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Original Research Article

Evaluation of Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) Spectroscopy for Characterization of *Trichosporon* Species Isolated from Clinical Samples

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Abstract

Background: *Trichosporon* is a medically important genus that includes causative agents of deep-seated, mucosa associated and superficial infections. Rapid and accurate identification is very important in institution of appropriate therapy, thereby reducing morbidity and mortality related to Trichosporonosis. The genotypic methods commonly used to discriminate these species are laborious and very expensive. Being cost effective, Fourier-transform infrared spectroscopy with attenuated total reflectance (ATR-FTIR) was evaluated in this study to discriminate between *Trichosporon* species. **Methods:** A collection of 75 clinical isolates pre-characterized by phenotypic and PCR-based sequencing techniques and belonging to 5 different *Trichosporon* and related species [*T. asahii* (n=62), *T. inkin* (8), *C. dermatis* (3), *T. asteroids* (1), *A. loubieri* (1)] of *Trichosporon* were subjected to ATR-FTIR. Standard strains from different genera were used as outliers. Spectral data acquired in the ATR mode under controlled microbiological and physical parameters for all measurements were compared. Infrared spectra were analyzed with Hierarchical cluster analysis (HCA) and Principal component analysis (PCA). A new mathematical operation, Reference & Threshold calculation (R&T) was proposed here for rapid & reliable identification of *Trichosporon* to species level. **Results:** The current study using ATR-FTIR method has sharply demonstrated 100% genus level discrimination with protein specific spectral window (1800-900 cm^{-1}). All the 5 different *Trichosporon* species could be successfully discriminated using second derivative spectra. Clustering data using statistical tools showed that the polysaccharide specific signatures (1200–900 cm^{-1}) were critical markers for species discrimination. The results have been further correlated using PCA. R&T operation applied on the genus and species level resulted in 100% identification concordant with PCR sequencing results. **Conclusions:** This study has confirmed that ATR-FTIR spectroscopy along with reference and threshold calculation is a promising diagnostic tool for rapid, reliable and cost-effective characterization of clinical *Trichosporon* species, compared to conventional/molecular techniques.

Keywords: *Trichosporon*, ATR-FTIR, HCA, PCA, Reference Spectra, Spectral Library.

Introduction

Trichosporon is a medically important genus that includes causative agents of deep-seated, mucosa-associated and superficial infections. It is the third most common cause of disseminated yeast infections in humans after Candida[1,2]. More than 50 species have been described in the genus *Trichosporon*, of which 16 are of medical importance, with *Trichosporon asahii* being the most common pathogen[1,3]. After repetitive taxonomic reclassifications, recently pathogenic species of *Trichosporon* were placed in three genera: *Trichosporon*, *Cutaneotrichosporon*,& *Apiotrichum*[4]. The reported mortality rates range from 50%-80%, eventually reaching 100% in onco-hematological patients with persistent neutropenia[1,5,6]. Rapid and accurate identification is very important to initiate appropriate therapy and thus reducing morbidity and mortality related to Trichosporonosis. Most medical laboratories still rely on automated commercial carbon-assimilation-based identification systems used along with conventional biochemical assays. While gene sequencing methods are considered the gold standard in microbiology, they are costly, time-consuming, and not readily available for routine identification at most clinical sites[7]. The genotypic methods, although widely accepted, have technical limits due to protocol complexities, reagent costs, choice of specific primers for each species, sensitivity to mutations, and are not applicable in routine identification[8-10].

Fourier transform infrared (FTIR) spectroscopy is a high-resolution, analytical technique. The underlying assumption is that related strains are more similar in their composition (e.g., cell wall components), which leads to the higher congruence of their FTIR spectra. The conventional transmission mode is a commonly used mode to take IR spectra of organisms with a tricky sample preparation method which can be greatly simplified by using the attenuated total reflectance (ATR) mode of spectral acquisition. In the ATR mode, the IR beam from the IR source is launched into an IR-transparent ATR crystal (made of a high-refractive-index material, such as Zinc selenide, germanium, or diamond) at a defined angle (exceeding the critical angle for internal reflection) whereby total internal reflection occurs within the crystal. Under these conditions, an evanescent wave (perpendicular to the propagating IR beam) forms at the surface of the ATR crystal and decays exponentially with distance from the surface. Interaction of the evanescent wave with a sample placed on the surface of the crystal results in partial attenuation of the total internally reflected IR beam at the wavelengths at which the sample absorbs IR energy as shown in **Figure:1**

