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REGULAR ARTICLE

# YEAST IN SOUTHWEST MONSOON RAINWATER

*Sheela Pal* *[1], *Nandkumar Kamat*[2]

***Address (es):***
[1] Mycological Laboratory, Department of Botany, Goa University, Taleigao,Goa, 403206, India
[2] Mycological Laboratory, Department of Botany, Goa University, Taleigao,Goa, 403206, India

*Corresponding author: nandkamat@gmail.com

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## ABSTRACT

Strong evidence of the presence of bacteria and fungi in the tropospheric boundary layer is available in the literature. We report successful isolation of unique morphotypes of wild ascomycetous yeasts from rainwater samples collected directly in sterile containers, taking extreme care to avoid ambient contamination. Direct and quick visualization of fresh rainwater samples under a phase contrast microscope indicated the sporadic presence of yeast cells. Further confirmation of the presence of yeast was obtained by plating of rainwater on a medium with antibiotics to generate pure colonies. We described their characteristics while molecular identification revealed it as *Candida tropicalis*. Yeast species could contribute valuable knowledge about yeast transportation in the atmosphere. However, knowledge is insufficient about the yeast deposited from the atmosphere and its transportation across the atmosphere. We report and discuss these interesting and exciting results which are useful in understanding the microbiological dimension of meteorology and the southwest monsoon rainfall in the light of present discourse on global warming and climate change. We offer a tentative model for a possible source, role, and fate of the yeasts in rainwater.

**Keywords:** *Candida tropicalis*, molecular identification, PCR, sequencing, rainwater microbial

## INTRODUCTION

The period June to September in India is considered the southwest monsoon period. It is the main rainy season of the Indian subcontinent. The whole country receives 75% of its rain in this season. The southwest monsoon originates in the general circulation of the atmosphere which is caused by a region of high pressure over the south Indian ocean and a region of low pressure which extends over the whole of central Asia (**Simpson. *et al*., 1921**). A controversial theory exists about the origin of the southwest monsoon. Many geologists believe that the monsoon first arose around 8 million years ago based on records from the Arabian sea and the record of wind-blown dust in the loess plateau of China (**Tambe**). The southwest monsoon first hit Kerala from the Arabian sea, which is a branch of the southwest monsoon and moves towards the northern region of India along the its western coast.

### Nucleation of cloud

Aerosols are an important factor of climate, directly scattering light, seeding warm clouds, and seeding ice-clouds. (**Brooks, 2019**). A marine aerosol population transits into cloud droplets and ice crystals. Aerosol rises from the marine environment and from continents, are formedby the gas phase (e.g.. DMS) emissionand from primary particles.

