

Compatible solutes in halophilic filamentous fungi

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ABSTRACT

Halophilic fungi combat the osmotic stress of their saline environment by the accumulation of compatible solutes, also termed as osmolytes. Filamentous fungi isolated from various athalassohaline, thalassohaline and polyhaline ecoiniches were selected from different genera and species, on the basis of their classification as obligate and facultative halophiles. The salt tolerance index of the isolates proved their euryhaline nature, able to adapt to a wide range of salt concentrations, the obligate halophiles however, showing a tendency to a stenohaline nature, with a narrow tolerance range. Examination of the osmolyte production indicated that sucrose, trehalose, arabitol, erythritol, inositol, mannitol, dulcitol, xylitol, galactose and glucose were present, the polyols being found in each of the isolates studied. The variations in concentrations of osmolyte pools *vis-à-vis* the salt tolerance characteristics in the different genera and species, did not indicate any correlation with the obligate or facultative halophilic nature of the fungi, or with the saline ecoiniches from which they were obtained, as well as with the different genera. However, the similarity in the different types of osmolytes between the different genera and species, indicated their notable role in osmoadaptation in fungi in general.

KEYWORDS: Osmolyte, halophilic, fungi, hypersaline, polyhaline

INTRODUCTION

Halophilic microorganisms are exposed to a high osmotic pressure of hypertonic saline environments and develop strategies of osmoregulation to maintain iso-osmotic balance with their surroundings, to enable growth and survival in the otherwise harsh conditions of hypersalinity and the resultant low water activity of their environment (Kunte *et al.*, 2002; Grant, 2004). While in archaea, osmoadaptation occurs mainly by a salt-in-cytoplasm mechanism, eubacteria and fungi survive by the build-up of compatible solutes or osmolytes, so termed because these do not interfere with the metabolic processes of the organism (DasSarma and Arora, 2002).

Halophilic fungi have been found to either have obligate requirement of sodium chloride for its growth, or are able to grow even without NaCl, and have been termed as obligate or true halophiles, and as facultative halophiles, respectively (Nazareth *et al.*, 2012). Reports on osmoadaptation in fungi and/or their survival in hypersaline environments, have focused on the synthesis of melanin in black yeasts and dematiaceous fungi (Kogej *et al.*, 2007), mycosporins in black yeasts, ascomycetes and basidiomycetes (Kogej *et al.*, 2006) and some compatible solutes such as glycerol, erythritol, arabitol, ribitol and mannitol, trehalose and glucose, in black yeasts and mycorrhizal fungi, as well as in a few filamentous fungi (Beever and Laracy, 1986; Kelavkar and Chhatpar, 1993; El-Kady *et al.*, 1995; Nesci *et al.*, 2004; Ramirez *et al.*, 2004; Roberts, 2005; Bois *et al.*, 2006; Kogej *et al.*, 2007). These fungi had been grown in the presence of salt but the halophilic nature was not determined (Nazareth, 2014). Moreover, only a single or a few select organisms have been studied from a specific sample.

This paper presents the types of osmolytes produced by halophilic fungi, and an analysis on the correlation of types of osmolytes accumulated in both obligate and facultative halophiles, and/or the habitat from which they have been isolated, namely, athalassohaline and thalassohaline,

hypersaline and polyhaline environments. An examination is also made with respect to osmolyte production by the different genera.

MATERIALS AND METHODS

Isolates: Thirteen halophilic fungi were selected, previously isolated from different saline environments: the Dead Sea water (DSw) and sediment (DSs) samples, with a salinity of 370‰ and 450‰, respectively (Nazareth *et al.*, 2012), from the estuary of Mandovi, Goa, on the West Coast of the Indian peninsula-from surface and bottom waters (EM_w and EM_b) and from sediment (EMs) samples, with a salinity range of 13‰-31‰ from upstream travelling towards the mouth, with the salinity of the sediment being 5‰-10‰ (Gonsalves *et al.*, 2012), from water samples of mangroves at Ribander (MRw) and sediment of solar salterns, Ribander, Goa (SRs), with salinity of 32‰ and 295‰, respectively (Nayak *et al.*, 2012). The fungi comprised the genera of *Aspergillus*, *Penicillium* and *Cladosporium*, of which some were obligate halophiles [*A. penicillioides* DSs40 (GenBank Accession number HQ702385), EM6s137 (JQ240645), EM8w_s146 and MRw207] and others were facultative halophiles [*A. restrictus* DSw14 (HQ702382), *A. penicillioides* EM7w_s138, *A. versicolor* SRs246, *A. wentii* SRs249, *P. corylophilum* DSw10, *P. waksmanii* DSw16, *P. corylophilum* EM5s132, *C. cladosporioides* DSw26 and *C. carpophilum* EM9w_b154] which were classified on the basis of their salt requirement for growth, as recorded earlier (Gonsalves *et al.*, 2012; Nayak *et al.*, 2012; Nazareth *et al.*, 2012).

