Ann Microbiol (2013) 63:887–895
DOI 10.1007/s13213-012-0540-6

ORIGINAL ARTICLE

Identification, phylogenetic analysis and characterization of obligate halophilic fungi isolated from a man-made solar saltern in Phetchaburi province, Thailand

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Received: 27 April 2012 / Accepted: 27 August 2012 / Published online: 22 September 2012

Abstract A total of 17 species from 43 isolates were obtained through serial dilutions of soil samples isolated from one of the man-made solar salterns located in Ban Laem district of Phetchaburi province, Thailand. Soil analysis of the sample revealed high salinity and moisture content, slight alkalinity and low amounts of nitrogen, total organic carbon and organic matter in the habitat. Morphological analysis was performed on all isolates, and molecular identification and phylogenetic analysis were carried out only on the halophilic fungi isolated. Six halophilic fungi, belonging to four species, were identified among the isolates, including five strains of *Aspergillus* genus [*Aspergillus flavus*, *A. gracilis*, *A. penicillioides* (2 strains) and *A. restrictus*]. One species was found to be a yeast, namely, *Sterigmatomyces halophilus*, which was the most frequent isolate found among the halophilic fungi. All other isolates were halotolerant fungi. Characterization of the halophilic fungal isolates showed that they were best adapted to conditions of 10–15 % NaCl (w/v), slight alkalinity (pH 7.0–7.5) and a temperature range of 30–35 °C.

Keywords Extremophiles · Halophilic fungi · Halotolerant fungi · Hypersaline environments

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Introduction

Extremophiles are microorganisms that can survive extreme physical conditions, such as temperature, pressure, pH and salinity. Organisms requiring salt for their growth are known as halophiles (Reed 1986; Madigan et al. 1997). Halophiles can be categorized into five major classes based on the amount of NaCl they require for their growth: (1) non-halophiles, growing in <0.2 M (approx. 1 %) NaCl, (2) mild halophiles, requiring 0.2–0.5 M (approx. 1–3 %) NaCl, (3) moderate halophiles, requiring 0.5–2.5 M (approx. 3–15 %) NaCl, (4) borderline extreme halophiles, requiring 1.5–4.0 M (approx. 9–23 %) NaCl and (5) extreme halophiles, requiring 2.5–5.2 M (approx. 15–32 %) NaCl (Kushner 1993).

Fungi were first reported to be inhabitants of solar salterns nearly a decade ago (Gunde-Cimerman et al. 2000), but very few fungi have been found to inhabit hypersaline environments (Gunde-Cimerman et al. 2009). However, some new species of fungi, as well as those species previously known only as food contaminants, have been isolated from hypersaline environments (Butinar et al. 2005; Zalar et al. 2005).

Hypersaline environments or solar salterns caused by the evaporation of sea water are also called thalassohaline environments (Oren 2002). Due to the evaporation of water in these environments, many minerals precipitate and sodium chloride (NaCl) salinity increases to levels of >300 psu (Gunde-Cimerman et al. 2000; Oren 2002). The salt compositions of hypersaline environments are similar to those of sea water, where sodium and chloride are the most abundant ions and the pH varies from neutral to slightly alkaline (Gostinčar et al. 2011). An example of an extreme hypersaline environment is the solar saltern or crystallization pond (Cantrell et al. 2006).

Many microbial communities have been found in salterns, including Archaea, bacteria, algae, protozoa, eubacteria and fungi (Gunde-Cimerman et al. 2004). As fungi have only

recently been isolated from hypersaline environments, their function in these extreme conditions is still not fully understood (Gunde-Cimerman et al. 2004). The halophilic behavior of fungi isolated from hypersaline environments is different from that of the majority of halophilic prokaryotes. With the exception of a few halophilic fungi, fungi can adopt and grow in a wide salinity range without requiring NaCl for their viability (Plemenitaš et al. 2008). Using the halophilic yeast model, researchers have identified a number of different mechanisms used by halophilic yeast to adapt to various salinities. The most well-known strategy is the presence of compatible solutes within the cellular system (Gostinčar et al. 2011) that do not interfere with regular cellular functioning (Oren 1999). The presence of these solutes helps the fungal cell withstand a wide range of salinity (Ventosa et al. 1998). The study of halophilic fungi contributes to a better understanding of interactions in simple ecosystems occupied by few inhabitants, increases our knowledge of stress responses and can help to identify genes that are able to enhance the functions of yeasts used in industries (Gunde-Cimerman et al. 2009).