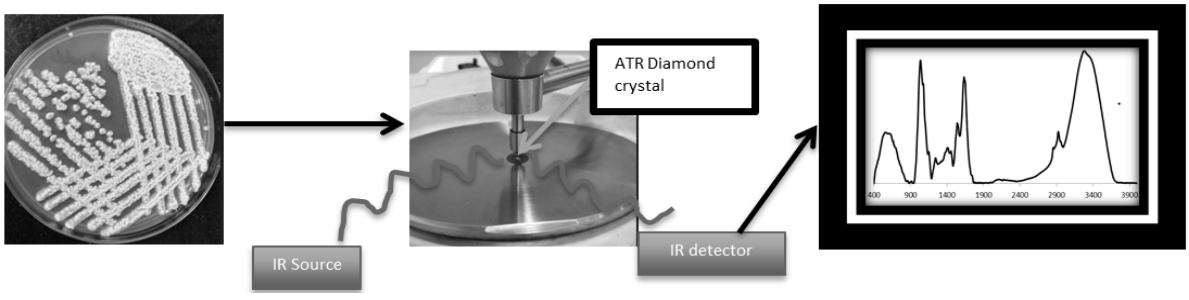

Figure 1: Data acquisition using ATR-FTIR system

An IR spectrum of the sample is then obtained by measuring the intensity of the total internally reflected beam reaching the detector as a function of wavelength that probes the molecular vibrations in cellular compounds (such as polysaccharides, lipids, carbohydrates, protein, nucleic acids) and is sensitive to minute structural change, the unique fingerprint spectra thus obtained enable analytical discrimination between micro-organisms at both species and sub-species level[11,12]. The present study aims to evaluate ATR-FTIR for discrimination between clinical *Trichosporon* spp. isolated from India, which has not been attempted so far.

Materials &Methods

1. Strains and Clinical source

A total of 75 *Trichosporon* isolates (*T. asahii* (62), *T. inkin* (8), *Cutaneotrichosporon dermatis(*formerly *T. dermatis)* (3), *Trichosporon asteroids* (1), *Apiotrichum loubieri (*formerly *T. loubieri*, 1) isolated from various clinical samples (Table S1) were obtained from various medical institutes of India viz. National culture collection of pathogenic fungi (NCCPF), PGIMER, Chandigarh, Government Medical College & Hospital, Chandigarh, Jawaharlal Nehru Medical College & Hospital, Ajmer (Rajasthan) & Sri Ramachandra Medical College and Research Institute, Chennai (Tamilnadu). Isolates were preserved in 10% glycerol saline at -80º C till use. PCR (IGS-1 region) confirmed isolates already submitted to GenBank & some got published (n=22)[13] were selected for this study. Details shown in **Table: 1**. Culture stocks were thawed and sub-cultured on SDA containing chloramphenicol and gentamicin and incubated at 30ºC temperature for 48 hours for ATR-FTIR spectroscopy. Standard strains from different genera (*S. aureus* ATCC 6538, *Candida albicans* ATCC 90028, *C. krusei* ATCC 6258) were used as outliers for genus level discrimination.