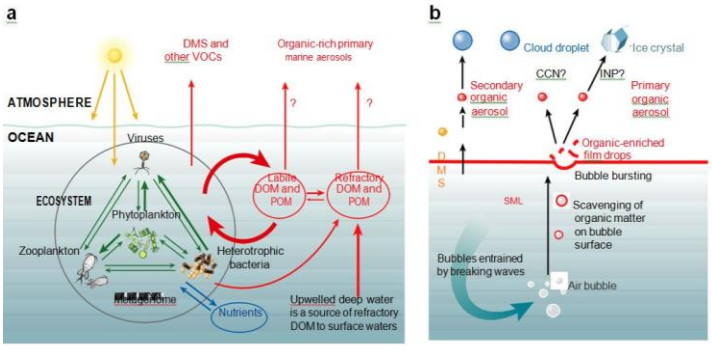



**Figure 1** Formation of marine aerosol and cloud nucleation. (Adapted from **Brooks et al., 2018**)

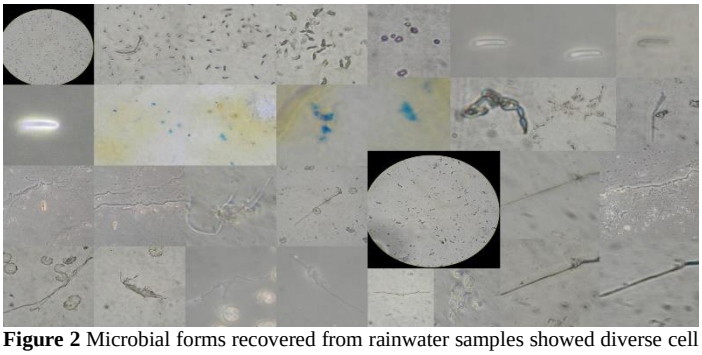


**Figure 2** Microbial forms recovered from rainwater samples showed diverse cell morphologies- Coccoid, Bacilloid bacterial cells; cyanobacteria and numerous fungal spores and hyphae- all these are getting a piggy-back ride in monsoon clouds and landing with rainfall. (**Kamat. N. *et al*, 2014**)

The composition of micro-organisms in the atmosphere, in the clouds or rainwater is not well defined yet. Though many studies of bacterial communities have been performed. Bacterial strains were isolated from the cloud and their role in the forming the chemical composition of the atmosphere was studied (**Tina *et al*, 2015**). **Morris. *et al*.**, (**2008**) suggested atmospheric dispersal may play an important role in bacterial biogeography patterns and later expressed bacteria in active form from an inactive state in a suitable environment. Fog and cloud provide protection to bacterial strains from ultraviolet rays and the provide nutrients for their survival ship (**Tina. *et al*, 2015**). Clouds help microbial transportation over a long distances (**Griffin., 2006**). **Baurer. *et al*, 2002** isolated bacterial and fungal spores from cloud water and tried to study the role of microbes in the contribution of carbon content. Strong evidence of the presence of bacteria and fungi in the tropospheric boundary layer, using dicarboxylic acid as a nutrient (**Amyot. *et al*, 2002**). The yeast biota in rain is less studied, so this study may help address an important gap in the study of yeast and its occurrence and ecological role in the atmosphere. An attempt has been made to collect a rainwater sample, isolation of microbeand to identify the isolates. The isolate is known to be *Candida tropicalis* after molecular identification.

*C. tropicalis* was considered yeast by **Kurtzman and Fell** (**2000**). Its habitat is quite broad and it can be isolated from various substrates and samples (bark, roots, leaves, gut, marine sediments, mud, waters, skin etc.). *C. tropicalis* is classed as fungi in the order of sachromycetales, and in the family of debaryomycetaceae. It is a common pathogen and can infect individuals with low immunity. Moreover this species is also reported in Kawasaki disease in Japan, which spreads from China as a windborne agent.Recently it has been found that *C. tropicalis* is of

biological and biotechnological importance (**Zhenming, 2010**). Numerous studies of *C. tropicalis* have been carried out, including substrate inhibition of phenol oxidation (**Stephenson, 1990**), a protoplast fusion technique (**Chang *et al*., 1995**) and degradation of phenol in the presence of other derivatives (**Komarkova *et al*., 2003**). Xylose as the sole carbon source can be produced by *C. tropicalis* . It has been employed to convert corncob hydrolysate to xylitol (**Cheng *et al*., 2009; Rao *et al*., 2006**). Strains of *C. tropicalis* have been used in the removal and recovery of zirconium (**Akhtar *et al*., 2008**). Differing *C. tropicalis* strains from a variety of marine environments have been proven for their potential applications in agriculture, in the fermentation industry and in the chemical industry. However, currently little is known about the distribution and diversity of *C. tropicalis* in various marine, soil, and air environments across the globe.
**Zhenming** (**2010**) studied the distribution and diversity of *C. tropicalis* strains from multiple marine environments and discussed the relevant potential applications in China. The marine yeast strains of *C. tropicalis* have many potential applications in biodiesel production (**Meng *et al*., 2009**), bioremediation (**Ukrit *et al*., 2009; Sonali 2008; Varma *et al* Komarkova *et al*., 2003** in the production of polyol sweeteners (**Rao *et al*., 2004; Cheng *et al*., 2009; Rao *et al*., 2006**), and in ethanol fermentation (**Ukrit *et al*., 2009**). **Chaves, *et al* (2017**) studied and reported that a strain of *C. tropicalis* isolated from the root of the mangrove tree *Rhizophora stylosa* in Zhanjiang, China could accumulate over 50% of oil during batch cultivation from glucose and hydrolysate of cassava starch. The authors concluded that the strain can be used in biodiesel production. C. *tropicalis* has been considered an osmotolerant microorganism and this ability to survive high salt concentrations may be important for fungal persistence in saline environments. This physiological characteristic makes this species suitable for use in biotechnology processes (**Chaves M. 2017**).