In Thailand, a surface area of 3.61 million ha is covered by saline soil. Areas of saline soil area in Thailand are found primarily in two regions, namely, the inland northeast region and along the coastal areas (comprising 0.58 million ha; Ghassemi et al. 1995). Many solar salterns have been constructed for the extraction of NaCl in the coastal belt of Phetchaburi province. It is generally believed in the solar salt industry that microorganisms and microbial products can affect the quantity and quality of salt (Gunde-Cimerman et al. 2009). Physical processes, such as evaporation and deposition of minerals and salt, are actively linked to biological systems which can increase or decrease the production of salt (Javor 2002).

The aim of this study was to investigate the diversity of halophilic fungi in man-made solar salterns in Phetchaburi province, Thailand, and gain an understanding of their characteristics, which are a consequence of external physical factors.

Methodology

Site description

The area studied in this work is located in the Ban Laem district (13°13′14.61″ N, 99°58′32.92″ E), Phetchaburi province, situated near the central part of Thailand (Fig. 1). The area is famous for its sea salt production systems, and numerous man-made solar salterns are present in the area (see Fig. 2). Higher plants are not present near the salterns, but many species of mangroves are present in the nearby areas (Fig. 3). The climate of the area can be categorized into a rainy period (May–November) and a dry period (December–April). Total average rainfall in the rainy and dry periods is about 950 and 85 mm, respectively. The average temperature throughout the year does not change much, with an average minimum and maximum temperature of about 24 and 32 °C, respectively. Salt production through man-made solar salterns is carried out in the dry season.

Soil sampling

Soil sampling was performed from a semi-precipitated, easily accessible man-made solar saltern (Figs. 2, 3) at the end of dry season during the last week of April 2010. The temperature of the sampling site was recorded as 33.4 °C. A part of the soil sample used for fungal diversity was suspended in 15 % NaCl solution (w/v) after sampling, while the remaining soil sample was utilized for soil analysis.

Soil analysis

Soil pH was measured by a pH meter (pH 91; WTW GmbH, Weilheim, Germany). Moisture content was determined by calculating the difference in weight between a fresh soil sample and a soil sample dried in the oven overnight. Soil salinity was measured using a salinity meter (EcoScan salt 6; Thermo Fisher Scientific, Waltham, MA). Organic matter and total organic carbon were measured by the dichromate titration method (Walkley 1947). Total nitrogen was determined using the Kjeldahl digestion method with modifications suggested in previous texts (Bremner and Mulvaney 1982; Buresh et al. 1982) using a digester (Tecator DS-6) and distillation apparatus (Tecator 1002).

Fungal isolation

Fungi were isolated by serial dilution on potato dextrose agar (PDA) supplemented with 15 % of NaCl (w/v). After 7 days of incubation, the colonies obtained were further purified and selected on the basis of morphospecies observations. Halophilic fungi were separated from halotolerant ones by checking and comparing the growth of the isolates on normal PDA medium with no supplemented NaCl.

Morphological study

Morphological studies were performed as described previously (Ellis 1971; Subramanian 1976; Carmichael et al. 1980; Klich and Pitt 1988). Isolates were examined under a stereomicroscope (SZ30; Olympus, Tokyo, Japan) and by fluorescent microscopy (Olympus BX60).

Molecular identification of halophilic fungi

Only halophilic fungi were identified by molecular analysis. DNA was isolated using the NucleoSpin® Plant II kit (Macherey-Nagel, Düren, Germany) following the standard protocol for fungal DNA isolation provided with the kit.

