Diversity of Halotolerant Fungi in Solar Salterns from Chanthaburi Province

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Abstract

This study aims to identify the species diversity of halotolerant fungi in soil and water in the solar saltern area of Chanthaburi Province during the rainy and dry seasons from October 2017 to July 2018. The fungi were identified and isolated into axenic cultures. A total of 30 taxa were discovered. Based on morphological characteristics, the fungal genera were classified into a wide variety of species, including *Aspergillus* (5), *Cladosporium* (4), and *Penicillium* (4). The common species were *Cladosporium* sp.3 (14.61%), *Cladosporium* sp.4 (11.24%), *Bipolaris* sp. (6.74%), *Cladosporium* sp.1 (6.74%), *Acremonium* sp. (5.62%), and *Aspergillus* sp.4 (5.62%). The highest number of fungal species found were 28 species from the July 2018 sample. A similar fungal species community was detected in samples collected in October 2017, January 2018, and April 2018, which showed Sorensen's index of similarity (S) between 0.34-0.58. Nevertheless, it differed from the samples collected in July 2018 (S = 0.12-0.24). To determine that the amount of fungus found in July was highest, but the community's similarity to the community in other months was least, indicating that most of it was probably not true solar salted fungus. However, fungi are carried from nearby ecosystems, so they are more likely to be halotolerant fungi than halophilic fungi. Screening of fungi for various applications based on enzymes and bioactive compounds production, strain A5-1 found at 340 psu of the soil salinity is the best gelatinase, protease, lipase, and cellulase producer and showed antibacterial activity against *Vibrio parahaemolyticus* SV006, *Staphylococcus aureus* M371, and *Streptococcus agalactiae* F107 by plate screening method following strain A58-2. Molecular identification indicated that strains A5-1 and A58-2 are *Penicillium decumbens* and *Aspergillus terreus* that could be used in biotechnological applications.

Keywords : halotolerant fungi, diversity, solar-salterns

1. Introduction

Solar saltern, marine saltern, is a type of man-made ecosystem with an environment that is different from other ecosystems. Several environmental factors affect micro-organisms, including fungi that live in this area, such as salinity, pH, temperature, light intensity, oxygen level, and nutrient concentrations (Cantrell *et al*., 2006; Nayak *et al*., 2012). Salinity in salt ranges from 3% to over 30% (Oren, 2002), which is very high compared to normal seawater salinity. Therefore, it is classified as a hypersaline and thallassohaline-type extreme environment (Oren, 2002; Ventosa and Arahal, 2009). The water in the saltern is acid-base, in a middle and low bass level. (Oren, 2002) Biological factors that directly affect organic matter and nutrients, especially nitrogen and phosphorus (Gunde-Cimerman *et al*., 2009).

High salinity levels found in solar saltern make it difficult for life to exist there. Nevertheless, a wide variety of microorganisms that are present in these hostile settings are essential to the ecology and operation of solar saltern. The significance of microorganisms in solar saltern is examined in this essay, with particular attention paid to their function in the cycling of nutrients, ecological balance, and possible uses in a variety of contexts. Salt pan microorganisms include archaea, algae, bacteria, and fungi that have evolved specifically to survive in salty environments. These extremophiles have evolved particular defense mechanisms, like modified cell architectures and suitable solute synthesis, to resist high salt concentrations. Microorganisms are the cornerstone of this complex ecosystem, and solar saltern displays astounding biodiversity despite its harsh surroundings. The extremophilic nature of these microorganisms suggests that they may harbor unique biochemical pathways, leading to the discovery of novel therapeutic compounds. Understanding the importance of microorganisms in solar saltern is essential for the conservation and sustainable management of these ecosystems. Human activities, such as salt extraction and habitat alteration, can disrupt the delicate balance of microorganisms in solar saltern. Conservation efforts should consider the role of microorganisms in maintaining ecological health and biodiversity within these unique environments. Microorganisms in solar saltern represent resilient and adaptable life forms that have successfully carved out a niche in extreme environments. Their importance extends beyond their immediate habitat, influencing nutrient cycling, and ecological balance, and offering potential applications in biotechnology and medicine. Recognizing the significance of microorganisms in solar saltern is critical for both scientific understanding and the conservation of these remarkable ecosystems.

