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Extremophiles are considered a cornucopian source of chemically diverse and often novel metabolites. Determination of their growth and distribution pattern and their chemical nature is therefore vital to fully gauge their existential uniqueness and potential as a fount of novel compounds. Present study deals with growth characterization and chemoprofiling of halophilic fungi inhabiting a hitherto unexplored terrain of Little Rann of Kutch, India. Thirty five morphologically discrete fungi, primarily belonging to phylum Ascomycota, were isolated from the soil, suggestive of the region's low microbial diversity. Calculation of fungal salt tolerance index identified one isolate as a halophile with the remaining being halotolerant. Morphological characteristics and 18S rDNA sequencing established the halophile's identity as *Aspergillus versicolor*. *A. versicolor* was further characterized for its salt tolerance potential and degree of halophily. The fungus grew optimally at 7.5% NaCl, thereby classifying it as a moderate halophile. Macro-morphology of the fungus corresponded well with its micro-morphology. At 0% and 10% NaCl, fungal specimen exhibited mycelial distortions, loss of cytoplasm and sparse conidiation; healthy growth features were observed at 7.5% NaCl. For chemoprofiling, *A. versicolor* was cultured on Czapek Dox broth and Malt Extract broth and the crude extract was analysed by liquid chromatography mass spectrometry (LC-MS). A comparative chemoprofile analysis determined Czapek Dox broth to be a better choice for profiling of this particular strain as it exhibited higher compound diversity. The rich chemical contour of *A. versicolor* suggests the fungus to be a potential source of novel bioactive molecules of pharmaceutical significance.

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ABSTRACT

Extremophiles are considered a cornucopian source of chemically diverse and often novel metabolites. Determination of their growth and distribution pattern and their chemical nature is therefore vital to fully gauge their existential uniqueness and potential as a fount of novel compounds. Present study deals with growth characterization and chemoprofiling of halophilic fungi inhabiting a hitherto unexplored terrain of Little Rann of Kutch, India. Thirty five morphologically discrete fungi, primarily belonging to phylum Ascomycota, were isolated from the soil, suggestive of the region's low microbial diversity. Calculation of fungal salt tolerance index identified one isolate as a halophile with the remaining being halotolerant. Morphological characteristics and 18S rDNA sequencing established the halophile's identity as *Aspergillus versicolor*. *A. versicolor* was further characterized for its salt tolerance potential and degree of halophily. The fungus grew optimally at 7.5% NaCl, thereby classifying it as a moderate halophile. Macro-morphology of the fungus corresponded well with its micro-morphology. At 0% and 10% NaCl, fungal specimen exhibited mycelial distortions, loss of cytoplasm and sparse conidiation; healthy growth features were observed at 7.5% NaCl. For chemoprofiling, *A. versicolor* was cultured on Czapek Dox broth and Malt Extract broth and the crude extract was analysed by liquid chromatography mass spectrometry (LC-MS). A comparative chemoprofile analysis determined Czapek Dox broth to be a better choice for profiling of this particular strain as it exhibited higher compound diversity. The rich chemical contour of *A. versicolor* suggests the fungus to be a potential source of novel bioactive molecules of pharmaceutical significance.

INTRODUCTION

The kingdom fungi encompass an enormous diversity of microorganisms, approximately 1.5 million fungal species, which inhabit almost all known ecological niches (Hawksworth 2001). Survival in such diverse habitats has necessitated biochemical evolution and the development of complex metabolic pathways that has resulted in production of a diverse array of primary and secondary metabolites. Primary metabolites are produced by all living cells and are required for normal growth and functioning of cellular processes. Secondary metabolites are secreted only by a few members of the species, often a defining characteristic of the organism synthesizing those (Samson et al. 2014). Specifically, these are low molecular weight compounds, the secretions of which are linked to the organism's morphological differentiation process, and are synthesized as families of related compounds (Zain 2013). Although secondary metabolites are not essential for the organism's survival, yet their genes being maintained consistently suggests that these compounds do provide critical benefits to the producer (Frisvad 2005). Some of their documented roles are tolerance against abiotic and biotic stresses, metal transportation, as sex hormones, differentiation effectors, agents of symbiosis between microbes and plants, nematodes, insects and higher animals (Wang et al. 1995; Demain and Fang 2000); many other roles are yet to be discovered (Omura et al. 2001; Bentley 2002; Zain 2013).

A fungus' chemoprofile is often a reflection of its genetic and biochemical makeup (Thrane et al. 2007). This property has consequently been employed in fungal chemotaxonomy, particularly for fungal genera such as the *Penicillium*, *Aspergillus* and *Fusarium* and their perfect states, since these fungi produce consistent profile of secondary metabolites (Samson et al. 2014). A fungus' chemoprofile also depends on the physicochemical nature of its habitat. For instance, fungi growing under low water index (a_w) produce a class of compounds known as polyamines that act

as osmolytes (Laura and Jose 2014). Chemoprofile of fungi isolated from extreme ecological niches such as salt marshes and solar salterns are subjects of much interest as such fungi are expected to exhibit novel metabolic pathways and metabolites that are characteristic of that particular species or strain.

Hypersaline habitats are characterized by high salt concentrations, low water index (a_w), high light intensity, low oxygen concentrations, neutral pH and believed to be inhabited mostly by algae, protozoa, Archaea and bacteria. Halophilic fungi, a term coined for those that require some salinity for optimal growth, had previously been associated solely with foods treated with salt or sugar for preservation. Only at the onset of 21st century did researchers discover eukaryotes, particularly those belonging to kingdom Fungi, thriving in such extreme habitats (Gunde and Cimmerman 2000). Since then, a number of fungal species and strains inhabiting natural hypersaline environments have been described. Based on cardinal range, these can be categorized as halotolerant, extremely halotolerant and halophilic (Kushner 1978). Studies on fungal communities populating such extreme environments have been largely conducted in and around salterns along the Adriatic coast, the Mediterranean coast, the Red Sea coast, and the Atlantic coast, Gulf of California, Great Salt Lake, Utah and the West Coast of Indian peninsula (Gunde-Cimerman et al. 2005; Nazareth and Gonsalves 2014).

Rann of Kutch is a saline wasteland located between 22˚55” to 24˚35” North latitudes and 70˚30’’ to 71˚45’’ East longitudes, situated in the western state of Gujarat, India. The area is characterized by scant and seasonal rainfall and extreme temperatures. Rapid erosion through fluvial processes and capillary action has advanced the process of desertification by making the area saline with surface salinity ranging from a minimum of 281.6 ppm to a maximum of 12,094 ppm (Gupta and Ansari 2012). Given the harsh environmental conditions, it would be interesting

to study the halophilic mycobiota of the region for their growth and distribution patterns. Chemoprofiling of such fungi would give an invaluable insight into its chemical nature and possible mechanisms to counter abiotic stresses.

Present study was conducted to isolate and identify halophilic fungi inhabiting the soil of Little Rann of Kutch and prepare a chemical portrait of the fungi using LC-MS technique. To the best of authors' knowledge, this is first such attempt aimed at studying halophilic fungi inhabiting the region.

MATERIALS AND METHODS

Soil sampling and analysis

Soil cores were extracted from 5 different sites within an area of 4 sq mi in the southern zone of Little Rann of Kutch (23˚18' N, 71˚27' E). A total of 25 soil samples, 5 samples from each site, were collected in a zigzag pattern from a depth of 6 inches in March, 2014. Each soil sample measuring 0.5 L was extracted using a post-hole auger and collected in properly labeled zip-lock bags. Samples were immediately transported to the Department of Agrochemicals at Indian Agricultural Research Institute, New Delhi for further analysis. A composite sample was prepared by mixing soil cores followed by preparation of 4 sub-samples. Each sub-sample was analyzed for its sand, silt and clay content, pH, electrical conductivity (EC), organic carbon content, total nitrogen and phosphorus content and soluble Na^{+}, Ca^{2+}, Mg^{2+} and K^{+}. The methodology adopted for each has been summarized in Table 1. Field moist soil samples were used for all analytical tests. Soil pH and $EC_{1:1}$ was measured for a soil suspension containing soil: water ratio of 1:1. Temperature was maintained at 25°C during the tests.

Isolation of fungi

Fungal isolation was performed by serial dilution method. 10 g soil was dissolved in 100 mL phosphate buffered saline and shaken in an orbital shaker at 150 rpm for 15 minutes. The suspension was serially diluted to give concentrations of 10^{-1}, 10^{-3} and 10^{-5}. From each dilution, 50 µL was pipetted out and dispensed onto Czapek Yeast Autolysate agar (CYA), Malt Extract agar (MEA) and Potato Dextrose agar (PDA) plates. The experiment was performed in triplicates. Rifampicin (100 µg mL^{-1}) was added to prevent any bacterial growth. The inoculated plates were incubated reverse side up in dark at 25±1˚C for 7 d. Subsequently, each morphologically distinct fungal colony was sub-cultured on the above mentioned media for obtaining pure isolates. The pure isolates were annotated as BBKF1- BBKF35 in ascending order.