Fig. 1 Map of Thailand showing the location of Ban Laem district, in Phetchaburi province, Thailand

Isolated DNA was sent to the mycology lab at the National Science and Technology Development Agency (NSTDA), Pathumthani, Thailand for the retrieval of internal transcribed spacer (ITS) 1–4 sequences. Species and strains were identified, and sequence similarities were obtained by using BLAST tool from NCBI. Phylogenetic trees were reconstructed by the neighbor joining method using Editseq (DNASTAR Lasergene; DNASTAR, Madison, WI), Clustal X ver. 1.81 (Thompson et al. 1997) and MEGA ver. 4.0.2 (Tamura et al. 2007).

Fig. 2 Man-made solar saltern where sampling was performed, located at Ban Laem district, Phetchaburi province, Thailand

Characterization of halophilic fungi

The effects of all physical factors (halotolerance, pH, temperature) studied in the characterization analyses were determined by the dry weight estimation method suggested in previous studies (Sutton and Starzyk 1972; Phillips and Gordon 1989; El-Kassas and Khairy 2009). A time period of 15 days was allowed for the growth of all halophilic fungi maintained in an incubator shaker (Innova 44R) at 100 rpm. Strains were cultured in 50 ml of PD broth (PDB) (modified as required). The pH was maintained at 7.0 except in the pH factor study, the temperature was maintained at 35 °C except

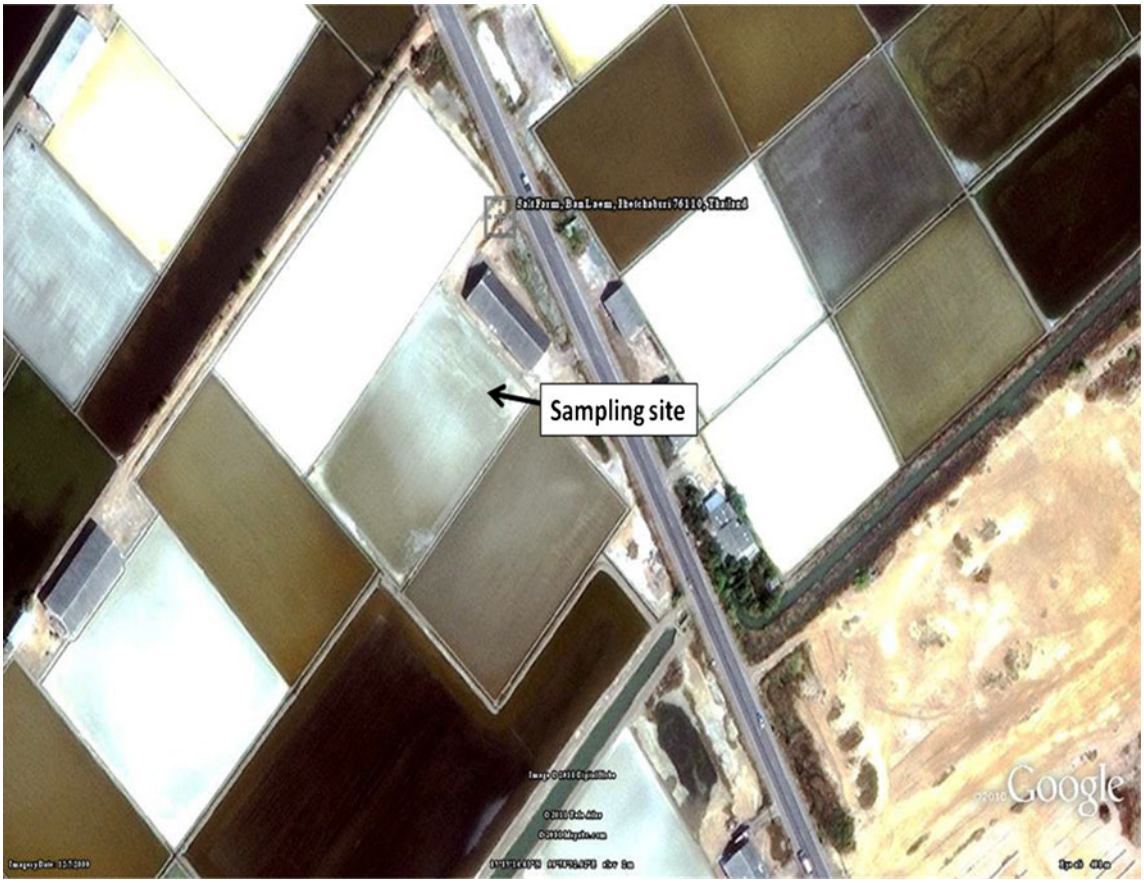

Fig. 3 Google earth Map; showing the sampling site and neighboring location at Ban Laem district, Phetchaburi province, Thailand (© 2010 digital globe; © 2010 google; ©2010 mapabc.com; ©2010 tele atlas)