**Genetic characteristics**

**Butler *et al*.** (**2009**) sequenced the genome of the diploid yeast *C. tropicalis*. **Doi *et al*.** (**1992**) reported 12 chromosomes per cell for *C. tropicalis* and stated it has a genomic size of 14.5 Mb, containing 6,258 genes encoding proteins and a guanine-cytosine content of 33.1%. The number of chromosomes is not known with precision. Prior to that *C. tropicalis* was considered as an asexual yeast, while some studies have reported that mating between diploid cells a and α, generating a/α tetraploid cells may occur (**Porman *et al*., 2011; Xie *et al*., 2012; Seervai *et al*., 2013**). Researchers have suggested that the cause of cells changing from white to opaque could be mating, which is regulated by colony phenotypic switching. **Seervai *et al*.** (**2013**) reported that strains of *C. tropicalis* can be induced to undergo a parasexual cycle without a meiotic reduction from a tetraploid state polyploidy, affecting cell gene expression and protein production (**Morrow, 2013**). *C. tropicalis* showed a reduction in ploidy and was considered a mechanism of adaptation; it may be associated with cell stress (**Bermane*t al*., 2012**). C. *tropicalis* has showed huge genetic similarity with *C. albicans* than other *Candida* species. (**Butler *et al*., 2009**).

**MATERIAL AND METHODS**

**Assembly of sterile, dust- and contamination-free PVC containers**

Pre-sterile plastic bottles were used to collect the rain water. Six times in every monsoon from 2016 to 2018.

**Selection of clear vegetation-free open spaces permitting direct collection**

Water was collected at Goa University campus at the same place during every sampling. The area was vegetation free and open in order to avoid contamination.

**Microscopy and photomicrography**

Each Raw water sample was analyzed under a light microscope with the help of a wet mount slide (bright field, dark field, phase contrast), followed by identification of sample components from morphology, optical properties, and published literature (Adapted from **Kamat, N. 2014**).

**Isolation of yeast purification and maintance of pure culture on slant**

Rain water samples were spread on MEA plates (containing 0.1mg/ml antibiotic) which were incubated for 48 hr at 25°C. The plates were observed under a microscope for the growth of yeast and colony morphology.

**Control plates**

Plates with nutrient agar and MEA (2%, with 0.1mg/ml antibiotics) were kept in the open air for one hour at the rain water collection site at the Goa university campus. Other plates were spread plated with water which was used for rinsingthe rainwater sampling apparatus. One plate with MEA (2%) was kept without spread plating rain water.

**Molecular identification of strain**

(By Triyat Scientific co., Nagpur,India)