Table 1: *Trichosporon* strains used in this study

S.N.	NCCPF No.	CLINICAL SOURCE	GEOGRAPHICAL REGION	MOLECULAR ID (IGS-1 region)	GENBANK ACCESSION NO.(IGS1 region) BankIt2463747
1	940120	Sputum	Ajmer	*T. asahii*	KT936601
2.	940122	Sputum	Ajmer	*T. asahii*	KT936603
3.	940013	Sputum	Kochi	*T. asahii*	KT936604
4.	940019	Sputum	Chandigarh	*T. asahii*	KT936605
5.	940123	Blood	Hyderabad	*T. asteroides*	KT936595
6.	940124	Blood	Hyderabad	*T. asahii*	KT936608
7.	940125	Blood	Hyderabad	*T. asahii*	KT936609
8.	940126	Blood	Delhi	*T. asahii*	KT936610
9.	940127	Blood	Lucknow	*T. asahii*	KT936612
10.	940128	Blood	Chennai	*T. asahii*	KT936613
11.	940129	Blood	Coimbatore	*T. asahii*	KT936614
12.	940130	Blood	Chandigarh	*T. inkin*	KT936596
13.	940131	Urine	Ajmer	*T. asahii*	KT936615
14.	940132	Blood	Hyderabad	*T. asahii*	KT936616
15.	940024	Hair	Chandigarh	*T. inkin*	KT936597
16.	940018	Blood	Chandigarh	*T. asahii*	KT936617
17.	940033	Wound tissue	Dehradun	*Cutaneotrichosporon dermatis*	KT936591
18.	940135	Hair	Thane. Maharashtra	*T. asahii*	KT936619
19.	940136	Nail	Delhi	*T. asahii*	KT936620
20.	940137	Blood	Chandigarh	*T. asahii*	MZ269003
21.	940005	Pus	Chandigarh	*T. asahii*	MZ269004
22.	940024	Hair	Hyderabad	*T. inkin*	-[1]
23.	940025	BAL	Hyderabad	*T. asahii*	MZ269005
24.	940068	Blood	Chandigarh	*T. asahii*	MZ269006
25.	940072	Blood	Chandigarh	*T. asahii*	MZ269007
26.	940138	Tissue	Pondicherry	*T. asahii*	MZ269008
27.	940139	Blood	Kolkata	*Cutaneotrichosporon dermatis*	MZ269055
28.	940140	BAL	Chandigarh	*T. asahii*	MZ269009
29.	940141	Blood	Chandigarh	*T. asahii*	MZ269010
30.	940142	Blood	Chennai	*T. asahii*	MZ269011
31.	940143	Urine	Chennai	*T. inkin*	MZ269050
32.	940144	Urine	Chennai	*T. asahii*	MZ269012
33.	940145	Urine	Chennai	*T. asahii*	MZ269013
34.	940146	Urine	Chennai	*T. inkin*	MZ269051
35.	940147	Urine	Chennai	*T. asahii*	MZ269014
36.	940148	Urine	Chennai	*T. asahii*	MZ269015
37.	940149	Sputum	Ajmer	*T. asahii*	MZ269016
38.	940150	Sputum	Ajmer	*T. asahii*	MZ269017
39.	940152	Urine	Ajmer	*T. asahii*	MZ269018
40.	940153	Sputum	Ajmer	*T. asahii*	MZ269019
41.	940154	Urine	Chandigarh	*T. asahii*	MZ269020
42.	940155	Urine	Chandigarh	*T. asahii*	MZ269021
43.	940156	Urine	Chandigarh	*T. asahii*	MZ269022
44.	940157	Urine	Chandigarh	*T. asahii*	MZ269023
45.	940158	Necrotized tissue	Chandigarh	*T. asahii*	MZ269024
46.	940160	Nail	Chandigarh	*Cutaneotrichosporon dermatis*	MZ269056
47.	940161	Nail	Chandigarh	*T. asahii*	MZ269026

[1] Based on BLASTn results, the isolate was identified as the species to which it was more closely related, although the percentage of reliability was 90.96% to type strain.