in the temperature factor study and salinity was maintained at 10 % (w/v) except in the halotolerance test. Three duplicates for each halophilic fungus were studied along with a blank to ensure accuracy. Mean results were expressed in milligrams.

Halotolerance test

The halotolerance test was performed following the procedure adapted by Tresner and Hyes (Tresner and Hayes 1971). Halophilic fungi were incubated in PDB supplemented with 5, 10, 15, 20 and 25 % NaCl (w/v).

pH

The method used by El-Said and Saleem (2008) was adopted for the pH study. The initial pH of the PDB for all halophilic fungal strains tested was adjusted to pH of 6.5, 7.0, 7.5 and 8.0, respectively, by 0.1 N NaOH or 0.1 N HCl.

Temperature

The temperature study was done following the procedure described in Astoreca et al. (2007). Halophilic fungi were incubated to temperatures of 25, 30, 35 and 40 °C by adjusting the temperature of the incubator shaker (Innova 44R; New Brunswick Scientific, Edison, NJ).

Results and discussion

Soil analysis

The results of the soil analysis carried out with sample are summarized in Table 1. The color of the soil ranged from green to gray. Dried soil appeared take the form of clay. The pH value showed that the soil samples were mildly alkaline, as

Table 1 Soil analysis results on samples collected at Ban Laem district in Phetchaburi province, Thailand

Parameter	Test result
pH	7.4
Salinity	13.11 %
Moisture content	27.16 %
Nitrogen content	0.108 %
Total organic carbon	0.39 %
Organic matter	0.67 %

Table 2 Species information on the halophiles screened in this study

Codes	Species	Strain	Accession number	Number of isolates
01 H	*Aspergillus flavus*	Strain 6830	HQ693703	1
02 H	*Aspergillus gracilis*	Isolate NRRL 4962	EF652045	2
03 H	*Aspergillus penicillioides*(1)	Strain ATCC 16910	AY373862	2
04 H	*Aspergillus penicillioides*(2)	Strain SCSGAF0031	JN850993	1
05 H	*Aspergillus restrictus*	Strain ATCC 16912	AY373864	1
06 H	*Sterigmatomyces halophilus*	Strain CBS 4609	AF444556	3

interpreted according to Bruce and Rayment (1982). As such, the samples showed the typical hypersaline condition of the site, as the pH of hypersaline environments ranges from neutral to slightly alkaline (Gostinčar et al. 2011). Moisture content in the soil was high because the precipitation process was under way in the man-made solar saltern for the production of salt.