**DNA extraction**

Cells grown in a monolayer were lysed by suspending 1-3 colonies aseptically and mixing with 450 µl of "B Cube" lysis buffer in a 2 ml micro centrifuge tube and lysing the cells by repeated pipetting.
2. 4 µl of RNAse A and 250 µl of "B Cube" neutralization buffer was added.
3. The content was vortexed and the tubes were incubated for 30 minutes at 65˚C in a water bath. To minimize shearing the DNA molecules, DNA solutions were mixed by inversion.
4. The tubes were centrifuged for 20 minutes at 14,000 rpm at 10 ˚C.
5. Following centrifugation, the resulting viscous supernatant was transferred into a fresh 2 ml micro centrifuged tube without disturbing the pellet.
6. 600 µl of "B Cube" binding buffer was added to the content and mixed thoroughly by pipetting and the content was incubated at room temperature for 5 minutes.
7. 600 µl of the contents was transferred to a spin column placed in a 2 ml collection tube.
8. Contents were centrifuged for 2 minutes at 14,000 rpm and flow-through was discarded.
9. The spin column and the collection tube were reassembled then the remaining 600 µl of the lysate was transferred .
10. The contents were centrifuged for 2 minutes at 14,000 rpm and the flow-through was discarded.
11. 500 µL "B Cube" washing buffer I was added to the spin column and centrifuged at 14,000 rpm for 2 mins. The flow-through was discarded.
12. The spin column was reassembled; 500 µl "B Cube" washing buffer II was added and centrifuged at 14,000 rpm for 2 mins. The flow-through was discarded.
13. The spin column was transferred to a sterile 1.5-ml microcentrifuge tube
14. 100 µl of "B Cube" elution buffer was added to the middle of spin column.
15. The tubes were incubated for 5 minutes at room temperature and centrifuged at 6000 rpm for 1 min.
16. Steps 14 and 15 were repeated for complete elution. The buffer in the microcentrifuge tube contained the DNA.
17. DNA concentrations were measured by running aliquots on 1% agarose gel.
18. The DNA samples were stored at -20°C until further use.

**PCR**

This is a process that uses primers to amplify specific cloned or genomic DNA sequences with the help of a very unique enzyme. PCR uses the enzyme DNA polymerase that directs the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA template. DNA polymerase adds nucleotides to the 3` end of a custom-designed oligonucleotide when it is annealed to a longer template DNA. Thus, if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide, DNA polymerase can use the oligonucleotide as a primer and elongate its 3` end to generate an extended region of double stranded.

**Purification of PCR product**

Unincorporated PCR primers were removed and dNTPs were removed from PCR products by using Montage PCR Clean up kit (Millipore).The PCR product was sequenced using the primers. Sequencing reactions were performed using ABI PRISM®BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems).

**Sequencing (Bioinformatics)**

Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

**RESULTS AND DISCUSSION**

A 10 to 200 ml rain water sample was collected (Fig.2), according to rain fall intensity. The rainwater was transparent and its pH temperature and TDS was recorded at 24°C, 6.92, and 9 ppm respectively. Six samples were taken from June to September. Isolation plates showed the same growth pattern of a wild colony during each sampling, on all triplicate plates. During plate observation it was noted that two dissimilar colonies had grown. The colonies were white and beige in color and their textures were rough and smooth. No growth was observed on the control plate, on which the rain water sample was not spreaded. Plates with nutrient agar kept at the sampling site showed a mixed growth of bacteria, mucor, penicilium, but yeast growth was not observed. While plates with MEA which were kept at the sampling site did not show bacterial and yeast growth, mucor and penicillium growth were observed. Molecular analysis proves isolate is *Candida tropicalis* yeast. So work which was undertaken to check that the southwest monsoon contains yeast has been proved. Further isolates were grown in the MEA media and modified agar media ( prepared with agar and rainwater) and studied for their morphological change in nutrient rich (MEA) and nutrient poor media (agar prepared with rain water).Morphology variation was observed on the MEA media prepared with sterile distilled water and on the agar media prepared with sterile rain water. Colonies were grown on the agar media with rain water plate in a countable number and were very tiny, while on the MEA plates, the colony sizes were large and growth was observed. Colonies showed morphological variation grown on nutrient rich and nutrient poor media.

**(a) (b) (c)**

**Figure 3** Wild colonies grown on isolation plate (a), Purified yeast culture (b) Magnified yeast colonies grown on MEA (c).

