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

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

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

Introduction

Hypersaline waters of solar saltens are termed as thalassohaline[1], have salt concentrations greater than 3.5%, that of sea water. Salinitiy of such saltens steadily increasing to saturation point, and variations in pH, temperature, light intensity, oxygen and nutrient concentrations. Hence facilitate the study of different microbial communities[2,3].

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

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

Materials and Methods

Collection of Samples

Samples of water (w) and sediment (s) were collected during the pre-monsoon summer season from Mangroves (M) and solar Salterns (S) at two sites in Goa: at Ribander (R), lying alongside the Mandovi estuary, Goa, leading into the Arabian Sea, and one interiorly at Santa Cruz (C), along the offshoot of the estuary. Water from mangroves are channeled into the salterns. Sampling of the mangrove water was done from five different sites about 15 cm apart, at the intertidal zone during low tide, at a distance of 1 m from the shore and a depth of 0.5 m, and pooled together. Sediment samples were also collected from the same points. Brine and sediment samples from salterns were obtained from five different saltpans at each site and pooled. The temperature of the mangroves and salterns was recorded in the morning (07.00 hr) and at mid-day (12.00 hr). Physicochemical conditions of pH and

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salinity of the samples were determined using a Cyberscan pH meter and Atago Handheld refractometer; for sediment samples, 1 g was shaken in 5 ml deionised water and the supernatent used for pH and salinity measurement.

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

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

Results

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

Fungal isolates

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

Halotolerance

Salt tolerance curves of the isolates, grouped according to the genus and then by the species, placing in priority, those having an absolute requirement of added salt for growth (Fig. 1). The salt tolerance range and concentrations for optimal growth of the isolates, together with their halophilic nature, are summarized in Table 1. *A. penicillioides* MRw201, MRw202, MRw203, MRw204, MRw207 and MRw208, required a minimum of 2% added salt for growth on CzA and could grow in presence of up to 20% salt, with MRw201 and MRw207 growing at salt concentrations of 2-25%, or even 30% in 15 days; SCw255 required a minimum of 5% added salt for growth and was able to grow at 20% salt, or at 25% salt in 15 days. All showed optimal growth at 10%