Salt tolerance index: Salt tolerance index of the isolates was obtained from the halotolerance curves performed in triplicate on Czapek Dox Agar (CzA) at 30°C (Gonsalves *et al.*, 2012; Nayak *et al.*, 2012; Nazareth *et al.*, 2012) and was calculated as the ratio of growth in terms of colony diameter obtained at different concentrations of solar salt, to the maximum growth obtained at the optimal salt concentration of 5% or 10%, after 7d incubation, as described earlier

(Nazareth and Gonsalves, 2014a).

Osmolyte analysis: The isolates were grown in Czapek Dox Broth supplemented with a salt concentration that supported maximal growth (10% salt for obligate halophiles and 5% or 10% salt for facultative halophiles). The cultures were harvested in the logarithmic growth phase, washed with deionised water and osmolytes were extracted by the method of Ramirez *et al.*, (2004). The fungal biomass (0.5g) was frozen, then homogenised in 1.5 mL deionised water, using a mortar and pestle, and sonicated with a B-Braun Labsonic sonicator, at 150 mv, 10 pulses for 20s with intervals of 10 s for 5 min, followed by heating for 5.5 min in a boiling water bath, and cooled. Samples were then centrifuged at 14,000 rpm for 15 min. The supernatant was filtered through a 0.22 µm Millipore filter and analyzed by HPLC (Waters) using a Sugar Pak column maintained at 70°C and refractive index detector; elution was carried out with calcium di-sodium EDTA (0.1 mM) at a flow rate of 0.4 mL min⁻¹. Data analysis of retention time of standards and samples was done using Millenium software. The peaks were compared with calibration curves of standard sugars galactose, glucose, sucrose, trehalose and sugar alcohols arabitol, dulcitol, erythritol, glycerol, inositol, mannitol, ribitol, sorbitol and xylitol (2 mg mL⁻¹). Sugar and polyol concentrations of the samples were calculated as mg g⁻¹ wet weight of mycelia.

RESULTS

Salt tolerance index: The salt tolerance indices (STI) of the isolates at different solar salt concentrations, given in **Fig. 1**, indicated that the obligate halophilic isolates of *Aspergillus penicillioides* DSs40, EM6s137, EM8ws146 and MRw207 were moderate halophiles, with optimal growth at 10% salt, and a varying minimum requirement of 2% to 10% solar salt for growth. The STI histograms were right-skewed and showed an extreme halotolerance level of 20% to 25% and even to 30% solar salt.

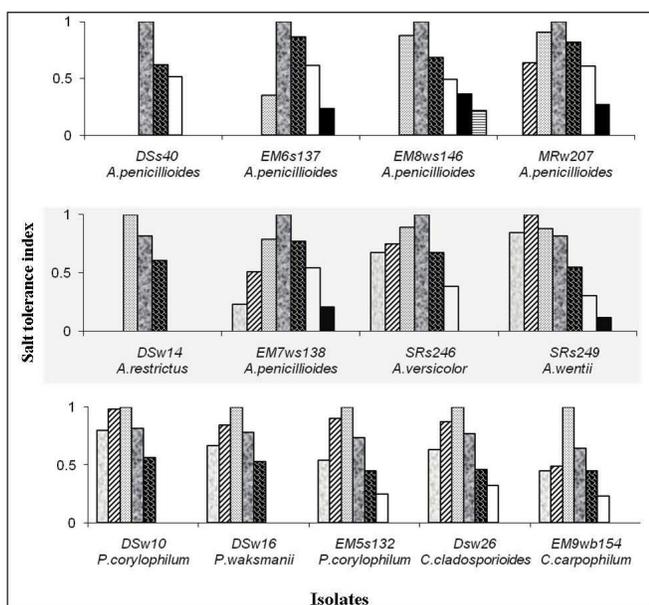


Fig. 1 Salt tolerance index of the halophilic isolates on CzA with solar salt: 0% (□), 2% (▨), 5% (▩), 10% (■), 15% (▣), 20% (▢), 25% (▤) and 30% (▥).