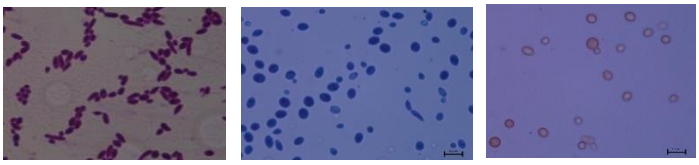


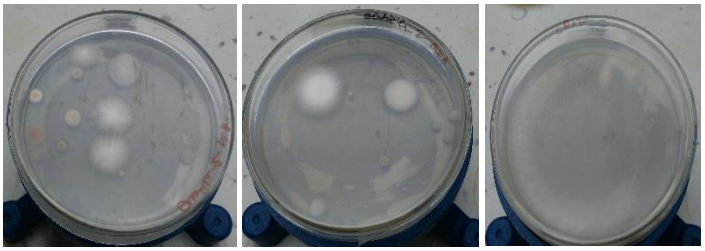
**Figure 4** Microscopy images of isolate. (a) Darkly stained cells in crystal violet (b) Cells mount in lactophenol cotton blue (c) Cells stained in congo red.

**Table 1** Characteristics of isolates

| Strain designation | No of colony | Color | Texture |
|---|---|---|---|
| Rw1a | 3 | White | Rough shiny |
| Rw1b | 2 | Beige | Smooth |
| Rw2a | 2 | Beige | Smooth |
| Rw2b | 1 | White | Smooth shiny |

**Table 2** Rainwater isolate showed colony characteristics on MEA and modified media

| | Growth Pattern | Colour | Texture | Size |
|---|---|---|---|---|
| Deionised water +MEA | Mat growth | White | Smooth | Large colony |
| Rain water+MEA | Mat growth | White | Smooth | Large colony |
| Rain water +Agar | Countable Colony | Gray | Slimy | Tiny colony |



**(a) (b) (c)**

**Figure 5** Control plates (a) Plate with nutrient agar kept outside for one hour, bacterial and fungal growth were observed, yeast was absent. (b) Plate with MEA (2% and antibiotics). No bacterial growth observed, mucor and pencilium were observed. No yeast growth was observed (c) Plate with MEA, spread plated withwater from sterile rinsed apparatus; no growthobserved.

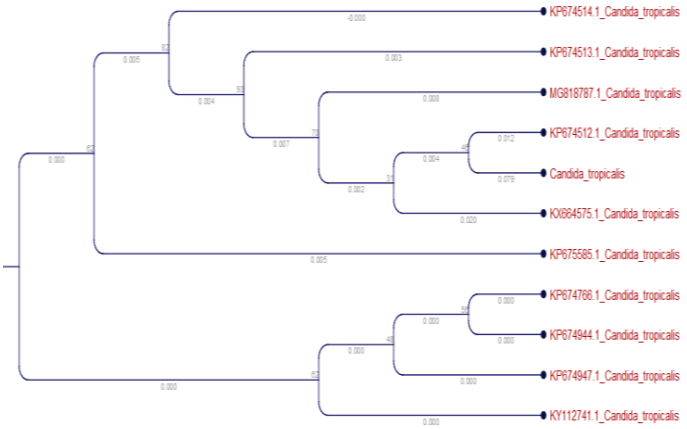



**Figure 6** Phylogeny analysis of *Candida tropicalis.* Isolate showed 0.012 dissimilarity with type species *Candida tropicalis* ( KP674512.1) obtained from gastric mucosa, China.