Determination of halophily

The isolated fungi were classified as halotolerant and halophilic on the basis of their salt olerance levels, represented as salt tolerance index (T_i). Each isolated fungus was cultured on plates containing CYA and CYA amended with 2.5% - 10% NaCl. Colony diameters were measured on 6^{th} day post inoculation. T_i was calculated as the ratio of colony diameters between CYA and CYA+NaCl (Frisvad, 2005). T_i values were inversely proportional to halophily, i.e. lower the index value higher the halophily. Fungi with index values lower than 1 were considered as halophilic, while those above 1 as halotolerant. Based on the aforementioned study, only one fungus was found to be halophilic, while the remaining isolates were found to be halotolerant.

Morphological and molecular identification

The halophilic fungus was further identified on the basis of colony morphology, microscopic observations and rDNA sequencing.

Morphological identification

Czapek Yeast Autolysate agar, a medium recommended as standard for *Aspergillus* characterization, was used for morphological identification (Samson et al. 2014). The medium was supplemented with 7.5% NaCl. Inoculated plates were incubated in dark at 25±1˚C for 8 d and observed for colony characteristics such as growth rate, extent of sporulation, mycelial color, production of cleistothecia and rear side colony color.

Microscopic structures were observed using Scanning Electron Microscope (SEM). Briefly, a 1 cm^2 section of 8 d old fungal culture plate was used as specimen. Sample was immersed in 2.5% glutaraldehyde and kept overnight at 4˚C. The tissue was rinsed thrice with 0.1 M phosphate buffer and subsequently immersed in 0.5% Osmium Tetroxide solution followed by washing in 0.1 M phosphate buffer. For dehydration, the tissue was transferred sequentially to 30%, 50%, 70%, 80%, 90%, 95%, and 100% alcohol. The sample was transferred into a 1:2 solution of hexamethyldisilazane (HMDS): 100% ethanol (EtOH) and kept for 20 min. Fresh solution of 2:1 HMDS: EtOH was added and further transferred into 100% HMDS. Each step was repeated twice for 20 min. After drying overnight, the sample was placed on a stub and sputter coated with gold-palladium followed by mounting on a Carl Zeiss EVO18 Scanning Electron Microscope. The specimen was observed under low vacuum mode for features such as shape of conidial heads, seriation, morphology and ornamentation (Raper and Fennell 1965; Samson et al. 2014).

Molecular identification

Molecular identification based on 18S rDNA sequencing was done to confirm the identity of the isolate (White et al. 1990; Diaz et al. 2012).

Culture preparation and DNA extraction: Fungal biomass for DNA extraction was prepared by inoculating 100 mL Czapek Dox broth with agar plug from a 10 d old culture. The broth was incubated in an incubator cum shaker for 8 d at 25±1˚C and 70 rpm. For obtaining genomic DNA, fungal mycelium of the halophilic fungal isolate (BBKF4) was harvested, washed thoroughly with distilled water and air dried. In a chilled mortar and pestle, 1 g biomass was homogenized in 3.5 mL CTAB buffer and 100 µL β-mercaptoethanol. The homogenate was collected in Eppendorf tubes, incubated in a water bath at 65˚C for 45 min and allowed to cool to room temperature. Equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added and mixed gently for 10 min. The suspension was centrifuged at 7000 rpm for 20 min at 25˚C and the supernatant was divided into three equal aliquots. 2/3rd volume of chilled isoamyl alcohol was added to each and mixed gently. The suspension was centrifuged at 8000 rpm for 15 min at 4˚C. The pellet was collected and washed with 70% ethanol and air dried. It was then dissolved in 500 µL TE buffer and stored at -20˚C till further use.

Primers and PCR Amplification: Two universal fungal primers, (ITS 1, 5'-TCC GTA GGT GAA CCT GCG G-3'; ITS 4, 5'-TCC TCC GCT TAT TGA TAT G-3'), described by White et al. (1990) were used for amplification of the ITS 1 and ITS 2 regions and the intervening 5.8S gene. Primers were synthesized by Sigma Aldrich Chemicals Pvt. Ltd. Bangalore. The PCR reaction mixture consisted of the following (Diaz et al. 2012): 5x Proof buffer (10 µL), dNTP mix (1 µL), Forward primer (10 µM, 2.5 µL), Reverse primer (10 mM, 2.5 µL), DNA template 50 ng, and DNA polymerase 0.5 µL. Final volume was made up to 50 µL with sterile water. Template DNA was amplified using the protocol proposed by White et al. (1990). Initial denaturation 94˚C, 60 s; denaturation 94˚C, 30 s; annealing 55˚C, 30 s; extension 72˚C, 90 s. The steps were repeated for 35 cycles followed by final extension at 72˚C for 5 min. PCR amplicon

quality was assessed. The PCR product was mixed with loading buffer and loaded onto 1% agarose gel stained with ethidium bromide. 1 kb ladder was used for size reference.

DNA Sequencing and analysis: The PCR amplicon was sequenced by Gujarat State Biotechnology Mission, India. The sequences were then used to perform individual nucleotide-nucleotide searches with the BLASTn algorithm.

Determination of growth pattern of the halophilic fungi and the impact of salinity on fungal morphology

Growth pattern of halophilic fungus at different NaCl concentrations and incubation period was elucidated to determine optimum salinity level, the corresponding degree of halophily, and growth rate. Fungus was grown in CYA plates supplemented with 0-10% NaCl and incubated at 25±1°C. Radius of the fungal colony growing at each salt concentration was measured 2, 4, 6 and 8 d post inoculation. A curve of growth against different time intervals was plotted. Radial growth rate (K_r) was calculated as $K_r = (R_1 - R_0)/ (t_1 - t_0)$, where R_0 and R_1 are colony radii at time t_0 = day 0 and t_1 = day 8 (Reeslev and Kjoller 1995). 3 replicate plates were included for each salt treatment. Based on growth characteristics at different salinities, the degree of halophily was determined. The fungus was classified as being slightly halophilic (optimum growth at 1.2 – 3% NaCl), moderately halophilic (optimum growth at 3 – 14.6%) or extremely halophilic (optimum growth at 14.6% and above) (Kushner 1978).

Effect of salinity on morphological features of the halophilic fungus was assessed. The fungus was grown on plates containing CYA without NaCl and CYA+7.5% NaCl. Colony characteristics were studied and the specimen was observed under scanning electron microscope for fungal topography.

Cultivation and extraction of fungal cultures

Mycelia of *A. versicolor* cultivated on Malt Extract broth (MEB) and Czapek Dox broth (CDB) was used for chemoprofiling and determining the influence of medium composition on the fungus' metabolite profile. Agar plug from 10 d old plate culture of the isolate was inoculated in 100 mL each of CDB and MEB in 250 mL Erlenmeyer flasks. Each medium was supplemented with 7.5% NaCl and the cultures were allowed to grow for 10 d in dark at room temperature. Fungal crude extracts were prepared by method proposed by VanderMolen et al. (2013) with slight modifications. Post 10 d incubation, mycelial mats were harvested from each medium, shredded and suspended in 100 mL 1:1 chloroform: methanol ($CHCl_3$:CH_3OH). Extraction was done overnight in a rotary shaker at 100 rpm and later filtered using 0.45 micron vacuum filter. To the filtrate, 90 mL chloroform and 100 mL distilled water was added and stirred for 1 h. The mixture was then transferred to a separatory funnel. Bttom organic layer was collected and evaporated to dryness in a rotavapor. To the dried organic extract, 100 mL 1:1 acetonitrile CH_3CN: MeOH and 100 mL hexane was added and stirred for 1 h. The solution was subsequently transferred to a separatory funnel and the bottom organic layer was collected and evaporated to dryness. The extract was stored at -20˚C till further analysis.

Analysis of fungal metabolite profiles by liquid chromatography-mass spectrometry (LC-MS)

Extracts of *A. versicolor* cultured on each of the two medium were examined by LC-MS. Analyses were performed using a Waters Acquity UPLC system utilizing a BEH C18 column (Waters; 50 mm× 2.1 mm i.d., 1.7 µm). The mobile phase consisted of CH_3CN and 0.1% formic acid-H_2O, increasing linearly from 2% CH_3CN at the time of injection to 90% CH_3CN at 15 min. The flow rate was 0.3 mL min^{-1} and column temperature was 35˚C. The UPLC system was

coupled to a Thermo Scientific LTQ Orbitrap XL equipped with electrospray ionization (ESI) source. Crude extracts were dissolved in 100% MeOH at a concentration of 2.0 mg mL^{-1}; 6.0 µL were injected, and the peak areas of each metabolite measured. Metlin Database was used for the tentative identification of the fungal metabolites from the m/z values obtained.

