 hours of treatment. However, the polyol content was not altered when the fungus was transferred to a fresh medium that was iso-osmotic. Hyper osmotic shock increased the polyol content irrespective of the solute (ionic or non-ionic) used to alter the solute concentration of the growth medium. The hypo osmotic shock treatment reduced the polyol content of the mycelium (Table 1). A time course study showed that the fall in polyols occurred within thirty minutes of the shock treatment. Colony transfer to a fresh medium of the same salinity did not alter the polyol level. Transfer of growth from 34 ‰ to 13.8 ‰ salinity decreased the polyol content by 50%, and transfer from 34.5 ‰ or 20.70 ‰ salinity to 6.90 ‰, reduced the polyol content by 75%. Furthermore, when the colony was transferred from a distilled water

Table 1—Effect of hypo-osmotic shock treatment on polyol content of *C. pygmea*

Transfer treatment	Polyol mg/g dry weight[+]	% decrease
[*]34.5 ‰ to 34.5 ‰	30.5	Nil
34.5 ‰ to 13.8 ‰	15.1	50.5
34.5 ‰ to 6.90 ‰	7.5	75.4
[*]20.70 ‰ to 20.70 ‰	20.0	Nil
20.70 ‰ to 6.90 ‰	4.7	76.5

[+] - 1 h after treatment
[*] - Control
(with permission from Kavaka)

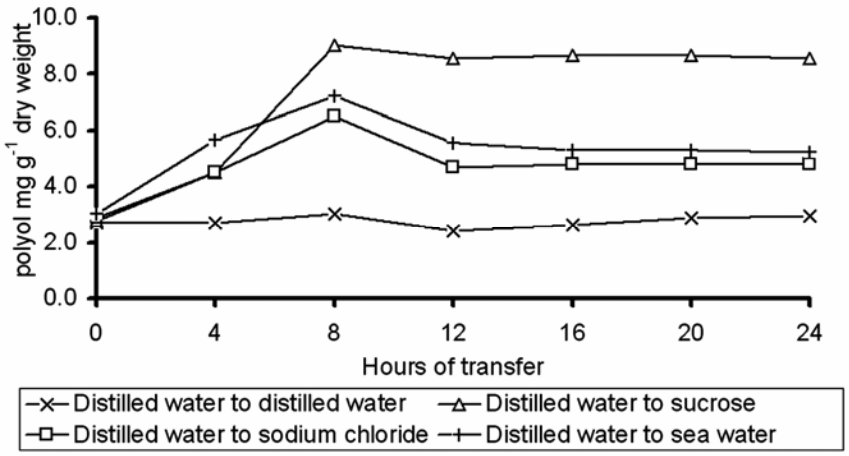

Fig. 1—Effect of hyper-osmotic shock on polyol contents of *Cirrenalia pygmea*

medium to a full strength seawater medium in the presence of cycloheximide (0.1 mmol), there was no increase in the polyol content (Table 2). Cycloheximide, an inhibitor of protein synthesis (translation) in eukaryotes, apparently prevented this polyol adjustment response to osmotic shock.

Qualitative estimation of polyols using GC showed that only erythritol was present in 8-day old mycelium grown on 20.70‰ salinity whereas the 15-day old mycelium harvested from the same medium had erythritol and dulcitol as main polyols. Mannitol was the predominant polyol in the 30-day old mycelium, although ribitol and inositol were also present (Table 3).

As the salinity of the medium increased, there was an appreciable increase in the concentration of glycogen and a decline in the concentration of total sugars of the mycelium (Table 4). Trehalose was detected in the mycelium of the fungus in the entire range of salinities tested.

The concentration of Na^+ in the mycelium increased nearly four times as the salinity was raised from 6.90‰ to 34.5‰ (Fig. 2). However, the concentration of K^+ did not alter with changes in salinity.

Our earlier work with this fungus showed that amino acid pool size was influenced by external salinity[18]. The amino acid pool size in the mycelium increased from 5.4 to 7 times as salinity of the medium was increased. The number of amino acids also increased from 13 to 18 when the fungus was grown on high saline medium. The concentration of glutamate and glycine increased 6 and 3.5, respectively, at 20.7‰ when compared with that at 6.90‰ salinity. Acidic amino acids (Asp + Glu) were present in high concentration in higher salinities (18.1% at 6.9 ‰ salinity to 21.8% at 20.7 ‰ salinity) than the basic amino acids (Lys + Arg + His – 0 and 2.2% at 6.9 ‰ and 20.7 ‰ salinities, respectively)[18]. Quartenary ammonium compounds (betaines) were not detected in the mycelium of the fungus growing at various salinities.