The diversity of fungi in solar saltern, as well as in other high salinity ecosystems such as the Dead Sea, might be less than in other areas. However, the halophile and halotolerant fungus can be found due to the physiological adaptation of the fungus in this area (Oren, 2002). The first halophile fungi reported from the Dead Sea was *Gymnascella marismortui* (Buchalo *et al*., 1998). At present, the study of fungal diversity in this very high salinity saltern is very few when compared to other fungus groups. A study of fungus in Thailand saltern is still very few too (Ali *et al*., 2013). The first report of saltern fungus from various countries in less than 20 years ago (Gunde-Cimerman *et al*., 2009). Notable halotolerant species in saltern were *Cladosporium cladosporioides*, *Aspergillus*, *Penicillium* (Cantrell *et al*., 2006; Cantrell and Baez-Félix, 2013). In Thailand, Ali *et al*. (2013) reported on the morphological and molecular genetics classification of fungi in Saltern, Phetchaburi Province. Among these, 4 species (6 breeds) are halophile fungus. At present, halophile fungi are getting more attention because they can produce biological polymers and bioactive compounds such as beta-carotene, ectoine, biosurfactants, exopolysaccharides, and compatible solutes. Including enzymes such as amylase, cellulase, and lipase that are active in the salty area (Margesin and Schinner, 2001; Ali *et al*., 2014).

Thailand's saltern area has a total area of approximately 81,485 rai (32,207.50 Acre), with Phetchaburi Province being the most area at 47.0%, followed by Samut Sakhon Province (43.1%), Samut Songkhram Province (7.7%). For salterns in the East, there is a salt-producing area that produces approximately 10% of all salt production in the country, with Chachoengsao Province

having a production area of approximately 0.2%. Because saltern fungus is one of the potential fungi for commercial use, the objective of this research is to study the biodiversity of saltern fungus in Chanthaburi Province. To serve as a database of biodiversity with natural habitats and for a salt fungus conservation plan in the future.

2. Materials and Methods

2.1 Sample sites

Soil and water samples were collected 4times from Thamai Subdistrict, Thamai District, Chanthaburi Province (12°37'18.59"N101°58'5.03"E) in rainy and dry seasons, which is October 2017, January 2018, April 2018, and July 2018. October 2017 and July 2018 represent the rainy season, which is not a salt farming season. The water in every saltern has the same physical characteristics. The other 2 times were collected in January 2018 and April 2018 in the dry season, which is the beginning and the end of the salt farming season. Samples were collected from the saltern with different physical characteristics such as watercolor, turbidity, and suspended sediment in the solar saltern (Fig.1). Three blocks were selected for each time and repeated 3 times. By collecting from the edge of the block, along with measuring the pH and water temperature in the sample block. Soil samples were collected by using a 5 cm diameter pipe to collect soil from the surface level to a depth of 15 cm and water samples were collected by using a 500 ml sample flask, collected at a depth of 5 cm from the surface. Soil and water samples were kept in plastic bags, and immersed in ice at about 4 °C. Brought the sample back to the laboratory to study the diversity of the fungus and measured salinity by diluting samples before measuring with a salinometer.

Figure 1 Physical characteristics of the salterns during the salt farming season, samples collection period

2.2 Isolation of fungi from soil samples

Diluted soil samples with sterile distilled water by using the tenfold dilution technique (Gunasekaran, 1995) until the soil solution was at the appropriate dilution level (10^{-1}-10^{-3}). Used pipette on soil suspension at every dilution level of 0.1 ml, then spread on potato dextrose agar (PDA) mixed

with 30 psu salinity seawater (PDA_{sw}). Three replicates of each dilution were made. The plates were transferred to a 28 ºC incubator and left for 3 days. Thereafter, each plate was screened daily and any developing fungal growth was recorded (colony type and number per plate). A small piece of hypha from a single colony was removed and transferred to new PDAsw and kept as a pure fungus for further classification.

2.3 Isolation of fungi from water samples

Each 100 ml water sample was filtered through a 0.45 µm HA-type bacterial filter (Millipore, USA). The filter was placed in PDAsw and incubated at 28 ºC for 3 days. Observed the growth and characteristics of the fungal colony. When the mycelium is visible, count the number of colonies on the petri dish. Separated the fungus by selecting only a single colony with different characteristics. Then kept as a pure fungus for further classification.

2.4 Identification of fungi by morphology

The morphology of each isolated fungi growing on the culture medium plates and slide culture was determined by observing the colony morphology, and the methods of spore production. Each fungal species was photographed order both a stereo and compound microscope at the relative magnifications (i.e. macroscopic and microscopic levels) to provide a record. Each fungus was identified using recognized keys. (Thom and Raper, 1945; Raper and Thom, 1949; Gilman, 1957; Raper and Fennel, 1965; Booth, 1971; Ellis, 1971; Barnett and Hunter, 2006).