RESULTS

Soil characteristics

Table 1 summarizes the physical and chemical characteristics of the soil. The soil had high EC value indicating high salinity. The N, C and P contents were found to be low, suggesting low nutrient content of the soil.

Isolation of fungi

86 soil fungi were isolated. Based on colony characteristics and digital microscopy, 35 distinct fungi were identified. These belonged to genus *Fusarium* (11), *Penicillium* (10), *Aspergillus* (8), *Curvularia* (2), *Rhizopus* (2) and *Dreschelera* (2) (Fig. 1). The isolates were designated as BBKF1 – BBKF35 in ascending order.

Determination of halophily

Based on tolerance index values, halophily of 35 fungi were determined. BBKF 4 was established as a halophile. The remaining isolates were halotolerant as they did not require NaCl for optimal growth. Isolate BBKF4 was selected for further identification and characterization studies.

Morphological identification

Growth characteristics on different media, conidial pigmentation, mycelia and rear side colony color were determined 8 d post inoculation. Thick mycelium with velvety texture and greyish green conidia suggested that the isolate BBKF4 belonged to genus *Aspergillus* (Table 2, Fig. 2 A-B). Scanning electron microscopy studies suggested the isolate could be *Aspergillus versicolor* (Table 3, Fig. 2 C-D).

Molecular identification

ITS 1- 5.8S- ITS 2 region of the rDNA was sequenced for molecular identification. Based on sequence homology and phylogenetic tree analysis, BBKF4 was identified as *Aspergillus versicolor*. The sequence has been submitted in NCBI GenBank (Accession no. KT164811) as *A. versicolor* KR87.

Growth pattern of halophilic fungi and impact of salinity on fungal morphology

A. versicolor KR87 was cultured on CYA having 2.5%, 5%, 7.5% and 10% salinity. Colony radius was measured and a curve of growth against salinity at regular time intervals was plotted to determine the optimal salinity and growth rate (Fig. 3). Based on colony radius on 8th d, it was established that *A. versicolor* KR87 grew optimally at 7.5% NaCl concentration. The growth of *A. versicolor* KR87 followed a linear trend. Radial growth rate was maximum at 7.5% salinity (6.96±0.07 mm d^{-1}), closely followed by 5% salinity (6.42±0.2 mm d^{-1}). Growth rate was least at 0% (3.33±0.06 mm d^{-1}) and 10% (3.45±0.07 mm d^{-1}) salinity. The fungus could therefore be considered as moderately halophilic (Kushner 1978).

To assess the impact of salinity on fungal morphology, *A. versicolor* KR87 was cultivated on CYA and CYA+7.5% NaCl. Remarkable differences in colony and micromorphology were observed. At 7.5% NaCl concentration, mycelial mat was thick with vibrant greyish green

pigmentation and showed characteristic radial grooves with profuse conidiophores. At 0% salinity, mycelia were thin and poorly developed. Microscopic observations revealed deformed hyphae and poorly developed conidial heads. As equated to the fungal morphology at 7.5% NaCl, fungal mycelia at 0% NaCl showed severe distortions indicating loss of cytoplasm (Fig. 4).

Liquid chromatography mass spectrometric analysis (LC-MS)

Crude extracts of *A. versicolor* KR87 grown on two different media were analyzed by LC-MS. LC-MS chromatograms displayed significantly different profiles for each growth medium (Fig. 5). Growth on CDB yielded 23 different metabolites belonging to 13 different compound classes. Majority of metabolites belonged to alkaloids (19%), amines (14%) and terpenes (9%) (Fig. 5A). When grown on MEB, the fungus revealed a thinner metabolite profile with 15 compounds belonging to 9 different compound classes (Fig. 5B). The metabolites mostly belonged to alkaloids, terpenes and flavonoid groups (20% each). Metabolites of certain compound classes such as polyketides, alkaloids, terpenes, amines and amino acids occurred in both the metabolite profiles (Fig. 6). Some of the important metabolites and their bioactivities have been summarized in Table 4.

DISCUSSION

Little Rann of Kutch is a unique saline- inundated wasteland that is parched by high temperatures in summers and flooded by tidal waters and seasonal rivers during monsoon. Evaporation of water in summers results in a monotonous flat saline surface composed of halite and gypsum crystals. These geomorphic features render the soil of Little Rann of Kutch high in salinity and low in nutrient content. Present study was conducted in the southern zone of Little

Rann of Kutch. In any soil sampling and analytical study due consideration should be given to the methodology adopted. Sampling methodology, sample area and design, instruments used, soil depth, soil volume collected and soil moisture content at the time of sampling are a few factors significantly influencing the measured parameters. Soil collected from the study region was analyzed for its chemical properties. Field moist soil samples were used as they are considered the most valid in terms of the existing soil-biological environment and the variability between in vivo and in vitro soil data is negligible (Jackson 1973). At a pH of 6.63±0.3, the soil was practically neutral. $EC_{1:1}$ value of the soil (8.61±0.28) classifies it as being highly saline. Low N, P and organic C contents indicated the soil's poor nutrient status. Various methods are available for soil chemical analysis. These methods vary among each other in terms of ease of performance, sensitivity, specificity and rapidness. For example, the Walkely- Black method adopted in this study is better suited for determining organic C content as it rules out extraneous sources of organic carbon such as graphite and charcoal, thereby ruling out any false measurement. Kjeldahl method, though laborious, still remains the most sensitive method of N determination. Chlorostannous-reduced molybdophosphoric blue color method is the most sensitive method of P estimation and therefore most suitable for infertile soil samples. Flame emission spectrophotometric method is advantageous over conventional gravimetric and titrimetric methods in terms of accuracy, simplicity and rapidity (Jackson 1973).

Identification based on colony morphology and microscopic studies revealed that the majority of isolates belonged to genus *Fusarium, Penicillium* and *Aspergillus*. Microbial diversity of the region was found to be low, in accordance with the studies conducted in other hypersaline habitats around the world. (Borut 1960; Ranzoni 1968; Salama et al. 1971; Khodair et al. 1991; Gunde-Cimmerman et al. 2000; Grishkan et al. 2003; Evans et al. 2013). Tolerance index of

fungi revealed the isolates as being predominantly halotolerant, with only *Aspergillus versicolor* KR87 being a halophile. Further growth characterization based on radial growth rate (K_r) confirmed *A. versicolor* KR87 as a moderate halophile. Impact of salinity on fungal morphology was evaluated. Distorted hyphae, poorly developed mycelium and low density of conidial heads were the characteristics of *A. versicolor* cultivated on a salt free medium, suggesting the fungus to be an obligate halophile. Certain metabolites such as polyamines play a pivotal role in fungal growth, development and differentiation processes (Laura et al. 2012; Laura and Jose 2014). In obligate halophiles, the pathway for polyamine metabolism is activated only in the presence of osmotic stress, which in this case, is triggered by high salinity. Lack of osmotic stress in a salt free medium might therefore lead to non-activation of polyamine metabolism pathway, ultimately resulting in poor fungal growth. Species of *Aspergillus* have usually been found to be halotolerant to varying degrees, irrespective of their source. Aspergilli are one of the most common fungal species and have been reported regularly from regions of extreme salinity (Buchalo et al. 1999; Molitoris et al. 2000; Kis-Papo et al. 2003), non-saline soils and house dust samples collected from around the world (Visagie et al., 2014). The outcomes of the present study support important observations made in earlier reports; prevalence of fungi in saline habitats that are halotolerant rather than halophilic (Hujslova et al. 2010; Nayak et al. 2012); halotolerant and halophilic traits are intrinsic characters of a particular species, rather than an adaptation in response to salt stress (Frisvad 2005); hypersaline environments do not harbor a characteristic fungal community of specialized taxa. It can hence be said that the fungi inhabiting hypersaline soils of Little Rann of Kutch have limited diversity, are mostly halotolerant and exhibit cosmopolitan nature.

Frisvad (2005) proposed that extrolites are produced by a species irrespective of its habitat. Further, metabolites are often produced in highest diversity and amounts at up to 5% NaCl concentration, beyond which there is a steady decline in metabolite profile. The metabolite profile of *A. versicolor* KR87 displayed chemically diverse compounds. A comparative analysis of the metabolites secreted by *A. versicolor* cultivated in CDB and MEB gave significantly different profiles. Fungus grown in CDB medium showed a greater chemical diversity with metabolites belonging to over 13 compound groups as compared to that of MEB which exhibited 9 compound groups. Polyketides, alkaloids, terpenes, amines and amino acids were the compound families shared by either growth medium. An organism's metabolome is greatly dependent on culture conditions such as nutrient source, light, temperature, pH etc. (Calvo et al. 2002; Yin and Keller 2011). Further detailed studies need to be carried out to formulate a medium that is ideal for secondary metabolite production. However, the findings of the current study suggest that CDB is the medium of choice for studying the fungi's metabolome. Earlier works also report CDB as an excellent media for metabolite production (Jennessen et al. 2005).