S.N.	NCCPF No.	CLINICAL SOURCE	GEOGRAPHICAL REGION	MOLECULAR ID (IGS-1 region)	GENBANK ACCESSION NO.(IGS1 region) BankIt2463747
48.	940162	Necrotized tissue	Chandigarh	*T. asahii*	MZ269027
49.	940163	Urine	Chandigarh	*T. asahii*	MZ269028
50.	940164	Nail	Chandigarh	*T. asahii*	MZ269029
51.	940165	Nail	Chandigarh	*T. inkin*	MZ269052
52.	940166	Pleural fluid	Chandigarh	*T. asahii*	MZ269030
53.	940167	Urine	Chandigarh	*T. asahii*	MZ269031
54.	940168	Urine	Chandigarh	*T. asahii*	MZ269032
55.	940169	Nail	Chandigarh	*T. inkin*	MZ269053
56.	940170	Urine	Chandigarh	*T. asahii*	MZ269033
57.	940171	Pleural fluid	Chandigarh	*T. asahii*	MZ269034
58.	940172	Sputum	Chandigarh	*T. asahii*	MZ269035
59.	940173	Mass left maxillary sinus	Chandigarh	*T. asahii*	MZ269036
60.	940175	Peritoneal fluid	Chandigarh	*T. asahii*	MZ269037
61.	940176	Sputum	Chandigarh	*T. asahii*	MZ269038
62.	940177	Tissue	Chandigarh	*T. asahii*	MZ269039
63.	940178	Sputum	Chandigarh	*T. asahii*	MZ269040
64.	940179	Tissue	Chandigarh	*T. asahii*	MZ269041
65.	940180	Nail	Chandigarh	*T. asahii*	MZ269042
66.	940181	Urine	Chandigarh	*T. asahii*	MZ269043
67.	940182	Nail	Chandigarh	*T. asahii*	MZ269044
68.	940183	Tissue	Chandigarh	*T. asahii*	MZ269045
69.	940184	Urine	Chandigarh	*T. asahii*	MZ269046
70.	940185	Pleural fluid	Chandigarh	*T. asahii*	MZ269047
71.	940186	Nail	Chandigarh	*T. inkin*	MZ269054
72.	940187	Pleural fluid	Chandigarh	*T. asahii*	MZ269048
73.	940188	Nail	Chandigarh	*T. asahii*	MZ269049
74.	940029	Wound discharge	Ajmer	*A. loubieri*	KT936693
75.	940189	Blood	Ahmedabad	*T. asahii*	KT936611

2. *Sample preparation for ATR-FTIR and spectral data acquisition:*

As FTIR spectra can reflect small variations due to culture parameters[14,15], the sample preparation conditions and culture method were standardized to maintain iso-ambient conditions during ATR-FTIR testing for all the isolates. A single colony was isolated using a sterile disposable loop and deposited directly onto the ATR sampling surface of ATR-FTIR spectrometer (Spectrum Two UATR, Perkin Elmer, Waltham, USA) **Fig.1**. A spectrum was immediately collected by co addition of 100 scans in the spectral range between 4,000-400 cm $^{-}$1 with a spectral resolution of 0.5 $cm^{-1}$and subtracted by a blank background spectrum previously collected from the clean sampling surface. Following the spectral acquisition, disinfection of the ATR sampling surface was achieved by wiping it with lint-free paper moistened with 70% ethanol. The process was repeated with three samples of each culture[16].

2.1 Pre- processing of raw spectra:

The Baseline Subtracted system-generated raw spectra (for each isolate in triplicate) were subjected to Pearson coefficient analysis for the removal of outlier spectrum among triplicates. Averaging the remaining spectra for the single isolate was done to obtain the final unsmoothed spectrum.

2.2 Spectral window selection:

Protein, lipids, and polysaccharides that make up the membrane, cell wall, and capsules have a significant impact on the systematics and phylogeny of the yeasts. The spectrum obtained in the 4000-400cm-^{1}region was divided into 4 windows viz. a). 900-400cm^{-1}, the fingerprint region. b). 1500-900cm^{-1} with dominant signatures of carbohydrates, c). 1800- 1500cm^{-1} the amide region, d). 4000-2800cm^{-1} representing water & lipid region and further analyzed.

2.3 Spectral smoothening and normalization:

The average spectra obtained in section 2.1 were smoothened using Savitsky-Golay algorithm with 9-point window along with second-degree polynomial (Jupyter notebook interface library versions- pandas 1.0.3, NumPy 1.19.5 & scipy 1.5.4in Python language) and then vector

normalized with the equation (Excel 2010).

Vector Normalization

The use of this method is widespread it is also called as 2-norm. In this, Mean-centered spectra are divided by the square root of the sum of the mean-centered intensities squared. In this way a spectrum is obtained in which the sum of all intensity values squared equals one. Practically, this mathematical operation is applied to a vector (row/column). This operation takes into account the calculation of the norm of a vector[16].