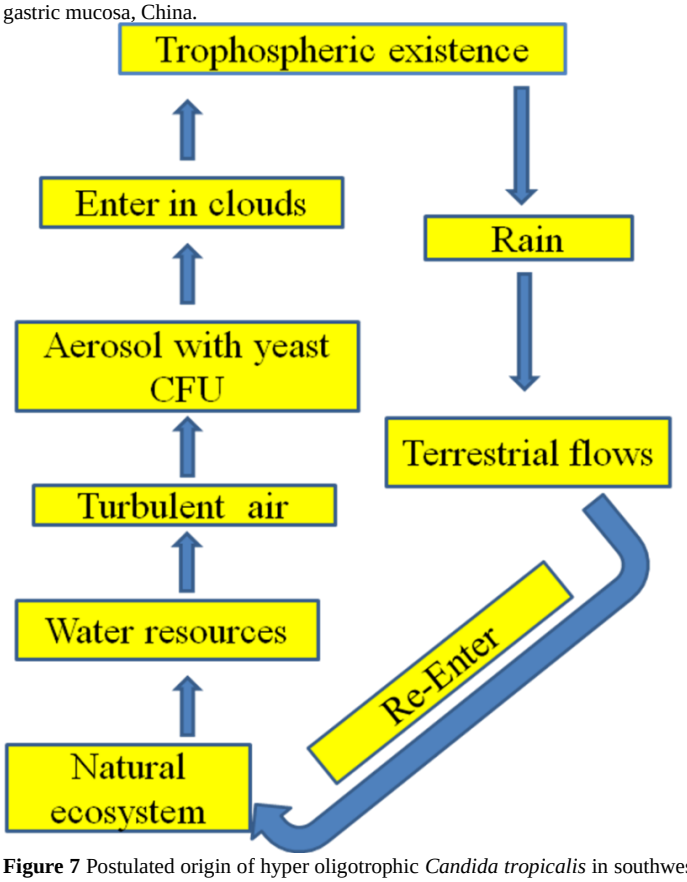



**Figure 7** Postulated origin of hyper oligotrophic *Candida tropicalis* in southwest monsoon clouds

**CONCLUSION**

This is the first report in the world identifying yeast from southwest monsoon rainwater. *Candida tropicalis* has never been suspected as entering the southwest monsoon airborne cloud environment, therefore this is the first report of *Candida* sp. and *Candida tropicalis* specifically in rainwater. As shown in fig.7 it could enter the turbulent air from terrestrial disturbances. These properties of the strain to grow on rainwater agar without nutrients indicates that it may exist in the airborne environment as a hyper oligotrophic yeast. This has important ecological implications for the distribution of terrestrial and aquatic yeast species. Besides biometerologically, it needs to be seen whether *Candida tropicalis* contributes to cloud condensation nuclei (CCN). Moreover, it is required in the future to study southwest monsoon regions to confirm the presence of such species in rainwater. Studies are required from various southwest monsoon regions to confirm the presence of such species in rainwater. Work comprises three parts: First, the field sampling for rainwater collection, second, the microbiology for yeast isolation and purification, and third the molecular analysis for identification of species. Rainwater samples were collected in pre-sterile plastic bottles and tightly capped during the monsoon season from June to September (3 years) on the Goa University campus. During each sampling, rain water was analyzed under the microscope and showed diverse and numerous cells of bacteria, fungal spores, and yeast cells. In this study yeasts have been isolated from rain water as previous work in our laboratory has given the sign of microbes in rain water (**Kamat, N. *et al*, 2014**). The same morphological types of wild colonies were obtained on the isolation plates. A heavy concentration of antibiotics does not allow bacteria to grow on a plate. Isolates were studied for their morphological characteristics and after purification colonies were maintained on slant. DNA extraction, PCR followed by phylogeny analysis of pure isolate was performed. Molecular analysis revealed the species as *candida tropicalis*. Further study can cover the co-relation of the yeast concentration and weather parameters. The diversity of microbes in clouds can be studied at various sea levels as well as their role in ecology in ambient environments. Their role could be investigated in the formation of cloud nucleation as it has been suggested (**Sarah *et al*., 2017**) that cloud formation initiates CCN due to the presence of atmospheric particles and microbes e.g., bacteria, fungi, and phytoplankton.

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