Ever since the discovery of Penicillin G 1, a secondary metabolite of *Penicillium notatum*, filamentous fungi have been voraciously researched and exploited for their pharmaceutical potential. Systematic drug discovery programs by pharmaceutical companies have led to discovery of compounds that have antibiotic, immunosuppresant, antineoplastic and lipid lowering activity, to name a few (Zain et al. 2013). Many of the metabolites secreted by *A. versicolor* KR87 have potential applications as antimicrobials, anti-cancer agents and lipid lowering drugs. For example, quinazoline acetic acid is an important component of pharmacologically active compounds with anticonvulsant, antibacterial and anti-diabetic activities (Pandeya et al. 1999). Tegafur, a chemotherapeutic fluorouracil prodrug inhibits DNA

and RNA synthesis and is used in cancer treatment (Ishikawa 2008). Likewise, Procarbazine, a benzene derivative, is an antineoplastic drug used for treatment of Hodgkin's lymphoma and certain brain cancers (Ogawa et al. 2003). Brefeldin A is a lactone antibiotic that inhibits protein transport from endoplasmic reticulum to Golgi apparatus indirectly by preventing formation of COPI-mediated transport vesicles (Klausner et al. 1992). 14-Hydroxy-6-O-methylerythromycin A, a polyketide, is known to have twice the antimicrobial efficacy of erythromycin (Fernandes et al. 1988). Dehydrofalcarinone, a carbonyl compound, is used as a fungicide, pesticide and phytoalexin. As mentioned earlier, polyamines play an important role in fungal growth and differentiation. They are also recognized as stress metabolites, aiding the organism's survival under abiotic stresses such as osmotic shock. N- carbamoyl putrescine is an important intermediate in polyamine biosynthetic pathway (Laura et al. 2012). Presence of this metabolite in *A. versicolor* KR87 metabolome sheds light on the probable mechanisms adopted by the fungi to survive and thrive in hypersaline conditions.

It is also imperative to acknowledge the role of halophiles in industrial and biotechnological arenas. Halophilic fungi have been used since ages for production of fermentation based foods such as soy sauce and fish sauce. Their modern day applications range from biorhodopsin production for optical computing to synthesizing biosurfactants and exopolysaccharides for oil recovery and compatible solutes as stress protectants (Margesin and Schinner 2001). Halophilic and halotolerant fungi can act as valuable sources of transgenes for imparting tolerance to industrially important microbes such as *Saccharomyces cerevisiae*, which are subjected to various abiotic stresses. Their genes can also be used for imparting halotolerance to plants, enabling them to grow in soils with high salinity and low water index (Munns 2002; Ashraf and Akram 2009).

Given the uniqueness of this extreme ecological niche, the saline wasteland of Rann of Kutch merits a more exhaustive and systematic study of the soil mycobiota. Such studies are bound to throw up certain atypical microorganisms that are imperative not just from academic perspective but are also of industrial significance.

CONCLUSION

Based on tolerance index and growth pattern at different salinities, *A. versicolor* KR87 was identified as a moderate halophile. Czapek Dox broth was found to be a preferred growth medium for chemoprofiling. The fungus exhibited diverse metabolites of pharmacological importance, indicating that it may be a promising source of novel drug lead compounds. Further research is being done to quantify the pharmacologically important compounds.

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REFERENCES

Ashraf M, Akram NA (2009) Improving salinity tolerance of plants through conventional breeding and genetic engineering: An analytical comparison. Biotechnol. Adv. 27: 744–752.

Arteaga S, Andrade-Cetto A, Cárdenas R (2005) Larrea tridentata (Creosote bush), an abundant plant of Mexican and US-American deserts and its metabolite nordihydroguaiaretic acid. J Ethnopharmacol 98 (3): 231–239.

Bentley SD, Chater K.F, Cerdeno-Tarraga AM, Challis GL, Thomson NR (2002) Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3 (2). Nature 417: 141-7.

399 Borel JF, Feurer C, Gubler HU, Stähelin H (1976) Biological effects of cyclosporin A: a new
400 antilymphocytic agent. Agents Actions 6(4):468-75.

401 Borut S (1960) An ecological and physiological study of soil fungi on the northern Negev
402 (Israel). Bulletin of the Research Council of Israel 8D: 65–80.

403 Bremner JM (1960) Determination of nitrogen in soil by the Kjeldahl method. J Agr Sci 55: 11-
404 33.

405 Buchalo AS, Wasser SP, Molitoris HP, Volz PA, Kurchenko I et al (1999) Species diversity and
406 biology of fungi isolated from the Dead Sea. In: SP Wasser (ed) Evolutionary Theory and
407 Processes: Modern Perspectives. Papers in Honour of Eviator Nevo. Kluwer Academic
408 Publishers, Dordrecht.

409 Calvo AM, Wilson RA, Bok JW, Keller NP (2002) Relationship between Secondary Metabolism
410 and Fungal Development. Microbiol and Mol Bio Rev 66 (3): 447-459.

411 Chu S, Gu J, Feng L, Liu J, Zhang M et al (2014) Ginsenoside Rg5 improves cognitive
412 dysfunction and beta-amyloid deposition in STZ-induced memory impaired rats via attenuating
413 neuroinflammatory responses. Int Immunopharmacol 19(2):317-26.

414 Dalboge H (1997) Expression cloning of fungal enzyme genes: A novel approach for efficient
415 isolation of enzyme genes of industrial relevance. FEMS Microbiol Rev 21: 29–42.

416 Demain AL, Fang A (2000) The natural functions of secondary metabolites. Adv Biochem
417 England Biotechnol 69: 1-39.

418 Diaz PL, Hennell JR, Sucher NJ (2012) Genomic DNA Extraction and Barcoding of Endophytic
419 Fungi. In: Sucher NJ, Hennell JR and Carles MC (eds) Plant DNA Fingerprinting and Barcoding:
420 Methods and Protocols. Humana Press.

421 Duraipandiyan V, Ignacimuthu S (2009) Antibacterial and antifungal activity of Flindersine
422 isolated from the traditional medicinal plant, *Toddalia asiatica* (L.). Lam J Ethno 123: 494-498.

423 Evans S, Hansen RW, Schneegurt MA (2013) Isolation and Characterization of Halotolerant Soil
424 Fungi from the Great Salt Plains of Oklahoma. Cryptogam Mycol 34(4): 329–341.

425 Fernandes PB, Ramer N, Rode RA, Freiberg L (1988) Bioassay for A-56268 (TE-031) and
426 identification of its major metabolite, 14-hydroxy-6-O-methyl erythromycin. Eur J Clin
427 Microbiol Infect Dis 7(1):73-6.

428 Frisvad JC (2005) Halotolerant and halophilic fungi and their extrolites Production. In: N.
429 Gunde-Cimerman et al (eds) Adaptation to Life at High Salt Concentrations in Archaea,
430 Bacteria, and Eukarya. Springer.

431 Grishkan I, Nevo E, Wasser SP (2003) Soil micromycete diversity in the hypersaline Dead Sea
432 coastal area, Israel. Mycol Prog 2:19–28.

433 Guerrero IC, Andres LS, Leon LG, Machin RP, Padron JM et al (2006) Abietane Diterpenoids
434 from *Salvia pachyphylla* and *S. clevelandii* with Cytotoxic Activity against Human Cancer Cell
435 Lines. J Nat Prod 69 (12): 1803–1805.

436 Gunde-Cimerman N, Zalar P, De Hoog S, Plemenitaš A (2000) Hypersaline waters in salterns –
437 natural ecological niches for halophilic black yeasts. FEMS Microbiol Ecol 32:235–240.

438 Gunde-Cimerman N, Oren A, Plemenitasˇ A (2005) Adaptation to Life in High Salt
439 Concentrations in Archaea, Bacteria, and Eukarya. Springer, Dordrecht, the Netherlands.

440 Gupta V, Ansari AA (2012) Geomorphic portrait of the Little Rann of Kutch. Arab J Geosci.

441 Ha BG, Yonezawa T, Son MJ, Woo JT, Ohba S et al (2014) Antidiabetic effect of nepodin, a
442 component of Rumex roots, and its modes of action in vitro and in vivo. Biofactors 40(4):436-
443 47.

444 Hawksworth D (2001) The magnitude of fungal diversity: The 1.5 million species estimate
445 revisited. Mycol Res 105: 1422-31.

446 Hufford CD, Lasswell WL Jr (1976) Uvaretin and isouvaretin. Two novel cytotoxic c-
447 benzylflavanones from *Uvaria chamae* L. J Org Chem 41(7):1297-8.