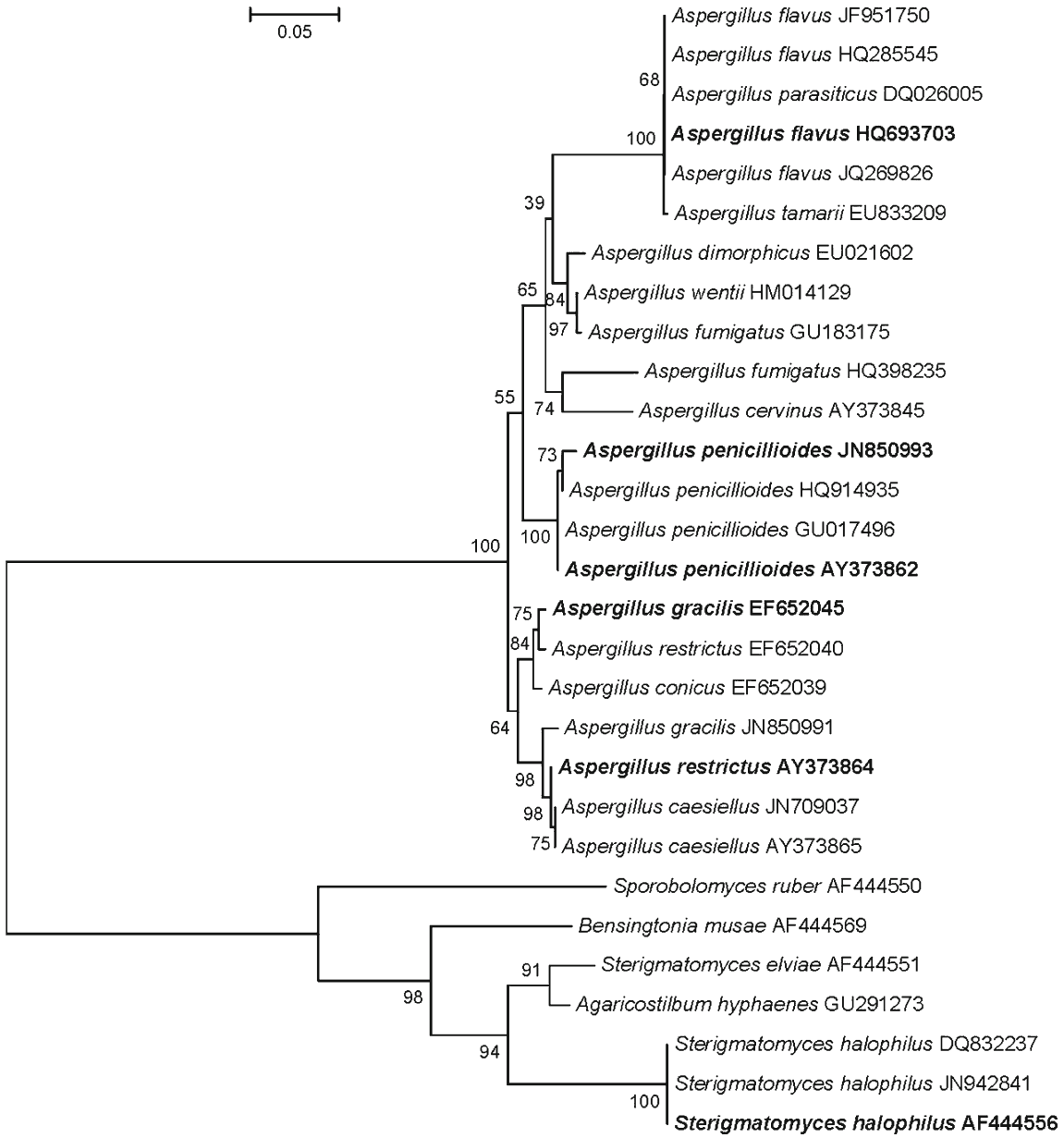

Fig. 4 Phylogenetic tree. The genus and species are followed by their accession number. Isolated fungi in the tree are given in *bold*

Soil salinity was high, but was in the range of the salt concentration present in the man-made salterns, which ranges from 3 to 30 % (Gunde-Cimerman et al. 2000). Nitrogen content, total organic carbon and organic matter were found to be lower than those reported to be normal in the literature (Bruce and Rayment 1982; Hach 1992; Hazelton and Murphy 2007).

Fungal isolation and morphological study

A total of 43 fungi were isolated on PDA supplemented with 15 % NaCl (w/v). All isolated fungi were able to grow on 15 % NaCl, which proves that they are all halotolerant. The initial quantity of NaCl for culture isolation was selected on the basis of the soil salinity test. Seventeen fungi were selected on the morphospecies basis in terms of color of colony/spore, growth of fungi, spore formation and spore size. Halophilic fungi were identified as being halotolerant based on their growth on PDA with no salt. The results revealed that halophilic fungi compromised nearly 24 % of the total halophilic/halotolerant fungal diversity in the man-made solar saltern under study (Table 2).*Sterigmatomyces halophilus* was the most frequent halophilic fungal isolate in the soil samples and was also the only yeast identified (Table 2).