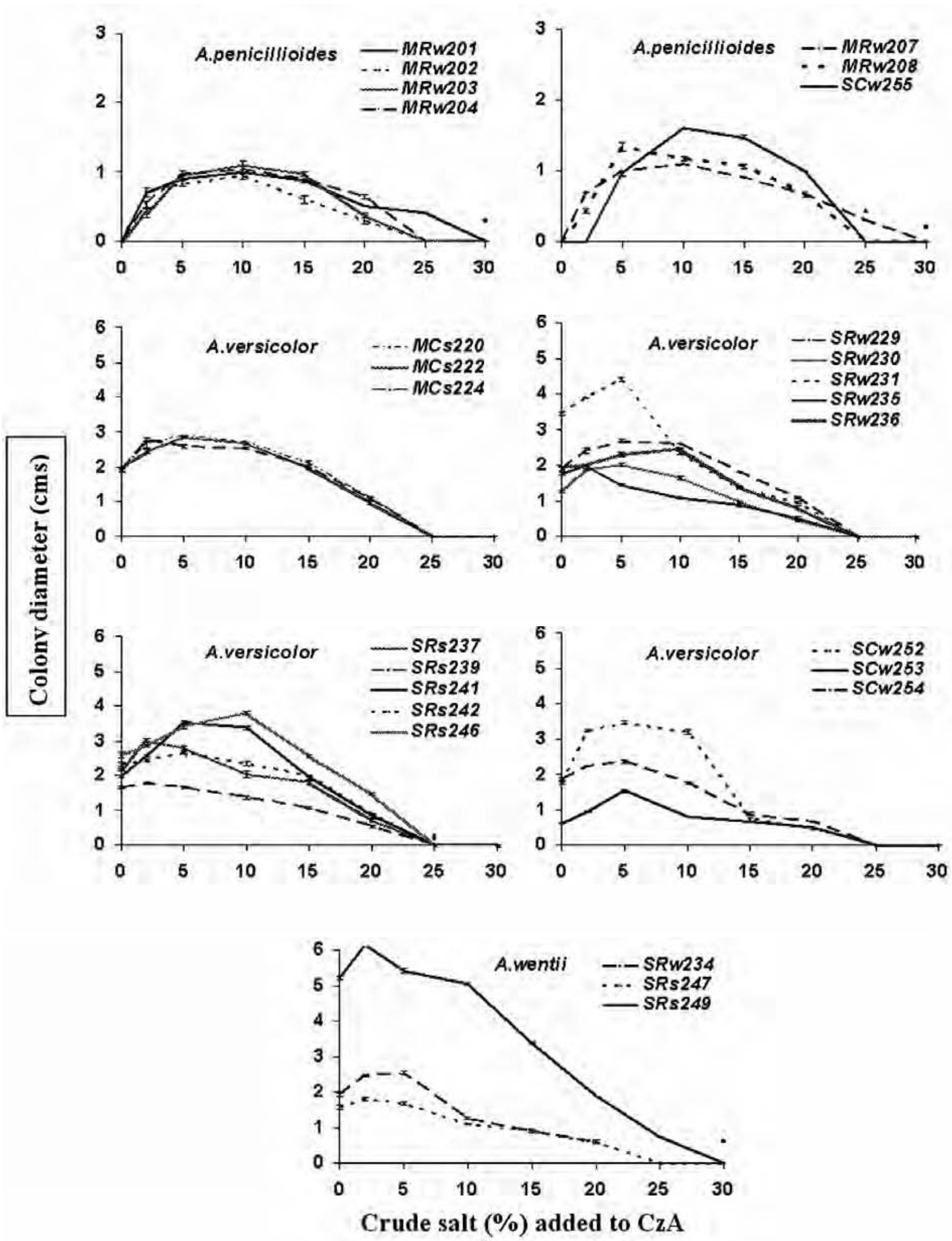

Fig. 1—Salt tolerance of isolates recorded after 7d incubation; unconnected points indicate delayed growth at the respective salt concentration, recorded after 15d incubation.