448 Hujslova M, Kubatova A, Chudickova M, Kolarik M (2010) Diversity of fungal communities in
449 saline and acidic soils in the Soos National Natural Reserve, Czech Republic. Mycol Prog 9: 1-5.

450 Isaa NM, Abdula AB, Abdelwahabb SI, Abdullahc R, Sukarid MA, Kamalidehghan B et al
451 (2013) Boesenbergin A, a chalcone from *Boesenbergia rotunda* induces apoptosis via
452 mitochondrial dysregulation and cytochrome c release in A549 cells in vitro: Involvement of
453 HSP70 and Bcl2/Bax signalling pathways. J Funct Foods 5(1):87–97.

454 Ishikawa T (2008) Chemotherapy with enteric-coated tegafur/uracil for advanced hepatocellular
455 carcinoma. World J Gastroenterol 14(18): 2797–2801.

456 Jackson ML (1973) Soil chemical Analysis. Prentice Hall India, New Delhi.

457 Jennessen J, Nielsen KF, Houbraken J, Lyhne EK, Schnurer J et al (2005) Secondary metabolite
458 and mycotoxin production by the Rhizopus microsporus group. J Agr Food Chem 53: 1833–
459 1840.

460 Khodair AA, Ramadani AS, Aggab AM (1991) Occurrence and density of alkalophilic bacteria
461 and fungi in saline soils of Makkah District, Saudi Arabia. Arab Gulf Journal of Scientific
462 Research 9:119–132.

463 Kis-Papo T, Grishkan I, Oren A, Wasser SP, Nevo E (2003) Survival of filamentous fungi in
464 hypersaline Dead Sea water. Microb Ecol 45: 183-190.

465 Klausner RD, Donaldson JG, Lippincott-Schwartz J (1992) Brefeldin A: insights into the control
466 of membrane traffic and organelle structure. J Cell Biol 116(5):1071-80.

467 Kushner DJ (1978) Life in high salt and solute concentrations, In: Kushner DJ (ed), Microbial
468 Life in Extreme Environments. Academic Press, London.

469 Mann J (2001) Natural products as immunosuppressive agents. Nat Prod Rep 18(4):417-30.

470 Margesin R, Schinner F (2001) Potential of halotolerant and halophilic microorganisms for
471 biotechnology. Extremophiles 5: 73-83.

472 Molitoris HP, Buchalo AS, Kurchenko I, Nevo E, Rawal BS et al (2000) Physiological diversity
473 of the first filamentous fungi isolated from the hypersaline Dead Sea. Fungal Divers 5: 55-70.

474 Munns R (2002) Comparative physiology of salt and water stress. Plant Cell Environ 25: 239–
475 250.

476 Nayak SS, Gonsalves V, Nazareth SW (2012) Isolation and salt tolerance of halophilic fungi
477 from mangroves and solar salterns in Goa - India. Indian J Mar Sci 41:164–172.

478 Nazareth SW, Gonsalves V (2014) Halophilic Aspergillus penicillioides from athalassohaline,
479 thalassohaline, and polyhaline environments. Front Microbiol 5(412): 1-5.

480 Ogawa K, Hiraku Y, Oikawa S, Murata M, Sugimura Y et al (2003) Molecular mechanisms of
481 DNA damage induced by procarbazine in the presence of Cu(II). Mutat Res 539(1-2):145-55.

482 Omura S, Ikeda H, Ishikawa J, Hanamoto A, Takahashi C, Shinose M (2001) Genome sequence
483 of an industrial microorganism *Streptomyces avermitilis*: Deducing the ability of producing
484 secondary metabolites. Proc Natl Acad Sci USA 98: 12215-20.

485 Pandeya SN, Sriram D, Nath G, Clercq ED (1999) Synthesis antibacterial antifungal and anti-
486 HIV evaluation of Schiff and Mannich bases of isatin derivatives with 3-amino-2-
487 methylmercapto quinazolin-4(3H)-one. Pharmaceutica Acta Helvetiae. 74: 11–17.

488 Pitt JI (1979) The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*.
489 Academic Press Inc, London.

490 Ranzoni FV (1968) Fungi isolated in culture from soils of the Sonoran Desert. Mycologia
491 60:356–371.

492 Raper KB, Fennell DI (1965) The genus Aspergillus. Williams & Wilkins, Baltimore.

493 Reeslev M, Kjoller A (1995) Comparison of biomass dry weights and radial growth rates of
494 fungal colonies on media solidified with different gelling compounds. Appl Environ Microbiol
495 61(12): 4236–4239.

496 Salama AM, Elbatanoni K, Ali MI (1971) Studies on the fungal flora of Egyptian soils. I.
497 Western Mediterranean coast and Lybian Desert. UAR J Bot 14:99–114.

498 Samson RA, Houbraken J, Thrane U (2010) Food and indoor fungi. CBS KNAW Biodiversity
499 Center, Utrecht.

500 Samson RA, Visagie CM, Houbraken J, Hong S-B, Hubka V et al (2014) Phylogeny,
501 identification and nomenclature of the genus *Aspergillus*. Stud Mycol 78: 141–173.

502 Smith JL, Doran JW (1996) Measurement and use of pH and electrical conductivity for soil
503 quality analysis. In: Doran JW & A.J. Jones (eds) Methods for assessing soil quality. Soil Sci
504 Soc Am Spec Publ, Madison, WI.

505 Tan KH (1996) Determination of soil texture. In: Tan KH (ed) Soil Sampling, Preparation, and
506 Analysis. Marcel Dekker, NY.

507 Thrane U, Andersen B, Frisvad JC, Smedgaard J (2007) The exo-metabolome in filamentous
508 fungi. In: Nielsen J and Jewett MC (eds) Topics in Current Genetics: Metabolomics. Springer-
509 Verlag Berlin, Heidelberg.

510 Todd PA, Goa KL (1990) Simvastatin. A review of its pharmacological properties and
511 therapeutic potential in hypercholesterolaemia. Drugs 40(4):583-607.

512 Valdés-Santiago L, Cervantes-Chávez JA, León-Ramírez CG, Ruiz-Herrera J (2012) Polyamine
513 metabolism in fungi with emphasis on phytopathogenic species. J Amino Acids. 837932.

514 Valdés-Santiago L, Ruiz-Herrera J (2014) Stress and polyamine metabolism in fungi. Front
515 Chem 1: 1–10.

516 VanderMolen KM, Raja HA, El-Elimat T, Oberlies NH (2013) Evaluation of culture media for
517 the production of secondary metabolites in a natural products screening program. AMB Express
518 3:71.

519 Visagie CM, Hirooka Y, Tanney JB, Whitfield E, Mwange K et al (2014). *Aspergillus*,
520 *Penicillium* and *Talaromyces* isolated from house dust samples collected around the world. Stud
521 Mycol 78: 63–139.

522 Walkley A (1947) A Critical Examination of a Rapid Method for Determination of Organic
523 Carbon in Soils - Effect of Variations in Digestion Conditions and of Inorganic Soil
524 Constituents. Soil Sci. 63:251-257.

525 Wang H, Gloer JB, Wicklow DT, Dowb PF (1995) Aflvinines and other antiinesctan metabolites
526 from the ascostroma of *Eupenicillium crustaceum* and related species. Appl Environ Microbiol
527 61: 4429-35.