Molecular identification and phylogenetic analysis

The halophilic fungal strains isolated in the soil samples were subjected to molecular analysis. When the ITS 1–4 sequences of these isolates were compared using the BLAST analysis tool on NCBI, the samples with code numbers 01 H, 02 H, 03 H, 04 H, 05 H and 06 H were found to be *Aspergillus flavus* strain 6830, *A. gracilis* isolate NRRL 4962, *A. penicillioides* strain ATCC 16910, *A. penicillioides* strain SCSGAF0031, *A. restrictus* strain ATCC 16912 and *Sterigmatomyces halophilus* strain CBS 4609, with similarity values of 100, 100, 99, 99, 97 and 99 % respectively (Table 2). Morphological observations were rechecked for more accuracy.

The phylogenetic tree, which presents the interrelationships among these halophiles (Fig. 4), was constructed using the neighbor joining method, using Editseq (DNASTAR Lasergene), Clustal X ver. 1.81 (Thompson et al. 1997) and MEGA ver. 4.0.2 (Tamura et al. 2007). Phylogenetic analysis shows branching of two basic nodes, one ascending for genus *Aspergillus* and the other descending for yeasts. The tree shows more detail for genus *Aspergillus*, as 83 % of the isolated halophilic fungal species belonged to genus *Aspergillus*. None of the six halophilic fungi under study shared a direct (immediate) common ancestor or the same direct common clade. Even the two strains from the same species of *A. penicillioides* do not share the immediate ancestor as *A. penicillioides* strain SCSGAF0031 is found to have a separate new clade. *A. gracilis* isolate NRRL 4962 and *A. restrictus* strain ATCC 16912 are found to have a common node at bootstrap value of 64 % (Fig. 4). Characterization steps further highlighted the relationship between *A. gracilis* isolate NRRL 4962 and *A. restrictus* strain ATCC 16912.

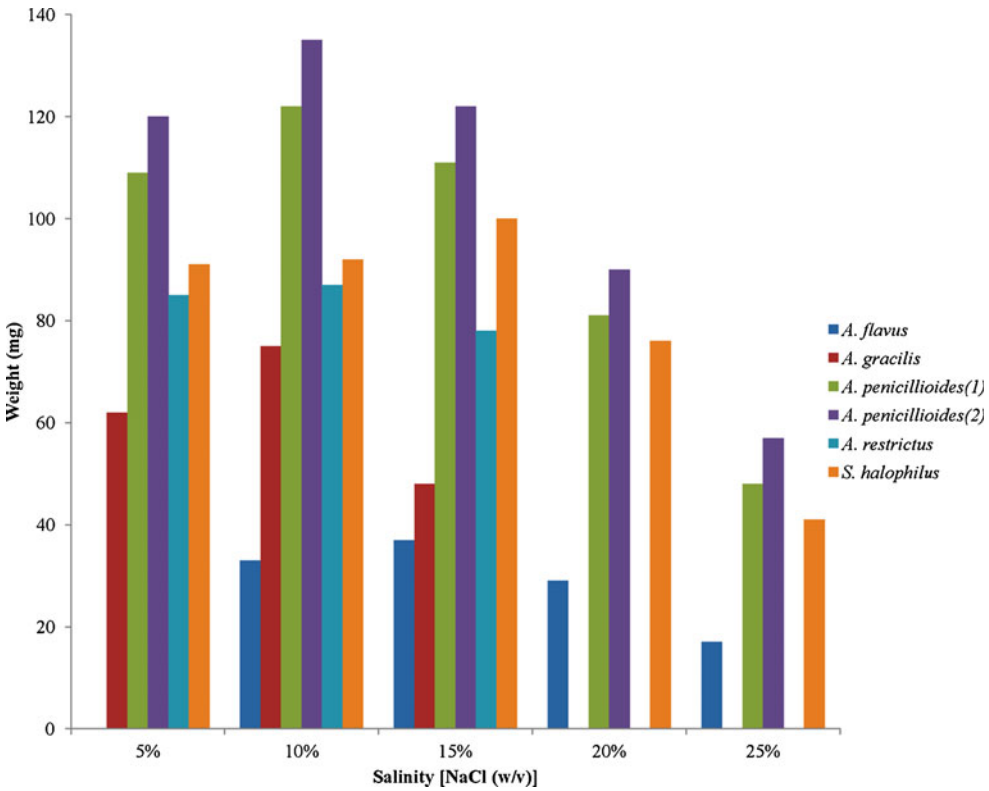

Fig. 5 Halotolerance test results based on final weight after 15 days of growth as a function of salt concentration (5–25 %); pH and temperature were held constant