2.5 Data analysis

The number of species and the frequencies of occurrence of each species were recorded and calculated. Frequency of occurrence was calculated as the number of samples on which a particular fungal species occurred divided by the number of samples examined, expressed as a percentage for each species.

To compare the similarity of the species composition between sites with different months, Sorensen's index of similarity (S) was applied (Magurran, 2004). The index was calculated with the formula: S = 2c/a+b where a = total number of species in site 1, b = total number of species in site 2, and c = number of species common to both sites. Similarity is expressed with values between 0 (no similarity) and 1 (absolute similarity) (Tsui and Hyde, 2004).

Divided the fungus founding frequency into 4 levels by using the following criteria. Frequencies ranging from 7.50% are considered very often. Frequencies ranging from 5.50% are considered often. Frequencies ranging from 3.50% are considered common. Frequencies ranging from 1.50% are considered few. Less than 1.50%, are classified as rare (Gunde-Cimerman *et al*., 2009).

2.6 Plate screening method for enzyme activity

Ninety fungal strain cultures were prepared by cork-borer (Ø =6 mm) and spotted on modified starch agar (1% (w/v) soluble starch, 3% (w/v) gelatin agar), skim milk agar (2.8% (w/v) casein), 1% (w/v) tween-80 agar, and cellulose agar (1% (w/v) carboxymethylcellulose (CMC) sodium salt) mixing sea water (10 psu) for amylase, gelatinase, protease, lipase, and cellulase tests, respectively. After 7 days of incubation at 30 ºC, determine the activity of fungal enzymes to hydrolyze substrates. Diameters

of a fungal colony, clear zones, and a visible precipitate zone were recorded (mm) and calculated as the activity ratio (AR) (AR = Ø clear zone/Ø colony or a visible precipitate zone) (Kasana *et al*., 2008; Ali *et al*., 2014).

2.7 Fungal culture and solvent extraction

Five discs of actively growing fungal colonies prepared by a 6 mm diameter sterile cork borer were added to 100 ml of potato dextrose broth (PDBSW30). After 7 days of incubation at 30 °C, fungal mycelium was harvested by filtration using a Buchner funnel with Whatman™ paper discs. Fifty milliliters of crude were kept for ethyl acetate extraction two times. Removed the ethyl acetate from the crude extract by using a condenser rotary evaporator (Buchi R-210, Japan). The obtained residues were dissolved in a mixing of sterile water, methanol, and dimethyl sulfoxide for two millimeters, and the control without the obtained residues and then kept for antibacterial activity.

2.8 Antibacterial activity of ethyl acetate-extracted fungal crude

Antibacterial activity screening by agar well diffusion technique suggested by the National Committee for Clinical Laboratory Standards (NCCLS, 2003). Centrifugation of bacterial cells in Tryptic soy broth (18 hours) at 6,000 rpm for 15 min, the bacterial cell was adjusted cell density as $1.5x10^6$ cfu/ml was applied on Mueller-Hinton agar by sterile cotton swab three times. Holes of 6 mm diameter were made in the plate using a sterile cock borer. Holes were filled with 80 µl of ethyl acetate-extracted fungal crude. Plates were incubated at 37 °C for 24 hours. The diameter of inhibition zones was recorded in centimeters (cm).

2.9 Morphological of the fungal colony and molecular identification

Fungal plates were checked with pure culture at least once a week and streaked again onto Sabouraud dextrose agar (SDASW30) and incubated again at 30 °C for 1 week (Fig. 3). The genomic DNA of all isolates was extracted by the method of the Macrogen Company (Republic of Korea). Molecular identification of fungal isolates A5-1 using 18S rRNA gene sequences. The 18S rRNA gene was amplified and sequenced using NS1 5’ (GTA GTC ATA TGC TTG TCT C) 3’ and NS24 5’ (TCC GCA GGT TCA CCT ACG GA) 3’ primers. Similarly, molecular identification of fungal isolates A58-2, A69, and A85 using the internal transcribed spacer gene (ITS) was amplified and sequenced using the primer sets ITS5 5’ (TCC GTA GGT GAA CCT GCG G) 3’ and ITS4 5’ (TCC TCC GCT TAT TGA TAT GC) 3’. All amplification reactions were sequenced at Macrogen Company. The nucleotide sequences were compared with the GenBank database using the Basic Local Alignment Search Tool (BLAST + 2.13.0) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the MEGA version 11 alignment search tool.