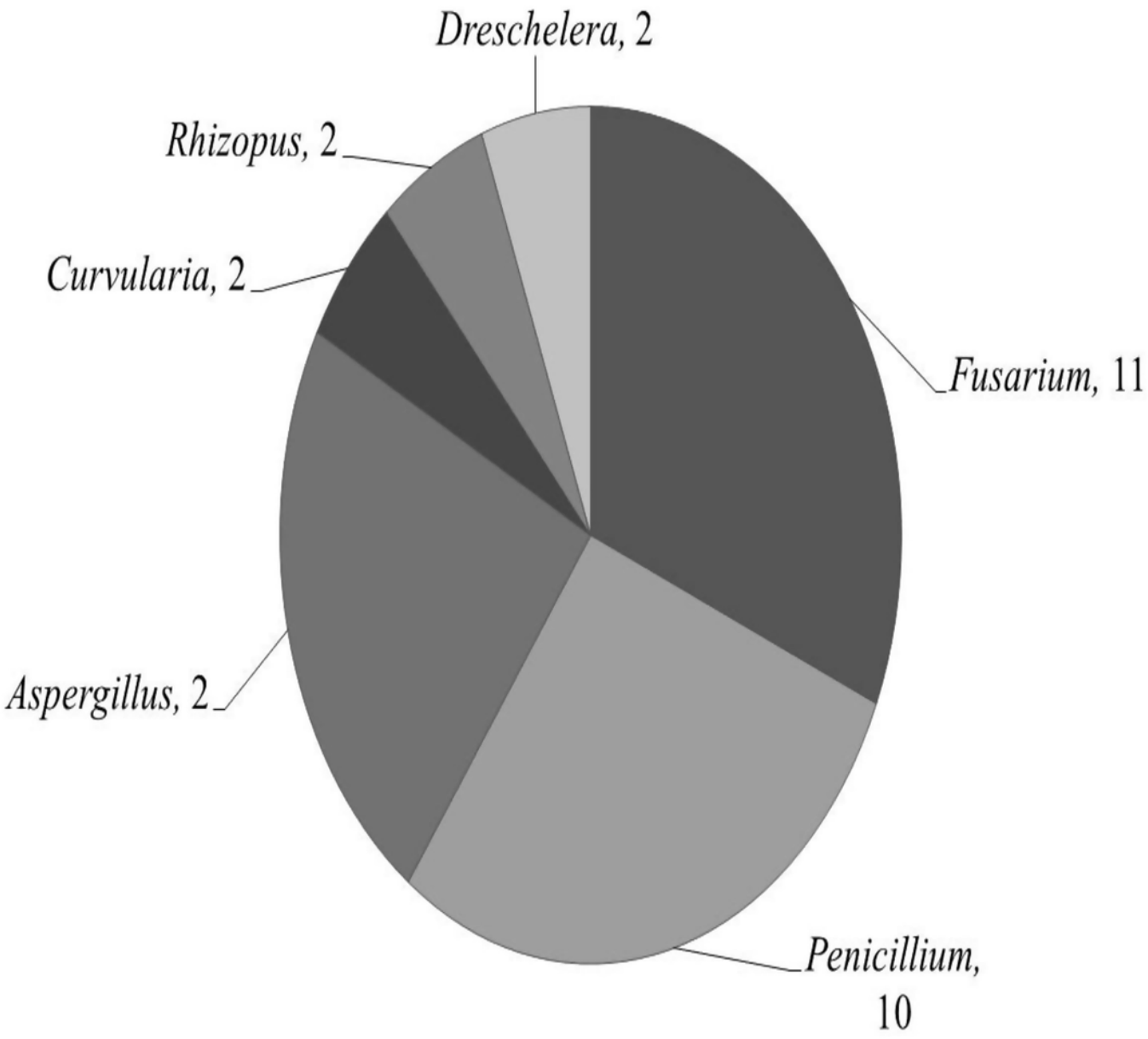
528 White T, Bruns T, Lee S (1990) Amplification and direct sequencing of fungal ribosomal RNA
529 genes for phylogenetics. In: Innis M, Gelfand D, Sninsky J, & White T (eds) PCR Protocols: A
530 Guide to Methods and Applications. Academic Press Inc, New York.

531 Yin W, Keller NP (2011) Transcriptional regulatory elements in fungal secondary metabolism. J
532 Microbiol 49:329-339.

533 Zain ME, Awaad AS, Al‐Othman MR, Alafeefy AM, El‐Meligy RM (2013) Biological activity
534 of fungal secondary metabolites. Intnl J Chem App Biol Sci 1 (1): 14-22.

1

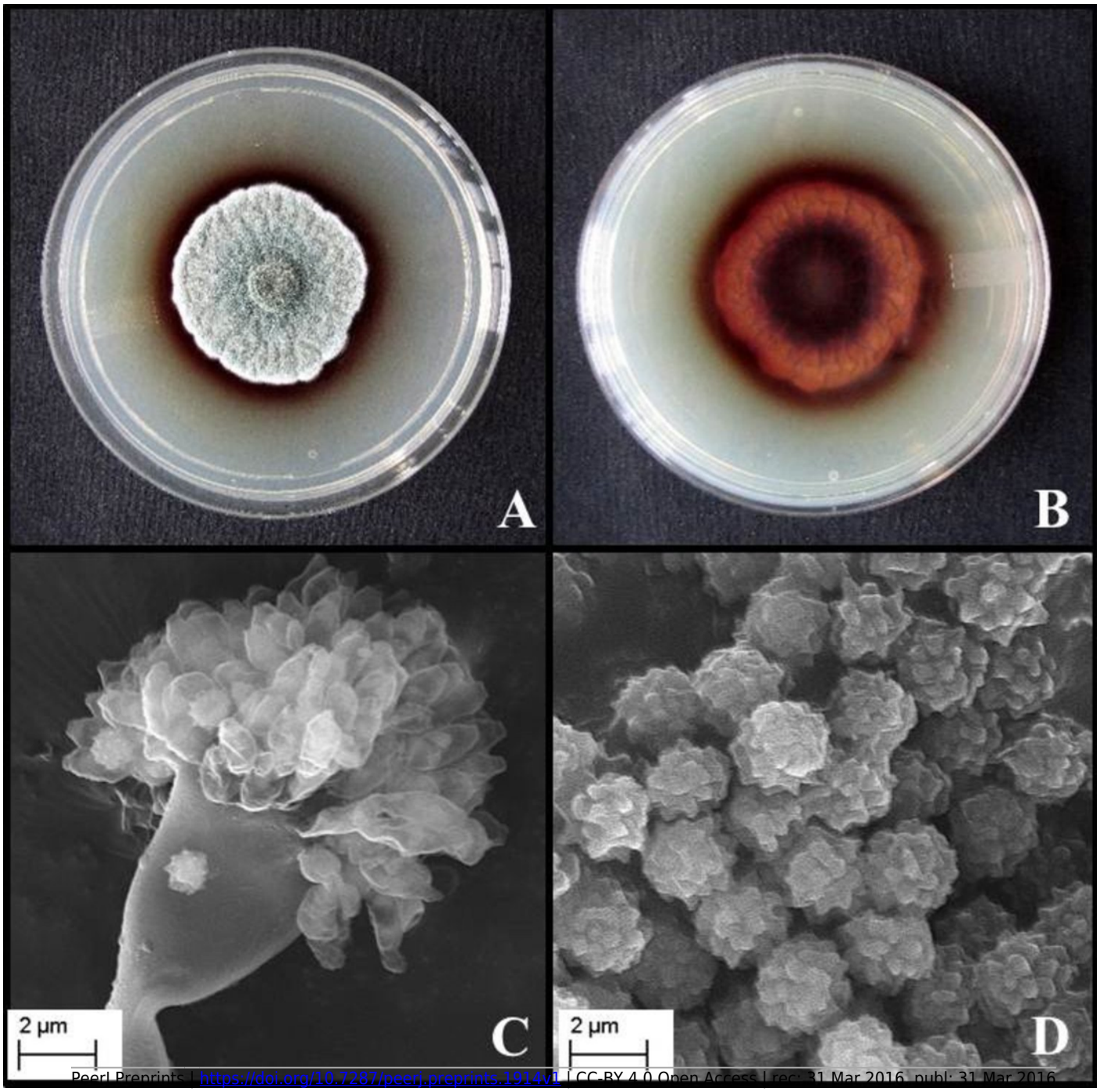
Species richness of the study area.