The sterol content of the fungus was also found to increase with increase in salinities (Fig. 2). As the

Table 2—Effect of cycloheximide (0.1 mmol) on polyol content of *C. pygmea*

Transfer treatment	Polyol content mg/ g dry weight
0‰ to 34.5‰	4.0
0‰ to 34.5‰ + cycloheximide	2.6
0‰ to 0‰ (control)	2.8

Table 3 — Retention times of standard polyols and mycelial samples tested

Polyol standard	Retention time	Sample retention time		
		8 days old culture	15 days old culture	30 days old culture
Dulcitol	22.57		22.41	22.92
Erythritol	17.45	17.47	17.54	17.79
Glycerol	13.72			
Inositol	22.19			22.34
Mannitol	23.79			23.67
Ribitol	19.87			19.82

Table 4 — Effect of salinity on total sugars and glycogen of *C. pygmea*

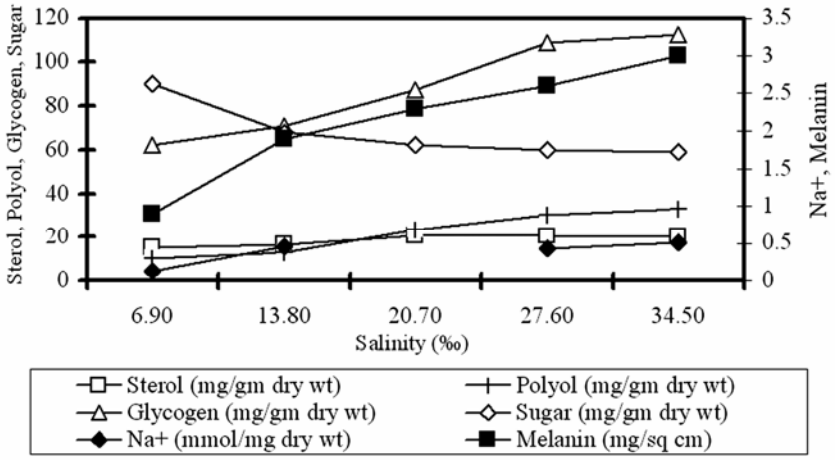

Fig. 2—Effect of salinity on sterol, polyol, glycogen, sugar, Na^{2+} and melalin content

Salinity (‰)	Total sugars (mg/g dry wt)	Glycogen (mg/g dry wt)
6.90	86.0	62.0
13.80	64.0	71.0
20.70	62.0	87.5
27.60	60.0	109.0
34.50	60.0	112.5

water potential of the medium decreased, the melanin content of the fungus was found to increase. Sucrose increased the melanin content much more than seawater of corresponding osmotic strength.

Discussion

Although halobacteria and anaerobic halophilic bacteria adapt to hyperosmotic conditions by building up high cytoplasmic salt concentrations[19], many osmophilic bacteria and eukaryotes including yeasts[20] synthesize solutes that do not perturb cell metabolism-compatible solutes[21]. Our results indicate that the polyol synthesis response was, depending more on osmotic stress than on the nature of the chemicals used to produce the stress. Thus, the hyphae of *C. pygmea* are both halotolerant and osmotolerant as has been demonstrated for another filamentous fungus, *Ashbya gossypii*[22]; they could sense sudden changes in the external osmoticum and synthesize polyols to adjust their turgor. Microorganisms growing in hyper osmotic environments synthesize sugar alcohols in order to regulate cell turgor. A high cytoplasmic concentration of polyols does not perturb enzyme activity as do the inorganic ions[23]. Therefore, polyols are termed compatible solutes along with amino acids, polyamines, sucrose and quartenary ammonium compounds[24]. Although osmotic adjustments by polyols occur in a number of terrestrial fungi including *Saccharomyces cerevisiae*, *S. rouxii*[25], *Penicillium chrysogenum*[6], *D. hansenii*[26], *Aspergillus nidulans*[27] and *Neocosmospora vasinfecta*[28], only one study reports polyol acting as a turgor regulator in a higher marine fungus[29] for *Dendryphiella salina*.