3. Results and Discussion

3.1 Physical and chemical properties of the sample site

The salinity of each sample site at different times has different values but possibly be in the same direction (Table 1). The average salinity throughout the study ranged from 140-360 psu. In the rainy season (October 2017 and July 2018), the average salinity ranged from 140-220 psu, lower than in the dry season (January 2018 to April 2018), which was at the beginning and end of the salt farming

season. In the dry season, the salinity ranges from 260-360 psu. Soil samples had average salinity throughout the year in the range of 160-340 psu, in the wet and dry seasons, ranging 160-220 psu and 260-340 psu respectively. For the salinity in this study was less than that of the Puerto Rico salterns between 250-600 psu (Cantrell *et al*., 2006). The average pH of the solar saltern's water is slightly alkaline as in normal seawater and the average throughout the year is not different. In the dry season, it is in the range of 7.3-7.6. The rainy season is in the range of 7.4-7.9 The average temperature of the water measured for each time throughout the year is similar at 36 °C - 39 °C.

Table 1 Average salinity and pH of soil and water samples from solar salterns, Chanthaburi Province

Sample/ site station	Average salinity and pH of soil and water samples							
	October 2017		January 2018		April 2018		July 2018	
	Salinity (psu)	pH	Salinity (psu)	pH	Salinity (psu)	pH	Salinity (psu)	pH
Soil								
Site 1	170	-	260	-	340	-	180	-
Site 2	190	-	260	-	300	-	160	-
Site 3	220	-	280	-	290	-	160	-
Water								
Site1	180	7.8	280	7.3	360	7.3	150	7.4
Site 2	190	7.9	270	7.4	340	7.6	140	7.4
Site 3	200	7.8	290	7.4	360	7.5	140	7.4

3.2 Analysis of total fungus in soil and water samples

The fungi from soil and water samples were found in a low number, with differences in each site. Most of the fungus lives in the soil. The study was able to estimate that the number of fungi found in soils in Chanthaburi Province was different from October 2017 to July 2018, between 1.40 and 5.00 cfu/g, and the number in water in the same period was 0.50 and 1.34 cfu/ml. The number of fungi in soil and water in solar salterns, including other highly salty areas such as the Dead Sea, has not been studied or reported. But when comparing the number of fungi in saltern soil and fungi in the studied site, they were found to be very low compared to those previously reported from natural fertile soils, which were approximately $2.5x10^5$- $8.0x10^5$ cfu/g (Vieira and Nahas, 2005; Parveen *et al*., 2011). Because salterns are classified as an extreme area with many restrictions. Therefore, only certain groups of fungi with high adaptability can live (Cantrell and Duval-Pérez, 2013). The result of the sample from July 2018 showed the highest number of fungi in the water, but the lowest soil fungi (Table 2) are probably due to the rainy season with a lot of precipitation, which made the soil quite soft. Some of the salterns fungi that live in the soil are washed up into the water. In the same way, the amount of fungus in the water was found during this time, probably due to the growth of fungus in salterns that have been washed up. Also, the low salinity of the water helps the growth and habitat for other types of fungus, including the plains that are washed up into salterns during this season.

3.3 Species diversity of fungi in solar salterns

After the separation of all the different types of fungi found, cultivated as pure fungus, and stored at the Fishery Division, Faculty of Agriculture and Natural Resources, Rajamangala University of Technology Tawan-ok, a total of 30 taxa were found, and the classifiable fungi were preliminary classified into 9 genera. Three species are coelomycete fungi. Five taxa of fungi cannot be classified as only mycelium is found, but no other structure (sterile). The other taxa cannot be classified at this time. Some of them are fungi that show incomplete reproductive structures (unidentified) (Table 3). However, the taxonomic number of the fungi found in this study is similar to the 43 taxa that Cantrell and Duval-Pérez (2013) reported from microbial mats in salterns in the wet season of Puerto Rico. And similar to the 38 taxa from the Dead Sea, Israel (Kis-Papo *et al*., 2001)

All fungi that can be classified are anamorphs. The genera found with the most diverse members are *Aspergillus*, *Penicillium*, and *Cladosporium* (5, 4, and 4 species, respectively). *Aspergillus* spp. and *Penicillium* spp. are the most reported fungi in salterns and high salinity areas. from various regions around the world (Kis-Papo *et al*., 2001; Cantrell *et al*., 2006, 2011; Cantrell and Duval-Pérez, 2013). In addition, it is likely to be a local fungus, and it has also been shown that these genus fungi may have mechanisms to adapt to many conditions, including extreme salinity conditions as well. Therefore, it can be found everywhere. From the preliminary classification of morphology, it was found that the fungus species in this research were similar to those previously reported. Especially Cladosporium spp. (Cantrell *et al*., 2006, 2011; Cantrell and Duval-Pérez, 2013).