2

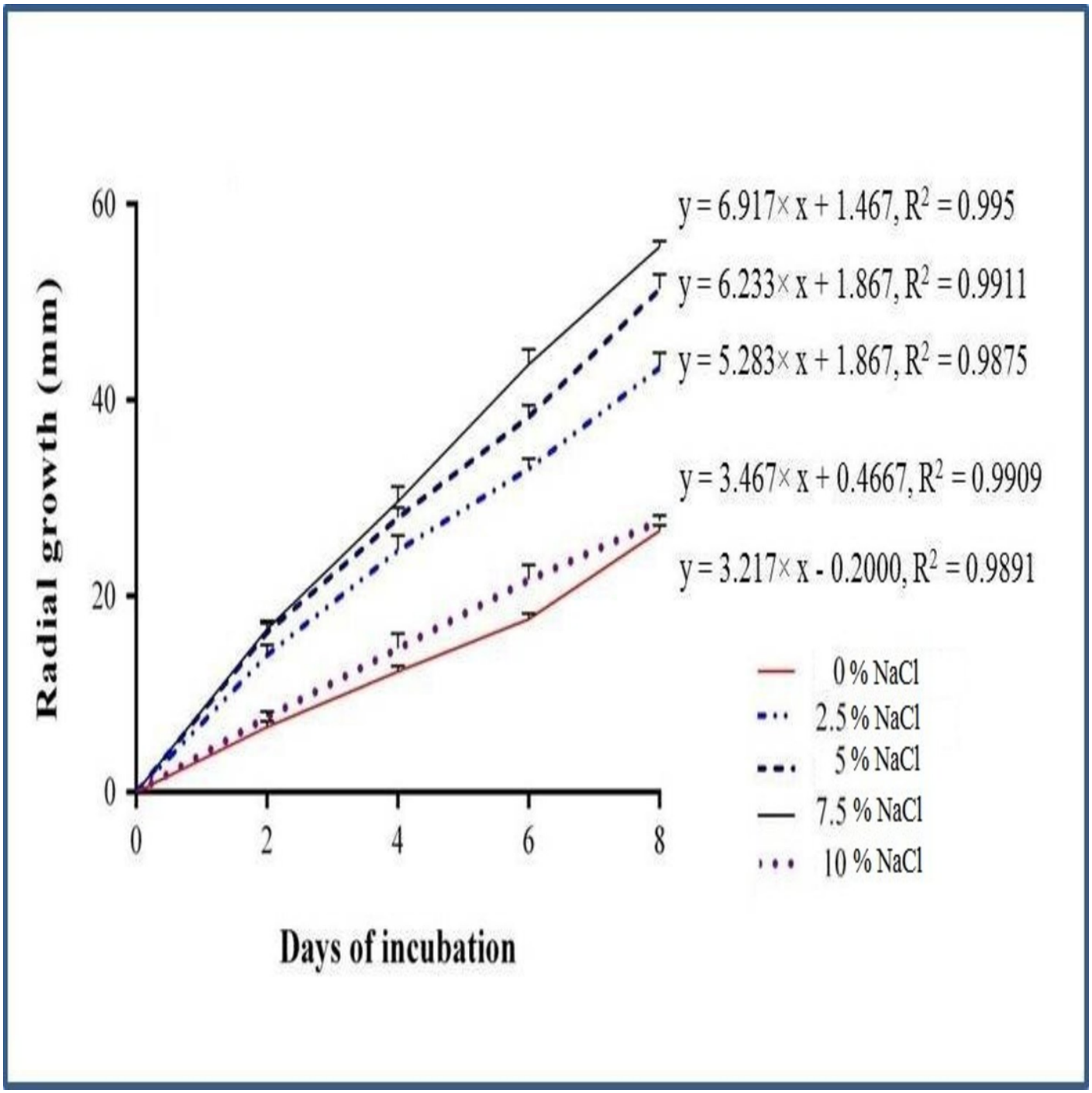
Morphological features of the halophilic isolate BBKF4.

(A) and (B) represent the front and reverse side colony morphology. Scanning electron micrograph of the conidial head (C) and conidia (D). The fungus was cultivated on CYA for 6 days at 25±1˚C.



3

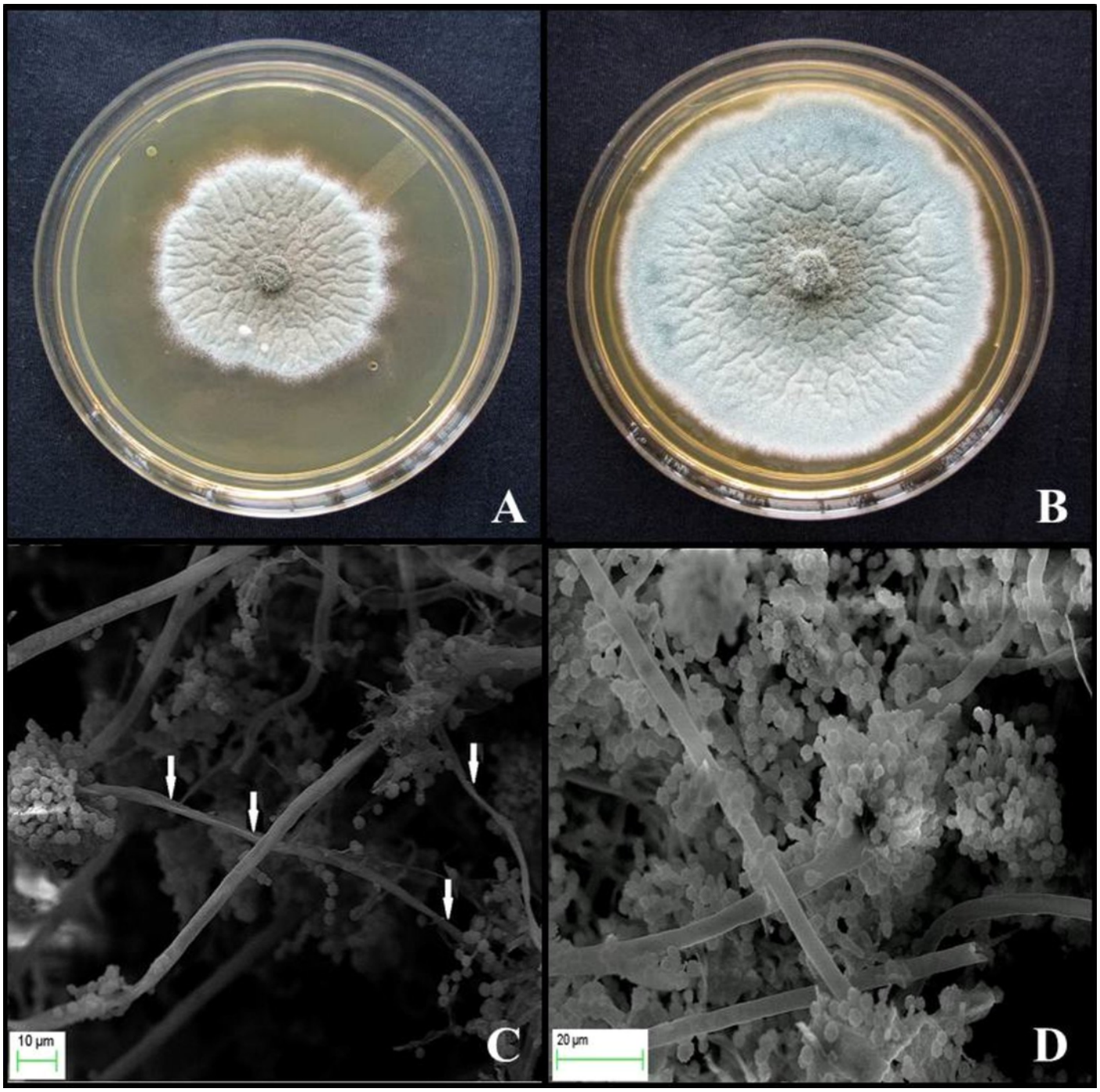
Radial growth of *A. versicolor* KR87 at different incubation periods and salt concentrations.



4

Impact of salinity on morphology of *A. versicolor* KR87.

Figures (A) and (B) represent the colony morphology of the fungus at 7.5% and 10% NaCl respectively. Figures (C) and (D) represent the micrographs of the fungus at 7.5% and 10% NaCl concentration respectively. The arrows in figure (D) indicate distortion in mycelia and loss of cytoplasm at 10% salinity. Conidiation is also scanty at 10% NaCl.



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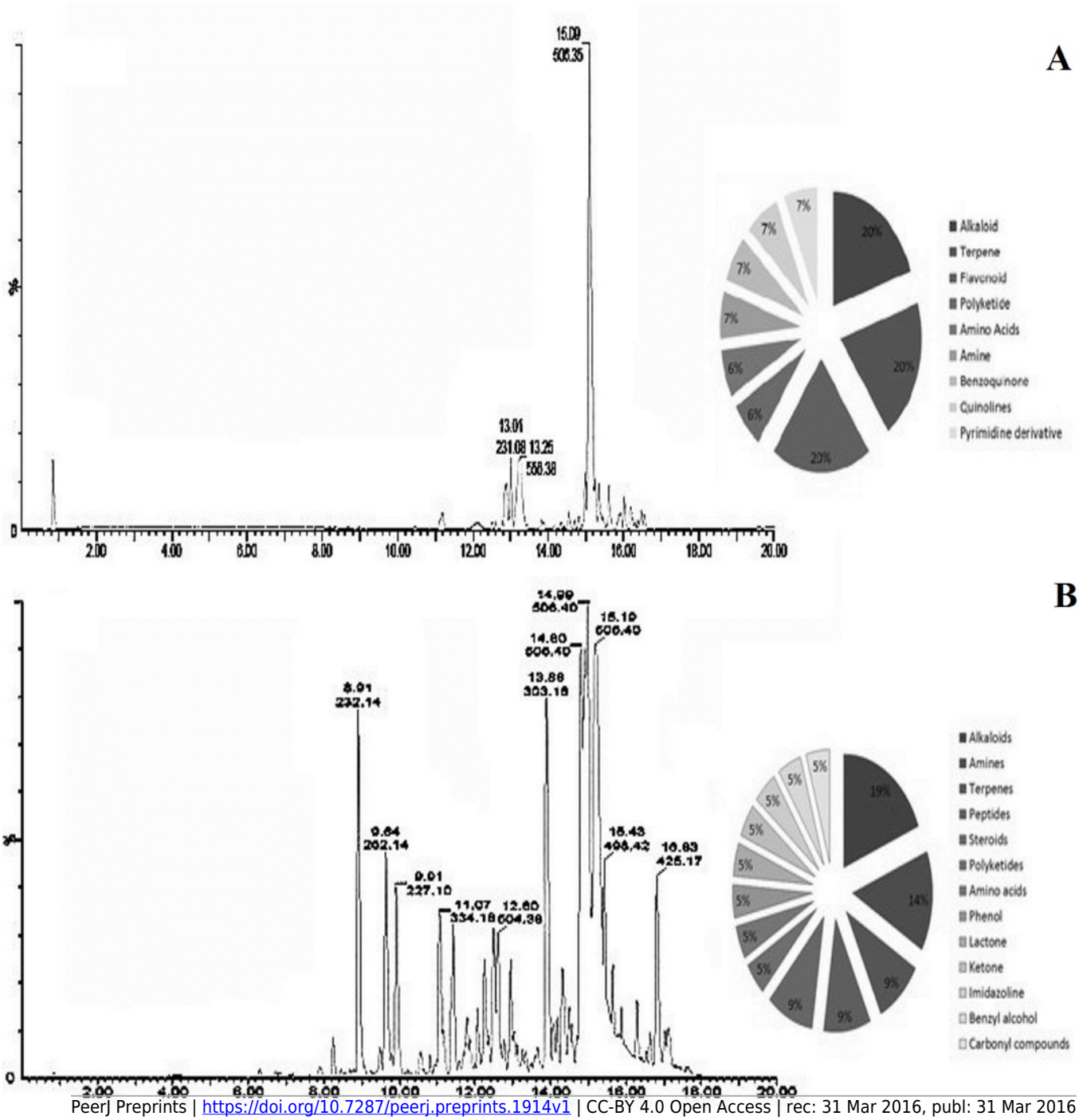
5

