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***Research Article** (Open Access)*

ISOLATION AND IDENTIFICATION OF DIVERSE MYCOTOXIC FUNGI OF TAPTI AND KHEWRA SALTERNS OF INDIA

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ABSTRACT
Certain salts which are either procured from salterns, or obtained from salt mines, are known to be infested with diverse fungal species. It is seen that some of these species, are identified as potential food spoilers, which may reduce the quality of the food. In this context, the present study has been attempted to identify isolate and study the fungal diversity of salt samples, collected from two different locations, such as Tapti and Khewra rivers in INDIA. Two salt samples i.e., Arabian salt, as it is commonly called is obtained from salterns, located near Tapti river, was collected from the local vendors, in Madhya Pradesh, and Himalayan salt sample was collected personally from the Khewra salt mine present in Jhelum district of Punjab. These samples, thus collected were processed for the study. The diverse fungi, were isolated using standard protocol and species were identified using PCR method, using appropriate primers. Both the salt samples, (Arabian and Himalayan) samples, showed the presence of different fungal species, such as *Aspergillus Cladosporium*. and *Penicillium*. Among, these strains, *Aspergillus*, and *Penicillium*, were identified to produce toxins, and are known to be potential food spoilers. The present investigation, yielded, the presence of nine different taxa of the fungal species, of which certain, species, are identified to degrade the food value as they are mycotoxic in nature. This study, suggests that the salts which are processed at these salterns, seems to be contaminated with fungi, and so may be treated further, with certain antifungal protocols, as employed in the diary industry. These measures, coupled with implementation of better handling practices along with improvised storage facilities, may to certain extent minimize the risk of fungal contamination, before the salts are packaged and marketed .

Keywords: *Salterns, PCR, Tapti, Khewra , Arabian Salt, Himalayan salt, Fungal diversity, Penicilium, Aspergillus, Cladosporium*

INTRODUCTION
The fields of industrial microbiology and solar salt production would appear to be antithetical, as salt is generally regarded as an antimicrobial agent. However, multi pond solar salterns include the full range of salinities from seawater to halite saturation, and they have always been popular environments for studies on halophilic microorganisms. The crystallized ponds, where NaCl saturation is reached, have been of particular interest. These ponds are colored red by a dense community of *Halobacteriaceae*, β-carotene-rich *Dunaliella* cells, and by red species of Bacteria (*Salinibacter*). Recent studies have shown that re crystallized and crystallized ponds also harbor a surprisingly rich diversity and abundance of halophilic and halotolerant fungi (Gunde-Cimerman *et al.*, 2000; Butinar *et al.*, 2005; Zalar *et al.*, 2005, 2007, 2008). It is generally accepted in the solar salt industry that microorganisms and their products in the evaporating ponds can affect both the quality and the quantity of the salt that is eventually produced (Javor, 2002). Physical phenomena such as evaporation and deposition of calcium carbonate, gypsum and salt are closely linked to biological systems, which can aid or harm salt production (Javor, 2002) and thus also contaminate the salt used for food preservation. Although it has long been recognized that

haloarchaea can be introduced into food via solar salt, resulting in the spoilage of heavily salted proteinaceous products (Norton & Grant, 1988; Grant, 2004), the contamination of food with fungi via salt has generally been overlooked. Sea salts have lately seen a surge in popularity (Parks, 2014). Salt is widely perceived a chemically pure and sterile food ingredient; however, sea salts may carry microbial contaminants (Butinar *et al*., 2011). Sea salt was shown to be the source of a mycotoxin-producing mold that spoiled dry-cured meat in a Slovenian production facility (Sonjak *et al.,* 2011). This finding, and the ways in which sea salts are produced and handled, raised the question of the presence of spoilage fungi in sea salts. One reason sea salt may contain viable fungi is the time-honored method by which salt is harvested from seawater by slow evaporation in shallow ponds called salterns. One third of salt for human consumption is produced this way (Butinar *et al*., 2011). Some organisms in saltern ecosystems may persist as viable propagules even when water activity becomes prohibitive to growth. Thus, sea salt may be enriched in microbes that can grow in high salt environments the very conditions relied on as an important principle of food preservation (Butinar *et al.*, 2011). Fungal spoilage may be characterized by highly visible, often pigmented growth, slime, fermented of sugars to form acid, gas or alcohol or odors/flavors. From these studies, it is quite evident, that, fungal species, which might be present, in the salterns, may cause food spoilage, if and when certain food stuffs are treated with fungal infected salt. Therefore, the present study is an attempt to identify and isolate different fungal species, present in the salt samples of Tapti and Khewra salterns of India.