2.4 Derivative:

To further increase the numbers of discriminative features present in the spectra, Section 2.3 was also applied on second derivative of the average spectra of Section 2.1.

2.5 Hierarchical clustering & Principal component analysis:

The obtained spectra in section 2.3 and 2.4 were subjected to hierarchical cluster analysis (HCA) and Principal component analysis (PCA) in PAST software 4.03 showing classification based on spectral similarity on genus level, species level and then compared with results of DNA sequencing data obtained earlier.

2.6 ATR-FTIR spectroscopy-based strategy for creation & validation of spectral library:

Novel mathematical operation- Reference & Threshold (R&T) calculation

The spectral library was built using the concept of Euclidean distance as obtained in HCA. It starts with reference spectra calculation. Interestingly, the method given here uses the same approach for this analogy in 2 dimension & extends to multidimensional space to find mid-point (reference spectra) or closest to mid-point/center of all the available spectra. The selected reference spectrum is one spectrum representing the desired group of spectra with all random effects (microbiological, physical parameters & instrumentation error). In general, the effects of randomness on a group of spectra revolve around the reference spectrum (standard value). For example, using standard spectra (ATCC/ MTCC) along with sufficiently large group of clinical spectra of same species, the reference spectra calculation will yield reference spectra as standard spectra. The clinical spectra revolve around the standard spectrum due to random effects concluding reference spectra to be located at mid-point or near to mid-point always, proven by mathematical algorithm given in this study. Finally, this method used here to find out the reference spectra (in absence of standard spectra) is equivalent to the standard spectra (MTCC/ATCC) assuming spectra was homogeneously distributed and unbiased in the multidimensional space. Following selection of reference spectra, Threshold (T) was calculated for each desired group of spectra. The threshold computed here is the equivalent of radius of the circle in 2D space.

3. Creation & validation of spectral library using R & T calculation

Creation

A total of 70 *Trichosporon* isolates that have been identified using inter-genic spacer 1 (IGS-1) sequences along with outliers (*ATCC C. albicans, ATCC C. krusei, ATCC S. aureus*) were used to create a spectral library for genus level. Whereas spectral library for species level, total 60 T. asahii & 6 T. inkin isolates were included. Five isolates (also identified by IGS-1 sequencing PCR) for genus level and 9 isolates for species level were taken as the test group for validation set as shown in **Table: 2**. The spectral library was created in Microsoft Excel 2010 for this study.

Table: 2 Trichosporon strains used for spectral creation & test group in this study

Strains used for spectral library creation GenBank ID/NCCPF ID		Strains used for validation of spectral library (test group) GenBank ID	
Genus level (n=70+ 3)	Species level (n=66)	Genus level (n=5)	Species level (n=9)
KT936601, KT936603, KT936604, KT936605,KT93660,KT936609,KT936610, KT936612, KT936614, KT936615, KT936616, KT936617, KT936619, KT936620, MZ269003, MZ269004, MZ269005, MZ269006, MZ26900, MZ269008,MZ269009,MZ269010,MZ269011, MZ269050, MZ269012, MZ269013, MZ269051, MZ269014, MZ269015, MZ269016, MZ269017, MZ269018, MZ269019, MZ269020, MZ269021, MZ269022, MZ269023, MZ269024, MZ269026, MZ269027,MZ269028,MZ269029,MZ269030, MZ269031, MZ269032, MZ269053,MZ269033, MZ269034, MZ269035, MZ269036, MZ269037, MZ269038,MZ269039,MZ269040,MZ269041, MZ269042, MZ269043,MZ269044,MZ269045, MZ269046, MZ269047, MZ269054, MZ269048, MZ269049, KT936611, MZ269055,KT936693 KT936595, MZ269056, NCCPF 94.24 **Outliers used** (n=3)- ATCC 6538, ATCC 90028, ATCC6258	KT936601, KT936603, KT936604, KT936605, KT93660, KT936609,KT936610, KT936612, KT936614, KT936615, KT936616, KT936617, KT936619, KT936620, MZ269003, MZ269004, MZ269005, MZ269006, MZ26900, MZ269008, MZ269009, MZ269010,MZ269011, MZ269050, MZ269012, MZ269013, MZ269051, MZ269014, MZ269015, MZ269016, MZ269017, MZ269018, MZ269019, MZ269020, MZ269021, MZ269022, MZ269023, MZ269024, MZ269026, MZ269027, MZ269028, MZ269029, MZ269030, MZ269031, MZ269032, MZ269053, MZ269033, MZ269034, MZ269035, MZ269036, MZ269037, MZ269038, MZ269039, MZ269040, MZ269041, MZ269042, MZ269043, MZ269044, MZ269045, MZ269046, MZ269047, MZ269054, MZ269048, MZ269049, KT936611, NCCPF 94.24	KT936613 KT936596 KT936597 MZ269052 KT936591	KT936613, KT936596, KT936597, MZ269052, KT936591, MZ269055, KT936693, KT936595, MZ269056