DSw14 *Aspergillus restrictus* was grouped under facultative halophiles, as it showed a delayed growth at 0-2% solar salt at 15 days incubation, which is not shown in the STI calculation. The facultative halophilic isolates of aspergilli (DSw14 *Aspergillus restrictus*, EM7w₁₃₈ *A. penicillioides* and SRs246 *A. versicolor*) penicilli (EM5s132 *P. corylophilum* and DSw16 *P. waksmanii*) and cladosporia (DSw26 *C. cladosporioides* and EM9w₁₅₄ *C. Carpophilum*) were also all moderate halophiles, with an optimal growth in the presence of 5% or 10% salt. The facultative halophilic fungi (SRs249 *Aspergillus wentii* and DSw10 *Penicillium corylophilum*) were slight halophiles with a growth optimum around 2% solar salt. All the facultative halophilic isolates showed an extreme halotolerance level of 15% to 25% solar salt, with either a bell-shaped or right-skewed histogram.

The obligate halophiles exhibited an STI of > 0.5 at salt concentrations of 10-20%, and of 5-15% and 2-20% for the facultative halophilic *A. restrictus* and *A. Penicillioides*, respectively, while that of the other facultatively halophilic aspergilli was ≥ 0.5 at salt concentrations of 0-15%. The STI at these concentrations of solar salt, by the different genera was in the order of cladosporia < penicilli < aspergilli.

Significant difference ($P < 0.05$) was obtained in the STI of each isolate at different salt concentrations, and the STI of isolates within the obligate aspergilli, within the facultative aspergilli and the cladosporia, as well as within all the isolates of *Aspergillus*; however, there was similarity ($P > 0.05$) in the STI of isolates of penicilli.

Osmolytes: The osmolytes detected in the given isolates were of four to six carbon chain, comprising sugars sucrose and trehalose, and polyols such as the four carbon chain erythritol, the five carbon chain ribitol, arabitol, xylitol, and the six carbon chain inositol, mannitol, and dulcitol, along with glucose and/or its epimer galactose, in the obligate halophilic *Aspergillus penicillioides* (DSs40, EM7ws137, EM8ws146 and MRw207), facultative isolates aspergilli (DSw14 *A. restrictus*, EM7ws138 *A. penicillioides*, SRs246 *A. versicolor* and SRs249 *A. wentii*), penicilli (DSw10 *P. corylophilum*, EM5s132 *P. corylophilum* and DSw16 *P. waksmanii*) and cladosporia (DSw26 *C. cladosporioides* and EM9w₁₅₄ *C. carpophilum*). There was a significant difference ($P < 0.5$) in the relative concentrations of the osmolytes in each isolate (**Fig. 2**).

In the obligate halophilic aspergilli *A. penicillioides*, both glucose and galactose were identified in DSs40, EM7ws137 and MRw207, while only galactose was detected in EM8ws146. The facultative halophilic aspergilli EM7ws138 *A. penicillioides* and SRs246 *A. versicolor* accumulated galactose, while glucose was found in SRs249 *A. wentii*. Glucose was also detected in all the penicilli, and to a lesser extent in cladosporia.

As shown in **Fig. 3**, the obligate aspergilli accumulated total of 4-14 mg sugars g⁻¹ mycelia wet weight and 14-37 mg polyols g⁻¹ mycelia wet weight. Between the different isolates there was a significant difference ($P < 0.5$) in the total sugars and total polyols accumulated. Amongst the facultative halophiles, aspergilli accumulated total of 5-11 mg sugars g⁻¹

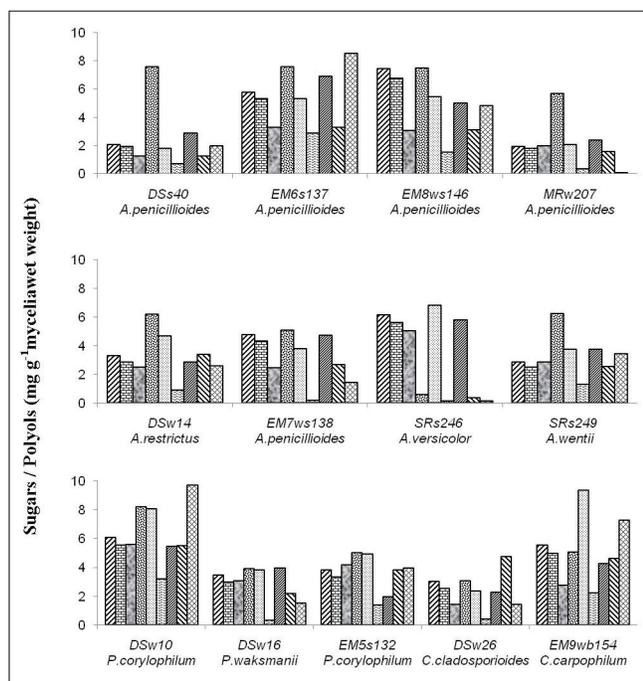


Fig. 2 Intracellular sugars and polyols in the halophilic isolates: Sucrose (▨), trehalose (▩), erythritol (▧), ribitol (▦), arabitol (▥), xylitol (▤), inositol (▣), mannitol (▢), dulcitol (□).