MATERIALS AND METHODS

***Sample I* (Arabian salt): Tapti river- Madhya Pradesh**.

The sample (Arabian salt) was procured from the locals, who collect, process, and market the package as Arabian sea salt.

***Sample II* (Himalayan salt): Khewra salt mine- Khewra, Jhelum district, Punjab.**

This sample was procured directly from the salt mine, travelling to Punjab.

This study is aimed to assess the fast growing, moderately xerophilic filamentous fungi(Jane Irwin 2020 and Rodríguez-Andrade *et al.*, 2019) that is present in these salt samples, which are likely to spoil a range of salted products. Arabian salt is composed of sea water and the Himalayan salt is from an ancient sea salt deposit: this distinction was noted throughout our analysis. The salts varied in their appearance and textures which were reported as follows: The Arabian sea (sample I) salt is a white, crystalline salt with the additive's calcium silicate, dextrose, potassium iodide and sodium bicarbonate. The other one mined salt (Him) (sample II) included was a very coarse, dark pink salt. The fungi were isolated from each of the samples of sea salts by following ITS (Internal Transcribed Region) DNA sequencing method.

Isolation

Fungi that were isolated from each of the samples of sea salts were processed by ITS (internal transcribed region) DNA sequencing method. Each sample was weighed as 10 grams and dissolved in 100 ml of distilled water and slowly the concentration of the sample was increased so that it reaches the salinity point. The salinity point of both the samples was 20 gm in 100 ml of distilled water and immediately filtered through disposable micro funnel filter funnels with super membranes (0.2 µm pore size), under aseptic conditions. As common salt is a refined salt it does not contain a fungus so sterile distilled water without added salt was used as a negative control. Four plates were prepared, two plates for one salt and rest two plates for another salt, each plate consisting of about 30 ml of dicloran glycerol *i.e.,* 3.8 grams of (DG 18) dicloran glycerol in 120 ml of distilled water and 26.4 ml of glycerol was added and dissolved. Then it was slowly poured in the petri plates without air bubbles in it and kept for a week so that the slowly growing xerophilic food spoilage fungi can grow. The filtered particles, including fungi, were trapped in the membranes which were removed and placed filtrate side up on dicloran glycerol (DG 18) enumeration medium supplemented with 30 µg ml -1 tetracycline dissolved in 1 ml of dimethyl sulphoxide (DMSO).This medium was developed to isolate moderately xerophilic food spoilage fungi. Plates were incubated at room temperature, 24 ^{o}C (±2_), for one week. Although the short incubation did not allow for

the detection of slow-growing xenophiles, it was ideal to detect fast-growing fungi more likely to induce spoilage concerns. Fungal colonies that emerged in seven days were counted; all were removed aseptically and sub cultured on DG18 to obtain unique individual isolates, determined by colony morphology and microscopic characteristics.

DNA extraction and PCR amplification

Mycelia harvested from DG18 plates were physically disrupted using a pestle in a 1.5 ml microcentrifuge tube containing sterile 0.5 mm glass beads (Biospec Products). Genomic DNA was extracted using the PrepMan Ultra Sample Preparation Reagent kit (Applied Biosystems by Life Technologies), E.N.Z.A. SP Fungal DNA Mini Kit (Omega Bio-Tek), or DNAs Plant Mini Kit (Qiagen) following recommended protocols. Extracted genomic DNA was stored in elution buffer at _20 _C. DNA from individual isolates was the target of PCR amplification of the internal transcribed spacer (ITS) regions of the rDNA repeat, a widely accepted barcode for fungi (Schoch *et al*., 2012). The forward ITS5 primer (50-GGAGTAAAAGTCGTAACAAGG -30) and reverse ITS4 primer (50 TCCTCCGCTTATTGATATGC-30) (White *et al.,* 1990) were used in 25 mL reactions with Quanta Accu StatTaq DNA polymerase or Sigma-Aldrich REDTaq genomic DNA polymerase with MgCl2. A DNA Engine PTC-200 thermal cycler (MJ Research) was programmed as follows for the RED Taq PCR: initial denaturation 95_C/ 2 min, 26 cycles of 94_C/1 min, 55_C/1min, and 72_C/3 min, and final extension of 72_C/15 min. For the Accu StatTaq PCR: initial denaturation 95_C/2 min, 20 cycles of 94_C/1 min, 55_C/30 s, and 72_C/1 min, 15 cycles of 94_C/30 s, 60_C/30 s, and 72_C/1 min, and final extension of 72_C/10 min