A sudden exposure of the fungus to higher salinities increased the polyol content (Fig. 1). A similar trend was noticed in the halotolerant yeast *D. hansenii*, which synthesizes glycerol within four hours of exposure to a salt stress[26]. Cycloheximide, an inhibitor of translation in eukaryotes, prevents this polyol adjustment response to osmotic shock in *N. vasinfecta* showing that such a response requires a translational process[30]. We extend this argument to *C. pygmea* as it had responded in a similar manner to the presence of cycloheximide in the growth medium (Table 2).

Irrespective of the nature of the solute present in the medium (ionic or non-ionic), there was an induction of polyols in *C. pygmea* while it was growing on a hyperosmotic medium. A similar response was noticed in *D. hansenii*[26] and *A. nidulans*[27]. Sucrose as an osmoticum induces more polyols perhaps due to its functioning as carbon skeleton for polyol synthesis. The polyol concentration in *C. pygmea* underwent changes in response to hyper and hypo osmotic treatments. Changes in the intra mycelial level of specific solutes, as a response to osmotic shock, are strong evidences for their role as osmotic adjusters[6]. Therefore, it could be concluded that polyols act as an osmo regulator in *C. pygmea*.

The response to hypo osmotic shock treatment was rapid with reference to polyol shifts occurring within thirty minutes. This could be due to excretion of polyols[6,30] or conversion of polyols to insoluble reserves. A time course study showed that the polyol content began to increase within thirty minutes upon exposure to up shock treatment and continued to increase up to 4 hours of treatment and stabilized in about 8 hours of incubation (Fig. 1). In the case of *Neurospora crassa*, turgor regulation after hyper osmotic challenge occurs more slowly[31]. The fact that morphologically similar, yet phylogenetically distinct organisms respond differently to a stress could be a reflection of the distinct ecological niches they occupy[31]. Thus, studies with more marine fungi are needed to understand the phenomenon of osmoregulation in them.

Fungi are known to accumulate arabitol, glycerol, sorbitol and other sugar alcohols to come to terms with their external hyperosmotic medium[32,33]. Many fungi accumulate a combination of different polyols in response to an osmotic stress. Adler *et al.*[26] reported that the polyol pool of *Debaryomyces hansenii* differs qualitatively and quantitatively with growth cycle and salinities. Here, there was a shift in the nature of the polyol accumulated from dulcitol to mannitol as the organism aged. In *Neocosmosporsa vasinfecta*, exposure to KCl increased the glycerol and erythritol content of the mycelium, and reduced the relative concentrations of cytoplasmic mannitol and glycerol[30]. Similarly, in *Eurotium rubrum*, the nature of polyols accumulated is influenced by the water activity of the growth medium[34]. *Cirrenalia pygmea* accumulates erythritol, dulcitol and also mannitol in response to salinity changes. *Dendryphiella salina* also accumulates polyols, particularly, glycerol in response to different salinities[9]. The osmo-protection roles of polyols are usually substantiated by measuring the activity of the relevant enzymes of polyol metabolism[21]. The role of polyols in *C. pygmea* as osmo regulators was further substantiated by the increased activities of polyol dehydrogenases and mannitol dehydrogenase as salinities in the medium increased[35].

In addition to polyols, trehalose was also detected in the mycelium of *C. pygmea*. Trehalose is reported from a variety of fungi and not in those which are marine[36] (but see Gonda *et al.*[37]). Trehalose boosts osmotic potential further than proline or any of the polyols tested osmometrically[38]. Trehalose confers additional benefits within the cytoplasm playing many roles even at low concentrations. The osmotic effects of trehalose accumulation may, therefore, be of secondary value to its other cellular functions. The decrease in the level of total sugars in *C. pygmea* with increasing salinity of the medium was not surprising since sugars are precursors of polyols. Storage of low molecular weight organic osmolytes in an osmotically inactive polymeric form, from which osmolytes could be generated through a few metabolic transformations, is an admirably suited adaptation for organisms growing in saline environments. The storage of polyols as glycogen polymer represents one such instance of maintaining an accessible source of compatible solute. The increased glycogen content of *C. pygmea* with increasing salinities and the interconversions of starch and glycerol in the halophilic alga *Dunaliella*[39] indicate the possibility of low molecular organic osmolytes being stored as osmotically inactive polymers. It is pertinent to mention here that *Saccharomyces cerevisiae* exhibits stress-induced recycling of glycogen and trehalose[40]. More detailed studies are needed however to confirm this with reference to *C. pygmea.*