The most frequently found fungus shows that it is a local fungus, which is *Cladosporium* sp.3 (14.61%) and *Cladosporium* sp.4 (11.24%). The commonly found fungi were *Acremonium* sp., *Aspergillus* sp.4, *Bipolaris* sp., *Cladosporium* sp.1, *Cladosporium* sp.2, *Cladosporium* sp.3, and *Cladosporium* sp.4. Rereading studies of the fungus genus in samples of hypersaline and related to the fungus genus found in this study showed that *Acremonium* sp. was found to be the most frequently reported fungus from direct detection in microbial mat specimens by the molecular genetics method. *Cladosporium dominicanum* was the most frequently reported fungus found in microbial mat samples in the Caribbean region (Cantrell and Duval-Pérez, 2013). Fungi in the genus *Cladosporium*, for example, *Cladosporium cladosporioides* and *C. sphaerospermum*, have been reported in water samples with high salinity, both tropical and temperate (Cantrell *et al*., 2006). However, the frequently found Bipolaris sp. has not been reported and may be a specific local fungus.

Table 2 Estimate the numbers of solar saltern fungi found in soil and water samples, Chanthaburi Province

Site sample	Estimate the numbers of solar saltern fungi							
	Soil samples (cfu/g)				Water samples (cfu/g)			
	October 2017	January 2018	April 2018	July 2018	October 2017	January 2018	April 2018	July 2018
1	5.00	1.62	3.20	1.40	1.10	1.24	1.18	1.34
2	2.40	2.80	2.46	2.10	0.80	0.60	0.50	1.00
3	2.12	2.00	1.94	1.90	0.80	0.90	1.02	1.12

Table 3 Frequency of fungi found in soil and water samples from salterns, Chanthaburi Province

No	Taxa	Occurrences of fungi					Frequency (%)
		October 2017	January 2018	April 2018	July 2018	Total number of occurrences	
1	*Acremonium* sp.	1	1	1	2	5	5.62
2	*Aspergillus* sp.1			1		1	1.12
3	*Aspergillus* sp.2	1	1	1		3	3.37
4	*Aspergillus* sp.3	1		1	1	3	3.37
5	*Aspergillus* sp.4	1	2	1	1	5	5.62
6	*Aspergillus* sp.5		2		1	3	3.37
7	*Bipolaris* sp.	1	1	2	2	6	6.74
8	*Cladosporium* sp.1	1	1	2	2	6	6.74
9	*Cladosporium* sp.2	1		1	1	3	3.37
10	*Cladosporium* sp.3	3	2	4	4	13	14.61
11	*Cladosporium* sp.4	3	2	3	2	10	11.24
12	*Curvularia* sp.1	1			2	3	3.37
13	*Curvularia* sp.2	1			2	3	3.37
14	*Curvularia lunata*	1			1	2	2.25
15	*Fusarium* sp.1			1	2	3	3.37
16	*Fusarium oxysporum*	1	1	1		3	3.37
17	*Nigrospora sphaerica*	1			2	3	3.37
18	*Penicillium* sp.1		1			1	1.12
19	*Penicillium* sp.2			1		1	1.12
20	*Penicillium* sp.3	1		1		2	1.12
21	*Penicillium* sp.4			1	1	2	2.25
22	Coelomycete 1				1	1	1.12
23	Coelomycete 2		1			1	1.12
24	Coelomycete 3	1				1	1.12
25	Unidentified 1		1			1	1.12
26	Sterile mycelium 1			1		1	1.12
27	Sterile mycelium 2		1			1	1.12
28	Sterile mycelium 3			1		1	1.12
29	Sterile mycelium 4				1	1	1.12
30	Sterile mycelium 5		1			1	1.12
	total	**20**	**19**	**23**	**28**	**90**	**100**
	Species richness (S)				**30**		

3.4 The abundance and similarity of the community's population

From the data in Table 3, when considering the number of species/taxa of solar saltern fungi separated at different times, it was found that they were not different. During each sample collection period, fungi between 14 and 17 taxa were found. Sorensen's index of similarity of the community at

different times (Table 4) shows that the community that grew in July is less similar to the community collected from samples in other months. The number of fungi in this month was higher than any other month, likely because the water has low salinity due to the heavy rains, which help the growth and habitat of other types of fungi. This includes the plains that have been washed out into the water in the salterns. It has been reported that water salinity is the principal environmental factor affecting the colonization of fungi (Alias and Jones, 2000; Sadaba, 1996; Jones, 2000)