Isolate identification

PCR amplicons were cleaned up using E.N.Z.A. Cycle-Pure (Omega Bio-tek) according to manufacturer instructions and sequenced with a PCR primer using an Applied Biosystems Automated 3730xl DNA analyzer. Isolate sequences were identified with NCBI nucleotide BLAST. Nucleotide sequences were first blasted (Mega blast) against all fungal (taxid: 4751) sequences, excluding uncultured/environmental sample sequences. Because of uncertainty about reputability of the identities of some NCBI accessions, *Aspergillus* and *Penicillium* sequences were identified by blasting against a "gold-standard" ITS barcode database assembled from GenBank accession numbers selected by the International Commission on *Penicillium* and *Aspergillus* (Samson *et al.*, 2014; Visagie *et al.*, 2014). *Cladosporium* isolate identities were inferred to the level of species complex by blasting. Isolate identities were inferred from among BLAST results with a zero E (expect) value, indicating a significant match, by selecting the match with the highest max score, which accounts for quality and length of the alignment.

RESULTS

Fungi in salt samples: Species composition varied among the two different sea salts of shared species mainly in the genera *Aspergillus* and *Cladosporium*. The most abundant genera were *Aspergillus* followed by *Cladosporium,* and *penicillium*, which each contributed multiple species. No *penicillium* species was common among two or more salts. Species composition varied among salts but *Aspergillus* species were found in both the salts. The nine taxa found in both the sea salts were: *Aspergillus tubingenesis, Aspergillus xerophilus, Aspergillus welwitschiae, Aspergillus unguis, Aspergillus niger, Penicillium camemberti, Penicillium oxalicum, Cladosporium herbarum complex, Cladosporium cladosporiodes* complex. Ordination analysis of taxa from two different salts were tested and no significant differences among fungal communities (Anova f=0.424, p =0.523)

Table I: This table shows the comparison between sample I and sample II

Groups	***Count***	***Sum***	***Average***	***Variance***
Column 1	9	111	12.33333333	104.25
Column 2	9	80	8.888888889	147.1111111