In the world of mathematics, the shortest distance between two points in any dimension is called the Euclidean distance. This is the square root of the sum of squares of the difference between two points.

$$(d)_{xy} = \sqrt{\sum_{i=1}^{n} (y_i - x_i)^2}$$

Where d_{xy} =Euclidean distance, n= n dimensional space, x &y are two points in Euclidean in n dimensional space, x_i *and* y_i are the i^{th} elements of the x and y points w.r.t origin of the multidimensional space.

However the concept was then extrapolated to a k dimensional space; k is the number of points on the spectrum of an isolate (1802 points in the spectrum of 1800-900 cm-1, 601 points in 1200-900 cm-1). As the first step according to the practical approach, a spectrum $X_j = \{x_{1j}, x_{2j} \ldots\ldots x_{kj}\}$ was taken among the n isolates in a cluster and Euclidean distances (d) is calculated with another spectrum $X_i = \{x_{1i}, x_{2i} \ldots\ldots x_{ki}\}$:

$$(d)_{ij} = \sqrt{\sum_{a=1}^{k} (x_{ai} - x_{aj})^2}$$

Where d= Euclidean distance, k= dimensions, a= index of absorbance, i, j= corresponds to index of isolates, x= absorbance value.

Finally, the calculated Euclidean distance for X_j(j^{th} isolate) will be squared for all the isolates and summed to get the Distance (D_j) for j^{th}isolate separately for all n isolates in k dimensional space (k= 1802 points on the spectrum of an isolate):

$$D_j = \sum_{i=1}^{n} (d)^2{}_{ij} \; where \; i \neq j \, , n = number \; of \; isolates$$

Form the value of distances D obtained for the isolates, the spectrum of the isolate with a minimum value of D was considered as reference spectra for that particular group, as the

mid-point corresponds to the minimum value of D_j. Once the reference spectrum was obtained in a cluster let's say $X_j = \{x_{1j}, x_{2j} \ldots\ldots x_k\}$, the threshold (T) was obtained as:

$$Threshold\ (T) = \max\big((Euclidean\ Distance)_{ij}\big); for\ i = 1 \longrightarrow n$$

In this way the maximum value of d_{ij} for the reference spectra was considered as a threshold value (T) for the isolate to be identified at genus/species level.

The designed process was validated in two tiers viz. genus &species level using spectra of the organisms from the test group. In the first-tier validation, the ATR-FTIR spectra (pre-processed-filtered, vector normalized, un-derived) of isolates belonging to the test group (n=5 along with 3 outlier groups -*S. aureus*, *C. albicans & C. krusei*) were used. Using the above-mentioned process, the Euclidean distance (d) of selected reference spectrum to the test group was computed. The calculated threshold value (T) of reference spectra was compared to the d values of the test group isolates w.r.t reference spectrum. It should be within that T value to be classified in that particular genus as shown in **Table. 5**. In the second-tier species level validation, the second derivative, pre-processed- filtered & vector normalized ATR-FTIR spectra were used to build spectral library (n=60 *T. asahii*, n= 6 *T. inkin*). The test group composed of (n= 9) isolates and the same process of calculating the d values of the test group isolates with reference spectra (available species in the spectral library) was performed. Finally, the d values of all the test isolates were compared with threshold values of each species as shown in **Table.5&6**. The d values under the threshold indicate the similarity to the reference spectra. In this manner, species-level identification can also be done with the help of this process.