mycelia wet weight and 18-23 mg polyols g^{-1} mycelia wet weight, penicilli accumulated total of 6-11 mg sugars and total of 18-45 mg polyols g^{-1} mycelia wet weight, while the amount of total sugars and polyols accumulated in *C. cladosporioides* DSw26 was 5.5 mg and 15.7 mg g^{-1} mycelial wet weight, respectively, and in *C. carpophilum* EM9wb154 it was 10.5 mg and 35.35 mg g^{-1} mycelia wet weight, respectively. There was no significant difference ($P>0.5$) between the facultative aspergilla, however, a significant difference ($P<0.5$) was seen amongst isolates of *Penicillium* and of *Cladosporium*, in the total sugars and total polyols accumulated.

DISCUSSION

The salt tolerance index (STI) of the isolates displayed their euryhaline nature, able to adapt to a wide range of salt concentrations, the obligate halophiles however, showing a narrower tolerance range below the optimal salt concentration as seen from the right skewed histogram, compared to that of the facultative halophiles. It has also been shown earlier that conidia of the obligate halophiles could germinate in the absence of solar salt, but could not grow thereafter (Nazareth and Gonsalves, 2014b). The STI also indicated the degree of halophilicity, the obligate halophiles having a greater STI value at a higher salt range, as compared to that of the facultative halophiles. Amongst the facultative halophiles, *A. restrictus* and *A. penicillioides*, so classified because of their ability to grow in the absence of solar salt, had a greater STI value at a higher salt range than that of the other facultative halophiles of *Aspergillus*, *Penicillium* and *Cladosporium*, which grew more readily in the absence of solar salt, thus showing the stronger halophilic nature of these two isolates over that of the latter.

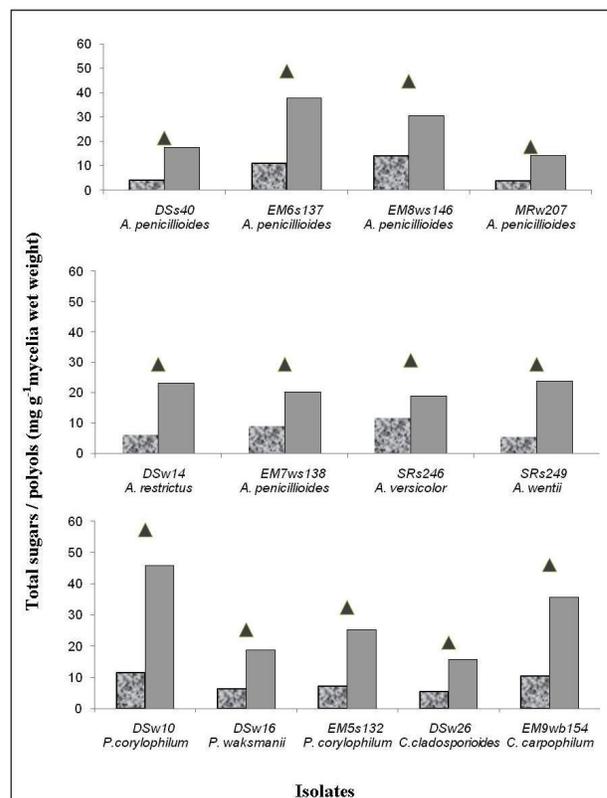


Fig. 3 Intracellular total sugars and total polyols in the halophilic isolates: sugars (▩), polyols (▧), sugars + polyols (▲)

The detection of a consortium of various polar uncharged compatible solutes produced by the isolates in response to osmotic stress of sodium chloride, which comprised sugars sucrose and trehalose, and polyols such as erythritol, ribitol, arabitol, and mannitol, corroborated earlier records, but were detected in higher concentrations than reported earlier (Beever and Laracy, 1986; Kelavkar and Chhatpar, 1993; El-Kady *et al.*, 1995; Davis *et al.*, 2000; Nesci *et al.*, 2004; Ramirez *et al.*, 2004; Roberts, 2005; Bois *et al.*, 2006, Dijksterhuis and Vries, 2006; Kogej *et al.*, 2007; Plemenitas *et al.*, 2008; Kumar and Gummadi, 2009); in addition xylitol, inositol and dulcitol were also detected.