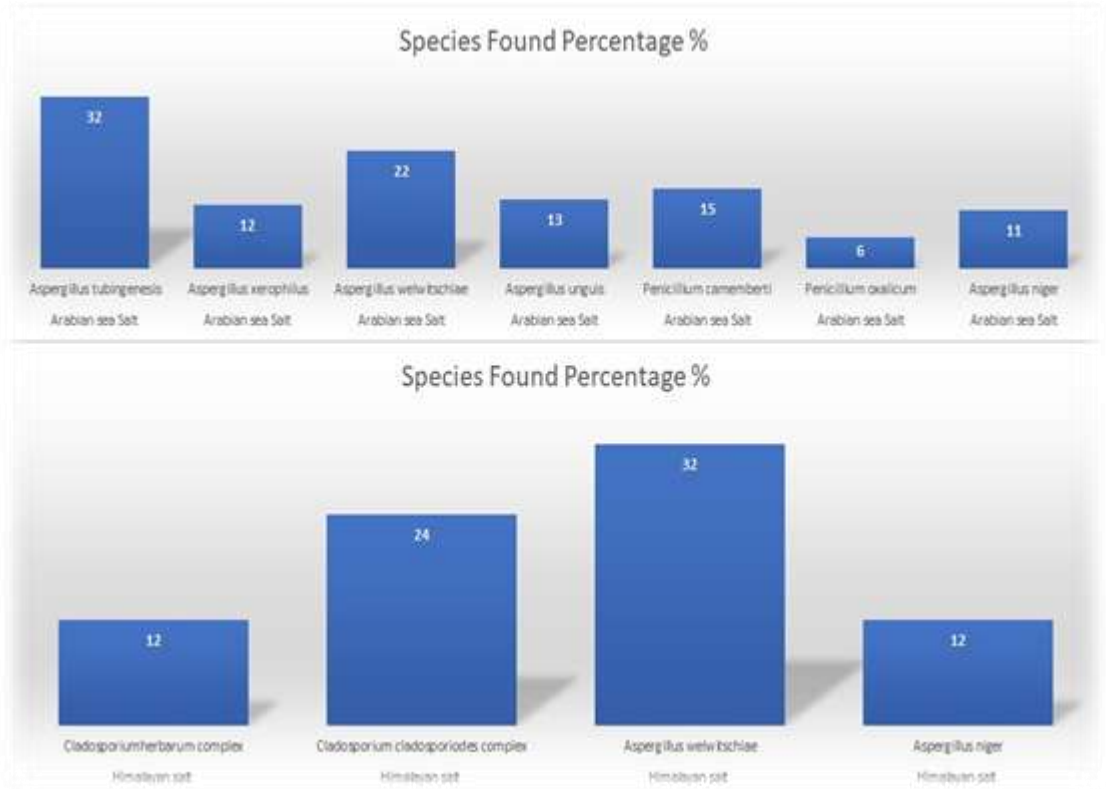

Figure 1: This figure represents the comparison of species with their percentage analysis

DISCUSSION

In the present, study, as anticipated, diverse fungal species, have been identified and species detected include *A. tubingenesis*, *A.welwitschiae* and *A. niger*, and *P.camemberti which* are capable of producing mycotoxins and might lead to food spoilage (Bräse *et al*., 2009). The origins of these sea salt fungi are unclear as they might arise during production directly from the saltern environment, or by introduction of fungi during processing, handling, or packing. Quite a number of fungal species, are known to have significant potential to spoil foods, and introduce mycotoxins or allergens when sea salt is used as a food ingredient. (Bennett and Klich, 2003; Butinar *et al*., 2011; Frisvad *et al*., 2004; Ponsone *et. al*., 2007; Steyn,1969*).* In the context of food spoilage, certain fungal species, such as *Penicillium oxalicum and Scopulariopsis `sphaerospora* are identified as important spoilers of sausages and other low water activity foods, and may be introduced to food through salt (Canel *et al*., 2013; Iacumin *et al*., 2009; Papagianni *et al*., 2007; Wolfe *et al*., 2014). *Penicillium camemberti* was isolated from the mined salt and is used to ripen cheese but some strains that may also be introduced *via* sea salt produced mycotoxins as suggested by Frisvad *et al*., (2004) and Ropars *et al*., (2012). Certain species, of *Cladosporium are known to* cause meat spoilage and sometimes, cause fungal infestations, when sea salts might be used as ingredients in meat preparations (Hammami *et al*., 2014; Mandeel, 2005; Sonjak *et al*., 2011). In this study, fungi were identified using ITS barcoding. Some of the detected fungi have the potential to cause food spoilage when sea salt is used as a food ingredient, especially in products that do not receive heat treatment, as is the case in dry-cured meats in which sea salts were shown to be the source of spoilage mold inoculum (Sonjak *et al*., 2011). The tested salts contained *Aspergilli* species already reported from global saltern communities, including *A.niger*, A. *tubingensis*, as shown in table 1 (Butinar *et al*., 2011). *Aspergillus wilwitcheae* are known in other low water activity environments, including foods and salterns (Gesheva

and Negoita, 2011; Vytrasov *et al*., 2002). Most *Aspergilli* species found in the two salts belong to *Aspergillus* section Nigri, a group of dark-spored species known for their global saltern distribution and ability to spoil low water activity foods (Butinar *et al*., 2011; Pitt and Hocking, 2009).