Results

IR spectra of the microbial spectrum under consideration exhibit absorption bands indicative of the molecular composition of cells, for which the tentative band assignments are given in **Table 3& Fig2.**

Table 3: Proposed functional group vibrational assignments for ATR-FTIR spectra of *Trichosporon, Candida* &*Staphylococcus* (as per literature)

Characteristic stretch/bend[a]	*Trichosporon*	*C. albicans* ATCC[44]	*S. aureus* ATCC [45]
C-O str. (Carbohydrate)	1035	1050	1033
PO_2 sym. str. (Nucleic acid)	1081	1089	1079
C-O-C str. (Glycosidic linkages)	1141	1150	-
C-N str. (Protein)	1404	1415	1403
CH deformation of >CH2 in lipids protein	-	-	1461
C=O str. Amide II (Protein)	1541	1550	1553
C=O str. Amide I Of β-pleated sheet structures of proteins	1630	1633,1652	1635
C=O str. (Lipid)	1740	1741	1733
CH_2, CH_3 str. sym (Lipids-fatty acids))	2849	2848	2863
CH2, CH3 str. asym (Lipids)	2917	2931, 2968	2943
C-H tr. (Lipids)	3000	3008	-
O-H str. Band of water	3286	3270	3275

a. asym. = asymmetric; sym. = symmetric; str. = stretching; def. = deformation.

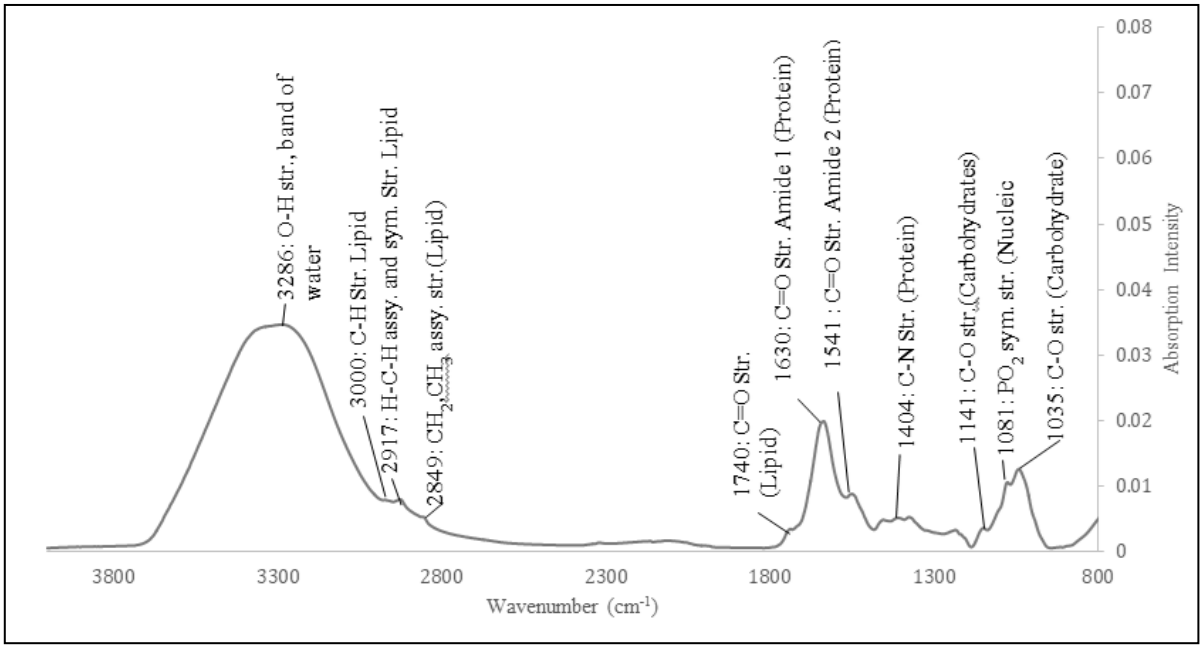

Figure 2: Proposed spectrum of *Trichosporon* in the region 4000-800 cm-1, after baseline correction and vector normalizations. The major absorption bands and their functional group are labelled