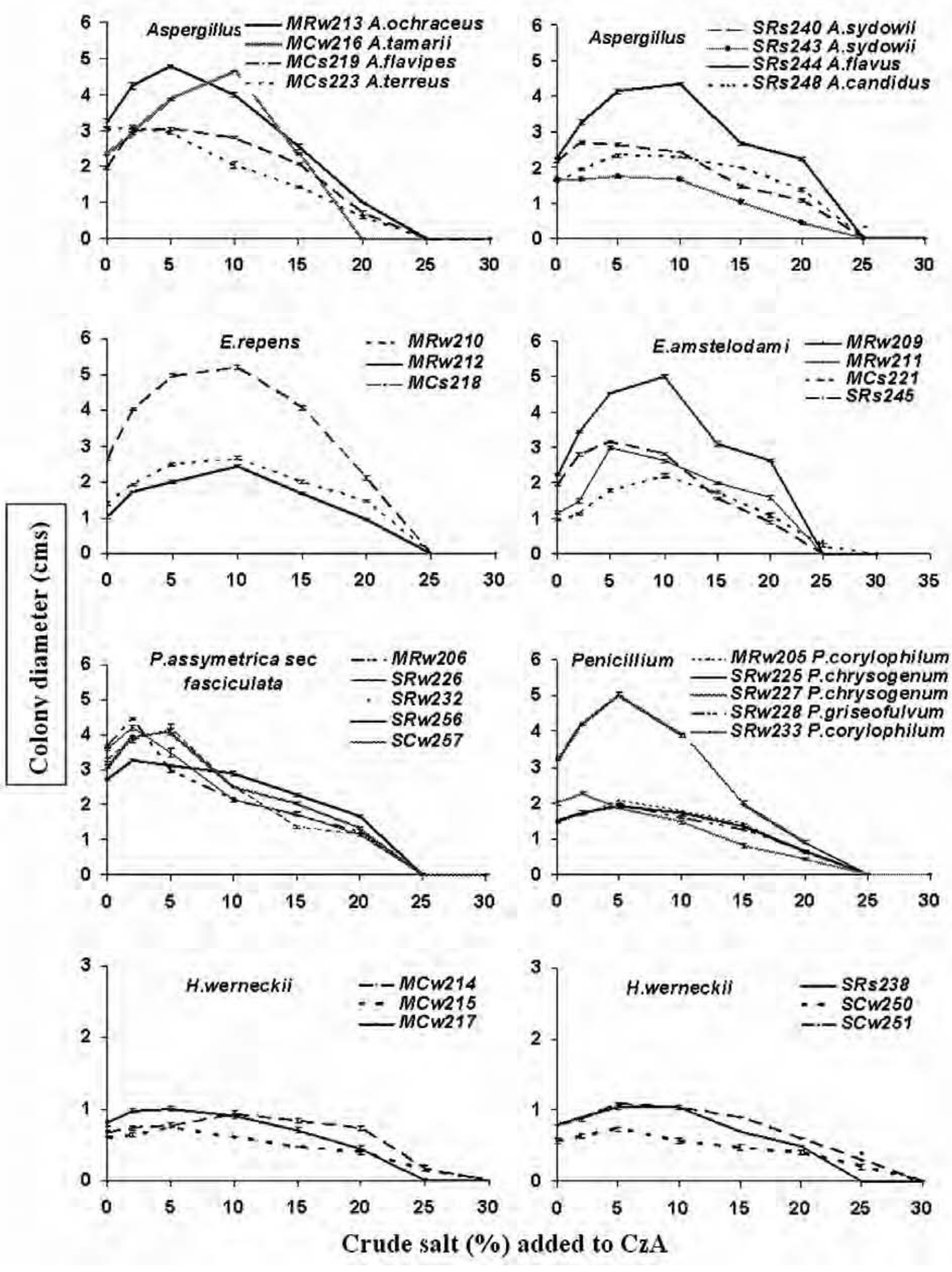

Fig. 1—(cont.). Salt tolerance of isolates recorded after 7d incubation; unconnected points indicate delayed growth at the respective salt concentration, recorded after 15d incubation.

Table 1—Obligate and Facultative Halophiles

Isolate		% Salt level supporting growth*		Halophile
Species	Number	Range	Optimal	
A. penicilloides	MRw201, MRw207	2 -25 (30)	10	Obligate
	MRw202, MRw203, MRw204, MRw208	2-20	10	Obligate
	SCw255	5-20 (25)	10	Obligate
A. candidus	SRs248	0-20 (25)	5	Facultative
A. flavipes	MCs219	0-20	2-5	Facultative
A. flavus	SRs244	0-20	10	Facultative
A. ochraceus	MRw213	0-20	5	Facultative
A. sydowii	SRs240	0-20	2	Facultative
	SRs243	0-20	5	Facultative
A. tamarii	MCw216	0-15	10	Facultative
A. terreus var terreus	MCs223	0-20	2	Facultative
A. versicolor	MCs220, MCs222,	0-20	10	Facultative
	MCs 224	0-20	2	Facultative
	SRw229, SRw230, SRw231, SCw254	0-20	5	Facultative
	SRw235, SRs239	0-20	2	Facultative
	SRw236	0-20	10	Facultative
	SRs237, SRs242	0-20	5	Facultative
	SRs241	0-20 (25)	5	Facultative
	SRs246	0-20 (25)	10	Facultative
	SCw252, SCw253	0-20	5	Facultative
A. wentii	SRw234	0-20	5	Facultative
	SRs247	0-20	2	Facultative
	SRs249	0-25 (30)	2	Facultative
E. amstelodami	MRw211	0-20	5	Facultative
	MCs221	0-25	10	Facultative
	SRs245	0-20 (25)	5	Facultative
E. repens	MRw209, MRw210, MRw212, MCs218	0-20	10	Facultative
P. asymmetrical sec fasciculata	MRw206, SRw226, SCw256	0-20	2	Facultative
	SRw232, SCw257	0-20	5	Facultative
P. chrysogenum	SRw225, SRw227	0-20	5	Facultative
P. corylophilum	MRw205	0-20	5	Facultative
	SRw233	0-20	2	Facultative
P. griseofulvum	SRw228	0-20	5	Facultative
H. werneckii	MCw214	0-25	10	Facultative
	MCw215	0-25	5	Facultative
	MCw217	0-20 (25)	5	Facultative
	SRs238	0-20 (25)	5-10	Facultative
	SCw250, SCw251	0-25	5	Facultative

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

salt concentration. The essential requirement of salt for growth of these isolates was further confirmed by inoculating the isolates on MEA, PDA and SA without salt and with 10% solar salt. No growth was seen on media without addition of solar salt; however, growth was obtained on all media with 10% salt, comparable to that obtained on CzA+10% salt (Fig. 2). The identification of MRw202, *A. penicillioides,* was confirmed by ITS sequence analysis, GenBank Accession No. HQ891824.