Table II: Primer designation and sequence with its annealing temperature and PCR product size

S. No.	Sample Name	Species found	Primer designation and sequence	Annealing temperature (^{o}C)	PCR product size (b p)
	Arabian sea salt (Sample I)	*Aspergillus tubingenesis*	5^1_AAC TCC CAA ACC CCT GTG AAC ATA_3^1 5^1_TTT AAC GGC GTC GCC GC_3^1	62	431
		Aspergillus xerophilus	5^1_TTT ACG AGG CGG CGA TGG GT_3^1 5^1_GGC CGT TTA CCT GGC TTC TT_3^1	65	561
		Aspergillus welwitschiae	5^1_GGC CAC TCA AGA GGC GAA AG_3^1 5^1_GTC AGA CCA GAG CAA TGG GC_3^1	64	445
		Aspergillus unguis	5^1_ATG GTG AAC TCG TCC TGG C_3^1 5^1_CCC TTC TTA GCG CAA TCT CG _3^1	62	570
		Penicillium camemberti	5^1_TTT TAG TGG AAC TTC TGA GTA T_3^1 5^1_AGT GCA GGA CTG CAG C_3^1	58	245
		Penicillium oxalicum	5^1_ACA GAT GAC AAG ATT CAG GCA CA_3^1 5^1_TTC TTT GAC ATC TGT TCA ACC CA_3^1	62	280
		Aspergillus niger	5^1_AGG GAC AAT AAG TGC AGA_3^1 5^1_ACT GTG CAC TGT CGC AAG TG_3^1	56	896
	Himalayan salt	*Cladosporium herbarum complex*	5^1_ACA TAC CTT TAT GTT GCC TCG_3^1 5^1_GGA GTA TCA GAC GAC AGC T_3^1	58	315

		Cladosporium cladosporiodes complex	5^1_ACA TAC CAC TTG TTG CCT CG_3^1	58	340
			5^1_CGC CAA TCA ATT TGA GGA ACG_3^1		
		Aspergillus welwitschiae	5^1_GGC CAC TCA AGA GGC GAA AG_3^1 5^1_GTC AGA CCA GAG CAA TGG GC_3^1	64	445
		Aspergillus niger	5^1_AGG GAC AAT AAG TGC AGA_3^1 5^1_ACT GTG CAC TGT CGC AAG TG_3^1	56	896

Table III: Showing percentage found in species and its standard deviation

S. No.	Sample name	Species found	Percentage %
	Arabian sea (Sample I)	*Aspergillus tubingenesis*	32
		Aspergillus xerophilus	12
		Aspergillus welwitschiae	22
		Aspergillus unguis	13
		Penicillium camemberti	15
		Penicillium oxalicum	6
		Aspergillus niger	11
		Standard deviation	± 10.21029
	Himalayan salt (Sample II)	*Cladosporium herbarum complex*	12
		Cladosporium cladosporiodes complex	24
		Aspergillus welwitschiae	32
		Aspergillus niger	12

The mycotoxic fungal species, detected in the present study, may be due to their cosmopolitan nature of distribution, or these species, may have invaded, due to airborne contaminants as suggested by Butinar *et al*., (2005); Visagie *et al*., (2014). *Aspergillus welwitschiae* was abundant in both salts and may be a previously unidentified saltern fungus. This is the first report of *Cladosporium* species from finished sea salt. *Cladosporium* is a prolific, cosmopolitan genus associated with solar salterns and other low water activity environments, as well as indoor and outdoor air (Butinar *et.al*., 2005; Cantrell *et al*., 2013;Gunde-Cimerman *et al*., 2003, 2000; Schubert *et al*., 2007; Zalar *et al*., 2007). Studies of Crous *et al*., (2015) and Rossman *et al*., (1999) suggest, that, based upon *Calostilbestriispora* and *Septoriella phragmites'* plant pathogenic ecology these fungi, may have been likely introduced from plant populations adjacent to salterns or salt storage facilities. Similarly, saprobes such as *Ulocladiumdauci* and *Aspergilluscaespitosus*, known as fungi of soil and decay communities, seem, to have been introduced from soil contact during harvest. (Runa *et al*., 2009; Samson and Mouchacca, 1975). Many *penicillia*, including *P. oxalicum*, are recognized as locally abundant saltern species that dominate their niche (Butinar *et al*., 2011), may have been introduced during salt storage after harvest. Himalayan salt, the only mined type of salt in this study, yielded the isolate which must have been introduced during processing or storage. In summary it may be said, that the diverse fungal species, which have been identified, in both the salt samples, which may be attributed, to the natural causes, or may be airborne, or contaminated in the process of packaging and storage. As these fungal species, have been identified to be potential food spoilers, caution may be administered when these salts, are used for food preservation. In conclusion, it may be suggested that, stringent safety, packaging and storage protocols, should be in place, in salterns, where the sea salt is being saturated and stored.

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