Table 4 The similarity index of the fungi community found in soil and water samples, in Chanthaburi Province at different times

Sampling month	October 2017	January 2018	April 2018	July 2018
October 2017	1.0			
January 2018	0.34	1.0		
April 2018	0.58	0.31	1.0	
July 2018	0.12	0.12	0.24	1.0

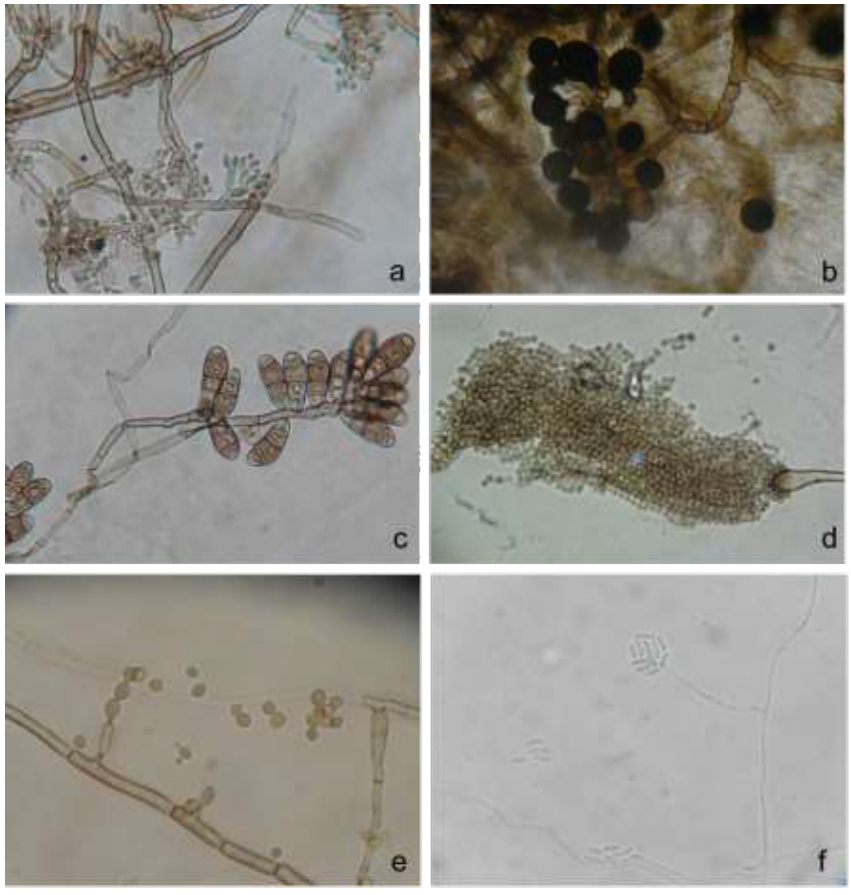

Figure 2 *Cladosporium* sp.3 (a), *Nigrospora sphaerica* (b), *Bipolaris* sp. (c), *Aspergillus* sp.2 (d), *Cladosporium* sp.4 (e), *Acremonium* sp. (f)

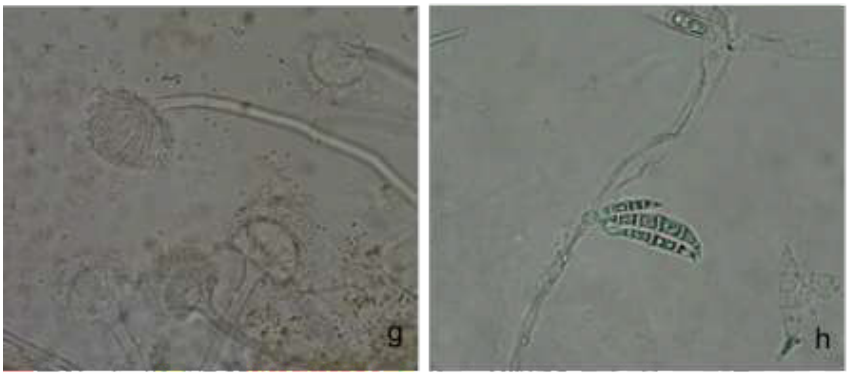

Figure 2 *Aspergillus* sp.3 (g) and *Fusarium* sp.1 (h)

Table 5 Enzyme activities of fungal isolates in media supplemented with specific substrates

Fungal strains code	Enzyme activities				
	Amylase	Gelatinase	Protease	Lipase	Cellulase
A5-1	-	+++	++	++	++
A58-2	-	+	++	++	++
A69	-	-	+	-	++
A85	-	++	-	-	++