LC-UV-MS chromatogram of crude extract of *A. versicolor* KR87 grown in MEB (A) and CDB (B).

Adjacent to each chromatogram is a pie-chart of the metabolite profile of *A. versicolor* KR87. Metabolite diversity is represented as % abundance of metabolites of each compound class.



6

Compound classes exhibited by *A. versicolor* KR87 grown in CDB (A) and MEB (B) and the compound classes found in either metabolite profile (C).

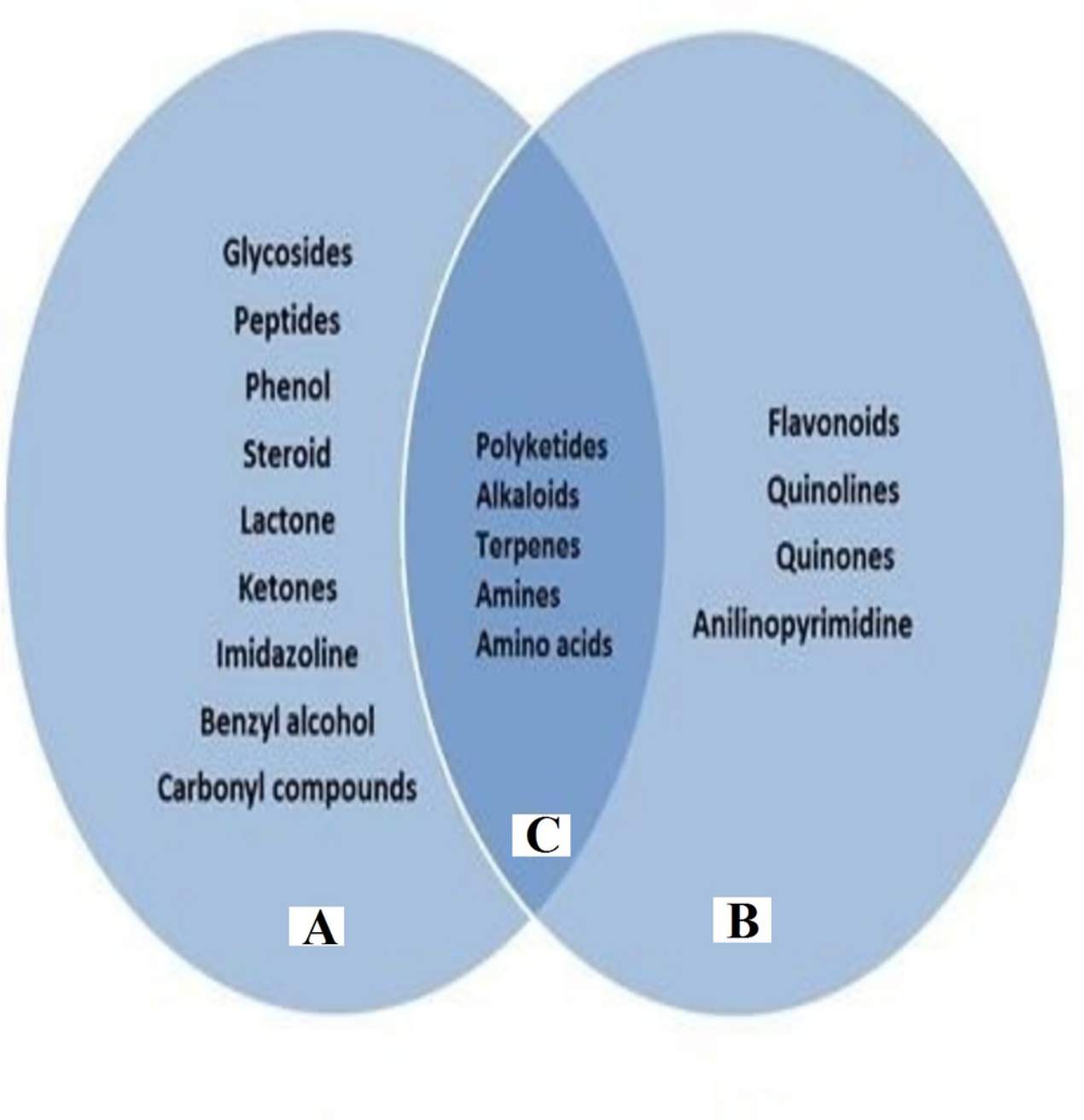


Table 1(on next page)

Physical and chemical properties of the soil samples collected from Little Rann of Kutch.

Property	Measured value	Methodology	Reference
Sand (g/Kg)	210	Hydrometer method	Tan (1996)
Silt (g/Kg)	650	"	"
Clay (g/Kg)	86	"	"
pH	6.63±0.3	Glass electrode	Smith and Doran (1996)
Electrical conductivity ($EC_{1:1}$) (dSm^{-1})	8.61±0.28	EC Probe	Jackson (1973)
Organic C content (%)	0.23	Walkley-Black method	Walkley (1947) Jackson (1973)
Total N content (%)	0.1	Modified Kjeldahl method	Bremner (1960) Jackson (1973)
Total P content (%)	0.2	Chlorostannous–reduced molybdophosphoric blue color method, in sulfuric acid system	Jackson (1973)
Elemental analyses (%) Na^+ Ca^{2+} Mg^{2+} K^+	 0.39 0.4 0.35 0.41	 Flame emission spectrophotometer	 Jackson (1973) " " "

1

Table 2(on next page)

Macroscopic characteristics of BBKF 4 grown on CYA for 8 days at 25±1˚C.

Color	Texture	Colony diameter (mm)	Colors on CYA		
			Conidia	Mycelia	Reverse
Greyish green	Suede-like with radial grooves	50±4	Greyish green	White	Red/ reddish brown

1

Table 3(on next page)

Microscopic features of BBKF4 as observed under scanning electron microscope.

Stipes			Vesicles			Conidia		
Length	Texture	Color	Diameter	Shape	Seriation	Diameter	Shape	Texture
300-400 µm	Smooth	Pale brown	10-12 µm	Pyriform	Biseriate	2-3 µm	Globose	Finely roughened

1

Table 4(on next page)

Biological activity of some metabolites secreted by *A. versicolor* KR87.

Metabolites	Potential applications	References
Carnosols, Isouvaretin	Anti-cancer agents	Hufford and Lasswell Jr. 1976
Flindersine	Antibacterial, antifungal	Duraipandiyan V et al. 2009
Nordihydroguaiaretic Acid	Antioxidant, anti-carcinogenic	Arteaga et al. 2009
Ginsenoside Rg5	Agent for the treatment of Alzheimer's disease	Chu et al. 2014
Boesenbergin A	Anti-inflammatory, cytotoxic and antioxidant activities	Isaa et al. 2013
Quinazoline acetic acid	Anticonvulsant, antibacterial, antidiabetic	Pandeya et al. 1999
Tegafur	Anticancer agent	Ishikawa, T 2008
Nepodin	Anti-diabetic	Ha et al. 2014
Simvastatin	Lipid lowering activity	Todd et al. 1990
Procarbazine	Anticancer activity, treatment of Hodgkin’s lymphoma	Ogawa et al. 2003

1