The rest of the cultures could grow in the absence of added salt, most of these growing at 0-20% added salt within 7d, with optimal growth at salt concentrations of 2/5/10% as shown below viz. *A. sydowii* SRs240, *A. terreus var terreus* MCs223, *A. versicolor* MCs224, SRw235 and SRs239,

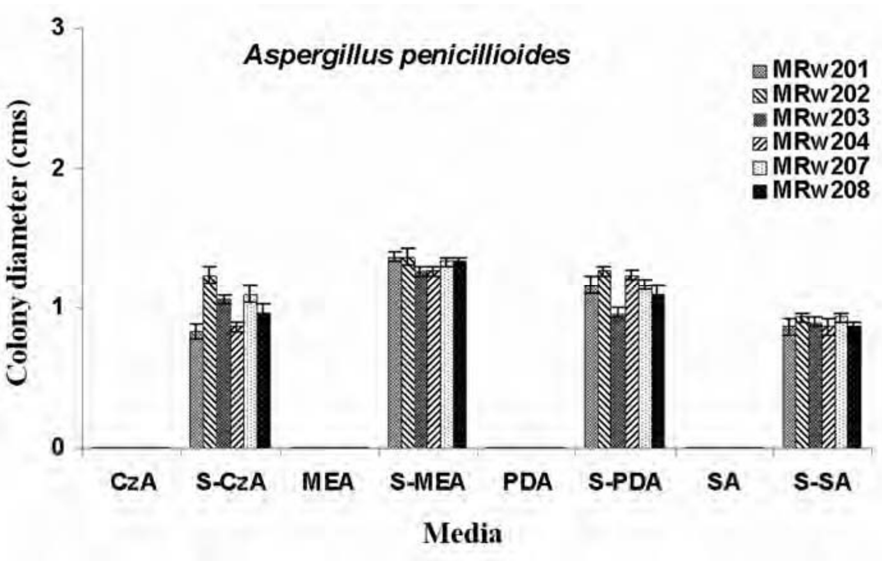

Fig. 2—Growth of *Aspergillus penicillioides* isolates in terms of colony diameter recorded after 7d incubation on CzA, S-CzA, MEA, S-MEA, PDA, S-PDA, SA and S-SA, where S represents 10% solar salt added to the medium.

A. wentii SRs247, *P.asymmetrica sec fasciculata* MRw206, SRw226 and SCw256 and *P. corylophilum* SRw233, optimum growth at 2% salt; *A. flavipes* MCs219, optimum at 2-5% salt; *A. ochraceus* MRw213, *A. sydowii* SRs 243, *A. versicolor* MCs220, MCs222, SRw229, SRw230, SRw231, SRs237, SRs242, SCw252, SCw253 and SCw254, *A. wentii* SRw234, *E. amstelodami* MRw211, *P. asymmetrica sec fasciculata* SRw232 and SCw257, *P. chrysogenum* SRw225 and SRw227, *P. corylophilum* MRw205 and *P. griseofulvum* SRw228, all growing optimally at 5% salt, *A. flavus* SRs244, *A. versicolor* SRw236, *E. amstelodami* MRw209, *E. repens* MRw210, MRw212 and MCs218 with optimal growth at 10% salt. *A. tamarii* MCw216 could grow at 0-15% salt, and optimally at 10% salt.

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

Discussion

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

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

There was a predominance of *Aspergillus* species, with *Penicillium* also in fairly high proportion while the telemorphic form *Eurotium*, as well as the black yeast *Hortaea werneckii*, were obtained in fewer numbers, amongst the present isolates. The predominance of *Aspergillus* and *Penicillium*, corroborates the report of Buchalo[34] that at low water potential the active mycota is dominated by species of these genera and is thus numerically the most common taxa. Cantrell *et al*.[29] and Gunde-Cimerman *et al*.[2] reported the presence of *Cladosporium clodosporioides* in salterns. In keeping with this finding, *Cladosporium* and *Alternaria* have also been isolated from these econiches, on isolation media with comparatively lower salt concentrations, and were found to be halotolerant[35,36]. However, the medium incorporating high solar salt concentration used in the present study did not favour the growth of these dematiaceous fungi. From amongst the diverse isolates obtained from the hypersaline waters of salterns of Slovenia[2,26,27] and of Cabo Rojo[28,29], the species *A. candidus, A. flavus, A. penicillioides, Eurotium, P. chrysogenum* and *H. werneckii* were amongst those also found in the present study.

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

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

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

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

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

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

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

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