Note : AR : activity ratio = (Ø activity/Ø colony), - : no activity, + : AR = 1, ++ : 1 < AR < 1.5, +++ : 1.5 < AR

Table 6 Antibacterial activity of fungal ethyl acetate-crude extract by agar well diffusion method

Bacterial strains code number	Bacterial sources	Ø Inhibition zones ± SD of fungal isolated (cm)			
		A5-1	A58-2	A69	A85
Staphylococcus aureus M371	Cow udder	1.87±0.09	1.77±0.09	1.36±0.11	1.41±0.02
Streptococcus agalactiae F107	*Oreochromis niloticus*	1.46±0.10	1.29±0.02	0.93±0.09	1.16±0.05
Bacillus amyloliquefaciens Y4	Mangrove soil	0.86±0.05	1.3±0.12	0.82±0.08	1.22±0.05
Vibrio parahaemolyticus SV006	*Litopenaeus vannamei*	1.92±0.05	1.29±0.11	1.17±0.06	1.19±0.02

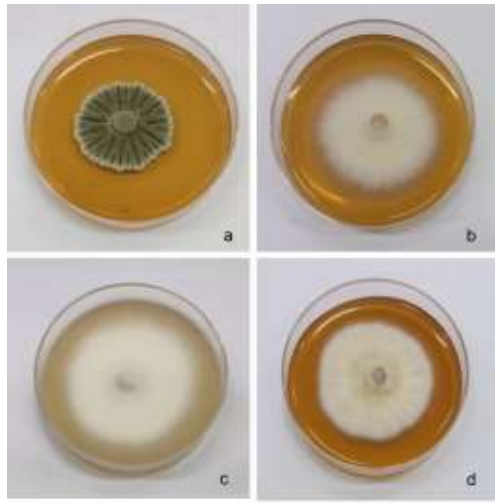

Figure 3 Morphology of fungal colony on Sabouraud dextrose agar mixing sea water (30 psu) for 7 days of incubation time at 30 °C. Fungal isolates code: a = *Penicillium* sp. A5-1, b = *Aspergillus* sp. A58-2, c = *Fusarium* sp. A69 and d = *Aspergillus* sp. A85

Table 7 Molecular identification of fungal isolates

Fungal strains code	Source	Month/Season	Highest salinity (psu)	Identification gene sequences	Primers	Fungal species (% identity)
A5-1	soil	April/summer	340	SSU and 18S rRNA	NS1/NS24	*Penicillium decumbens* (100 %)
A58-2	soil	January/winter	280	ITS	ITS5/ITS4	*Aspergillus terreus* (100 %)
A69	water	July/rainy	140	ITS	ITS5/ITS4	*Fusarium solani* (99.6 %)
A85	soil	April/summer	300	ITS	ITS5/ITS4	*Aspergillus terreus* (99.8 %)

3.5 Enzymes and antibacterial activity and molecular identification

Four strains screened from ninety fungal strains based on enzymatic activity and antibacterial activity displayed optimum growth at 0-1.5% NaCl concentrations (data not shown), but none could tolerate NaCl concentrations above 10%. They do not require salt for growth, but they survived in the high salt concentration range of 140-340 psu in this study. They are classified as solar saltern halotolerant fungi, a potential source of primary and secondary bioactive metabolites that can be used for various industrial processes such as textile, detergent, pharmaceuticals, food, cosmetics, paper, biofuel, and waste management (Margesin and Schinner, 2001; Zheng, *et al*., 2013; Patankar *et al*., 2021).

Strains A5-1, A58-2, A69, and A85 were found in April (340 psu), January (280 psu), July (140 psu), and April (300 psu), respectively. Three strains can survive in a salinity range of 280-340 psu. They can tolerate NaCl concentrations above 2.5 M (approximately 145 psu) and are classified as extremely halotolerant (Edbeib *et al*., 2016; Obeidat, 2017). It can grow quite well even without salt in the medium (data not shown). The unstable salinity is due to seasons and changes in environments.

Therefore, fungi in the solar saltern possess different adaptive mechanisms to protect cells, one mechanism is also capable of producing enzymes, antibiotics, antioxidants, anti-cancer compounds, hydrophobins, biosurfactants, pigments, polysaccharides, and nanoparticles (Patankar *et al*., 2021). In this study, fungi showed the ability to degrade substrates on agar plates such as starch, gelatin, skim milk, tween 80, and carboxymethylcellulose (CMC), as referred to as amylase, gelatinase, protease, lipase, and cellulase activity (Table 5).