Although amino acids are implicated in turgor regulation in halophilic algae[41], prokaryotes[42] and angiosperms[43], these compounds do not play a role in the marine fungus *D. salina*[29]. Our earlier study indicates that *C. pygmea*, in addition to polyols, uses amino acids as osmolyte[18]. Accumulation of negatively charged amino acids (glutamate and aspartate) as observed in *C. pygmea*[18] might be a prudent choice for the fungus to regulate its turgor, since, for each of such molecule accumulated, one counter ion (K^+ or Na^+) must be taken in to the cell - a metabolic strategy to overcome ion toxicity[44].

As in the case of *D. salina*, the mycelial sodium content of *C. pygmea* increased with salinity. Clipson & Jennings[29] argued that the accumulation of cations may not be the osmotic strategy adopted by *D. salina* since much of the cations of the fungus is located in the cell wall and not in the cytosol. For these reasons, we could not ascribe an unequivocal role to sodium in turgor regulation in *C. pygmea*.

Although Dragendorff-positive compounds regulate cell turgor in some halophilic bacteria and marine algae[45], there was no evidence of these compounds in the mycelium of *C. pygmea.* This was true of the marine fungus *D. salina* also[36].

The lipid composition of *C. pygmea* was influenced by salinity as revealed by the increase in the concentration of sterols as a response to salinity as well as qualitative and quantitative changes in the fatty acids[46]. The fall in the unsaturation index of fatty acids could be an adaptation to salinity as it renders the plasma membrane more rigid, thereby aiding the retention of osmolytes[46,47].

The melanin content of *C. pygmea* increased with increasing salinity. Ravishankar *et al*.[48] demonstrated that melanin deposition in the hyphae of *C. pygmea* protects the hyphae from bursting due to sudden and steep changes in the external osmoticum. Relevant to this is the fact that in halophilic black yeasts, synthesis and deposition of DHN melanin is dependent on salt concentration of the external medium; a higher salt concentration leads to a more compact arrangement of the pigment granules in the cell wall[49,50]. Furthermore, a melanised appressorial wall of *Magnaporthe grisea* limits glycerol loss creating a permeability barrier and it is hypothesized that this preplasmic trap may aid resorption of the osmolyte in appressoria[51]. Therefore, it is possible that melanin pigment of *C. pygmea* at higher salinities reorients its structure in such a way as to prevent the leakage of osmolytes, accumulated as response to osmotic shock. However, this needs confirmation at the ultrastructural level.

A marine fungus, like any other marine organism, should possess metabolic and structural strategies to come to terms with its external hypertonic environment. More commonly, this is achieved by synthesizing polyols to generate a cell turgor. Although synthesis of polyols for osmoregulation could mean a metabolically expensive strategy (as it diverts carbon skeleton from the main synthetic stream), it is advantageous over the alternative strategy of ion accumulation, since it does not perturb cell metabolism. Our results show that *C. pygmea* has, apart from polyol accumulation, other attendant features as osmoadaptation. A lower water potential could be generated by the 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. It is also likely that sodium ions are sequestered in the vacuole of the older regions and metabolically sluggish or inactive regions of the hyphae thereby aiding in the reduction of water potential (Fig. 2).

Both *Dendryphiella salina* and *Cirrenalia pygmea* have been isolated from the same habitat. *Dendryphiella salina* adjusts its osmotic potential solely by the accumulation of glycerol whereas *Cirrenalia pygmea* synthesizes different osmolytes in order to regulate its intracellular solute potential. There are many reasons why an organism might produce a mixture of osmolytes rather than accumulating a single compound. For example, 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. These factors probably underlie the complex patterns of osmolyte accumulation that have evolved among fungi[38]. *Cirrenalia pygmea*, a marine fungus that frequently encounters conditions of low water activity in its natural habitat appears to have evolved a panoply of adaptations to survive.

Acknowledgement

The authors thank Swami Satyapriyananda, Secretary, RKM Vivekananda College, and Chairman, Vivekananda Institute of Tropical Mycology, for facilities and Prof. H. P. Molitoris, University of Regensburg, Germany for reading a draft of the manuscript.

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