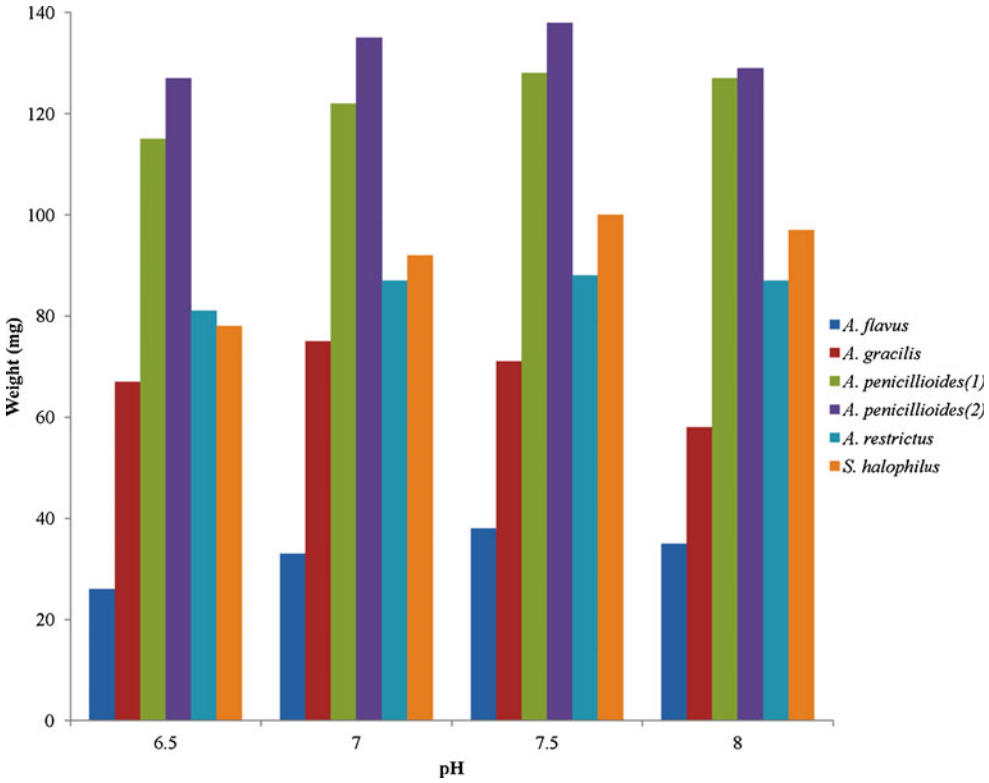

Fig. 6 pH test results based on final weight after 15 days of growth as a function of pH (6.5–8); salt concentration and temperature were held constant

Characterization of halophilic fungi

Three factors, namely, halotolerance, pH and temperature, were studied to gain a better understanding of the growth behavior (weight in mg), survival and adaptation of the halophilic fungal isolates. The results are shown in Figs. 5, 6 and 7. Both strains of *Aspergillus penicillioides* produced the maximum of dry weight under all conditions studied,