In this study, the best gelatinase and cellulase enzyme producer was strain A5-1, which can tolerate the maximum NaCl concentration at 340 psu. It displayed gelatinase activity within an activity ratio (AR) range of AR >1.5. The results of enzyme activity showed the relation with the antibacterial activity in both Gram-negative bacteria and Gram-positive bacteria that were isolated from various sources of bacterial habitat. The diameter of the clear zone showed 1.92±0.05 cm and 1.87±0.09 cm and 1.46±0.10 cm with *Vibrio parahaemolyticus* SV006 isolated from *Litopenaeus vannamei*, *Staphylococcus aureus* M371 isolated from mastitis cow, and *Streptococcus agalactiae* F107 isolated from *Oreochromis niloticus*, respectively. This is the result of low water activity in the medium, which induces the production of bioactive metabolites in halophilic and halotolerant fungi (Sepcic *et al*., 2011) (Table 6).

The fungal strain A5-1 was identified using morphology (Fig. 3) and molecular techniques to amplify SSU rRNA and 18s rRNA gene sequences by using NS1 and NS24 primers. Genetic alignment analysis and comparing sequences with the GenBank database indicated that strain A5-1 is *Penicillium decumbens*. For other strains, using the primer sets ITS5 and ITS4 to amplify the internal transcribed spacer gene (ITS), it was indicated that two isolates belonging to the genus *Aspergillus*, strains A58-2 and A85, are *Aspergillus terreus* and A69 *Fusarium solani*, respectively, and correlated with the study of Kopytina (2019), which reported that the identification of isolated fungi from different geographical regions proved the existence of large amounts of fungi of the genera *Aspergillus*, *Penicillium*, *Alternaria*, and *Cladosporium* (Dendouga and Belhamra, 2022). In consideration of the cellulase activity, all strains can produce cellulase enzyme within an activity ratio (AR) range of $1 < AR < 1.5$. These results were corroborated by the studies of Ali *et al*. (2014), Chamekh *et al*. (2019), and Dendouga and Belharma (2022), who also reported the production of cellulase from different halotolerant *Aspergillus* and *Penicillium* species at a 10% NaCl concentration on solid medium according to the same genus in this experiment (strains A5-1, A58-2, and A85).

On the other hand, almost all of them cannot produce amylase. Except, the fungal strain A69, which isolated from the solar saltern when the water increased and the salinity dropped to 140 psu, the lowest salinity in July of the rainy season. It has the ability to produce cellulase and a little protease activity (AR=1) due to high concentrations of sodium chloride, which can cause osmotic and ionic stress even for halotolerant species. In this study, 1% sodium chloride in the medium may be led to a reduction in water activity, which limited fungal growth and inhibited enzyme production (Ufot *et al*., 2022) and was related to the diameter of the clear zone. The results of antibacterial activity showed 1.36±0.11 cm, 1.17±0.06 cm, and 0.93±0.09 cm with *Staphylococcus aureus* M371, *Vibrio parahaemolyticus* SV006, and *Streptococcus agalactiae* F107, respectively (Table 6).

Besides, *P. decumbens* A5-1 and *F. solani* of A69 showed a slight of the diameter of the clear zone against *B. amyloliquefaciens* Y4 at 0.86±0.05 cm and 0.82±0.08 cm, respectively due to *B. amyloliquefaciens* Y4 being the probiotic strain from mangrove soil that can produce anti-*Aeromonas hydrophila* and anti-*Streptococcus agalactiae* substances (Tongjun and Rakseree, 2021). Further investigations could consider the ability of *P. decumbens* A5-1 to produce enzymes to degrade agro-industrial waste for biofuel production and should apply this strain for applications in medicine, food science, agriculture, and waste management.

4. Conclusion

Our studies presented the species diversity of fungi in solar salterns, in Chanthaburi Province. The number of fungi in July 2018 was the highest, but it had the least resemblance to the other fungus communities in other months, suggesting that the majority of the fungi discovered in this month were likely not true saltern fungi which are classified as halotolerant fungi. After that, we cultivated ninety halotolerant fungi from solar salterns. When testing the ability to produce enzymes and bioactive compounds, this study showed that four dominant isolates can produce enzymes and antibacterial substances against both pathogenic Gram-positive and Gram-negative bacteria. More research should be done on the ability of halophilic fungi to produce extracellular enzymes and antimicrobial compounds. Specifically, the investigation of fungal strains living in salinized environments may lead to novel industrial and biotechnological applications.

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