The production of high concentrations of compatible solutes would have contributed to the wide range of salt tolerance by the organisms, particularly of the facultative halophiles, as it is known that organisms employing the mechanism of osmolyte production as a means of osmoregulation, are more flexible, with a higher degree of adaptability than those employing the salt-in-cytoplasm mechanism, as the intracellular concentrations of organic osmolytes can be up- or down- regulated according to the salinity of the medium (Kunte *et al.*, 2002; Yancey, 2005).

The polyol osmolytes in the obligate halophiles were in greater concentration than the sugar osmolytes, and thus appear to have a significant role in the osmoadaptation mechanism of these fungi. The various polyol osmolytes produced by the isolates comprised 4 to 6 carbon chain with a corresponding increase in molecular weight (MW), namely,

the 4 carbon chain erythritol with MW 122 g mol⁻¹, the 5 carbon moieties ribitol, arabitol, xylitol with MW 152 g mol⁻¹ and the six carbon chain inositol, mannitol, and dulcitol, with MW 182 g mol⁻¹. It could be expected that the increase in the polyol chain, with its corresponding higher molecular size, would have an increasing capacity for osmoregulation. It was also noted that the isolates produced a higher total concentration of 5 carbon and 6 carbon polyols than that of the 4 carbon erythritol, which would also have contributed to a more efficient osmoprotection. Shen *et al.*, (1999) also reported that during salt stress, yeast cells had more of 6 carbon polyols than the 3 carbon glycerol. This is in accordance with the earlier records that polyols may be differentially active as compatible solutes, depending on their molecular weight, wherein larger molecular size polyols render higher osmotic pressures than smaller ones (Hallsworth and Magan, 1996; Davis *et al.*, 2000).

A consortium of compatible solutes has been noted to be more efficient in increasing water pressure than a single compound, since individual compounds are differentially active as compatible solutes (Ramirez *et al.*, 2004). A mixture also helps to avoid the toxicity associated with high concentration of a single osmolyte that can cause destabilization of proteins and interference in cellular processes such as protein - protein interaction and DNA - protein interaction, and also prevents feedback mechanisms that can down regulate metabolic pathways in the presence of a high concentration of the product (Kogej *et al.*, 2007; Singh *et al.*, 2011). Moreover the varied osmolytes do not compete with each other (Holthauzen and Bolen, 2007) and may in fact act synergistically (Davis *et al.*, 2000).

Among the sugars detected in the isolates, sucrose was dominant. It may be transported intracellularly from the medium, serving a transient role as a compatible solute (Beever and Laracy, 1986; Grant, 2004; Roberts, 2005), and serves as a good source for polyol production, eventually being replaced by other, more effective osmolytes. Reducing sugars, such as glucose and its epimer galactose, were also detected and may function as intermediates in the synthesis of osmolytes. Gaunt and Manners, (1973) reported that glucose serves for trehalose production. Roberts, (2005) reported that only a few carbohydrates are used for osmotic balance since those with reducing end are chemically reactive and can react with surface amino groups of proteins. Trehalose is used not only as a reserve carbohydrate but also as an osmoprotectant (Bois *et al.*, 2006), serving to counteract desiccation, and has been linked to longevity (Kuehn *et al.*, 1998; Dijksterhuis and De Vries, 2006; Uyar *et al.*, 2010), functioning also as a protective solute in stress conditions of high and low temperatures, as well as metal toxicity (Kuehn *et al.*, 1998).

Among the polyols detected, mannitol, erythritol, and arabitol have been well documented in reports as cited above. However, inositol, a polar uncharged solute, which was one of the major polyols detected in both obligate as well as facultative halophilic isolates, has been only tentatively identified in small amounts as an osmolyte in *Aspergillus nidulans* (Beever and Laracy, 1986). This paper also reports the presence of xylitol and dulcitol amongst the osmolytes,

hitherto unreported. Furthermore, a greater spectrum of osmolytes was detected in a given halophilic fungal isolate, than so far reported.

CONCLUSIONS

A commonality of the solutes was observed in both obligate and facultative halophiles, as well as in all three genera of the facultative halophiles, although they may have varied in relative concentrations, suggesting these may have a significant role in osmoadaptation in all halophilic filamentous fungi. The osmolytes did not appear to have a specific correlation to the type of saline niche: thalassohaline or athalassohaline, hypersaline or polyhaline from which they had been isolated, or the obligate or facultative halophilic nature and degree of halophilicity or tolerance of the isolate, nor to a specific genus or species. These results show that halophilic fungi can serve as valuable source of compatible solutes with a great potential for biotechnological applications.

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