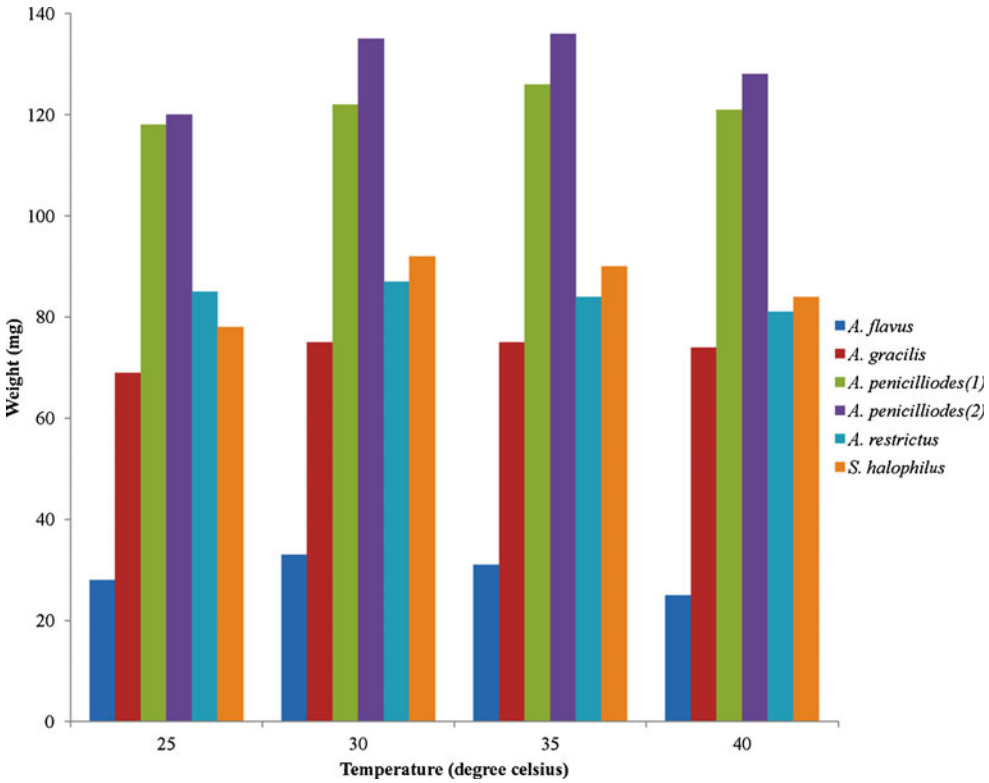

Fig. 7 Temperature test results based on final weight after 15 days of growth as a function of temperature (25–40 °C); salt concentration and pH were held constant

followed by *Sterigmatomyces halophilus*; *Aspergillus flavus* showed the least growth in terms of dry weight.

In the halotolerance test, all species, with the exception of *Aspergillus flavus*, were able to grow in medium containing 5 % NaCl (w/v), with *A. gracilis* and *A. restrictus* only able to grow in medium containing no more than 15 % NaCl (w/v). Most of the species were found to grow well in 10 and 15 % NaCl (w/v). Salt levels of 20 and 25 % NaCl (w/v) decreased the growth of all those fungal strains that were able to grow in that concentration of salt (Fig. 5).

All halophilic fungi, except for *A. gracilis*, were found to have optimum growth in a slightly alkaline pH of 7.5; *A. gracilis* showed the best growth at pH 7.0. This growth behavior from halophilic fungi shows their adaptation to their natural habitat, as based on the soil analysis results, the pH of the soil samples was also near 7.5 (Table 1). Exposure to a higher alkalinity (8.0) and acidity (6.5) decreased the growth of all of the halophilic fungi studied (Fig. 6).

Most of the species were found to grow best at 30 °C, but showed little difference in dry weight at 35 °C. This behavior also indicates that these strains have all adapted to the natural habitat of solar salterns: the average temperature of the area throughout the year is >30 °C. Lower (25 °C) or higher (40 °C) temperatures did not favor good growth of the halophilic fungal strains tested (Fig. 7).

Conclusion

Even though it is difficult to find halophilic fungi in nature, our recent screening study revealed six strains. The harsh habitat conditions in man-made solar salterns, as shown in the soil analysis, and the adaptation to those conditions prove that our isolated halophilic fungi are extremophiles. Our collective study of phylogenetics and characteristics indicates the evolution and interconnections amongst our halophilic fungal isolates. An in-depth analysis of each halophilic fungus in our study will provide more information about the adaptations and responses of individual fungal strains to their respective environment. These halophilic fungi are also potential sources of important biological compounds and are currently being studied further.

Acknowledgment We thank all of the people who helped us in this study, especially the lab staff of Fungal Diversity Lab KMUTT, Thailand.

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