Classification of *Trichosporon* strains by ATR-FTIR spectroscopy using HCA &PCA

The results of unsupervised agglomerative clustering are depicted in the form of a dendrogram. Hierarchical clustering analysis (HCA) of smoothened vector normalized spectra of *Trichosporon* yeast over the complete range of 4000cm^{-1}-400cm^{-1} (especially due to 1800-900 cm-1) shows 3 distinct clusters which conclude at the genus level clustering of the organisms as analyzed by DNA sequencing methods as shown in **Fig.3**. The IR region 4000 cm^{-1}- 400 cm^{-1} is contributed by all the components of the cell and returns data along 7208 points when run on a bandwidth of 0.5 cm-1. To reduce the dimensionality of the dataset and understand the major spectral features contributing to clustering in the 1800-900 cm-1 region, the data were subjected to Principal Component Analysis (PCA). PCA score plot of the first and second component of ATR-FTIR spectra of fungal biomass grown on SDA is shown in **Fig.3** which shows a clear genus-specific clustering with the bacterium and the other yeasts lying outside the cluster. The explained variance by PC1 and PC2 is 64.5% & 27.7%.

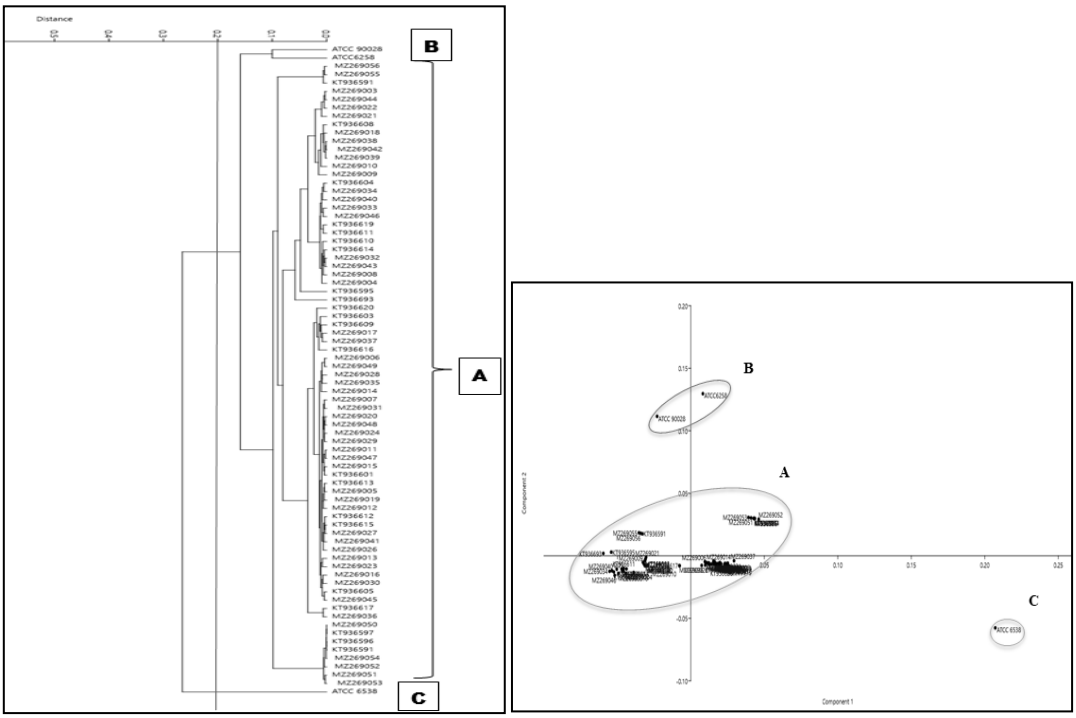

Figure 3: Hierarchical clustering analysis (HCA) & Principal component analysis (PC1, PC2-*score plot) of* smoothened vector normalized spectra of *Trichosporon* yeast over the range of 1800-900 cm^{-1} showing 3 main distinct clusters: [A] *all clinical isolates of Trichosporon,* [B] *ATCC Candida yeast,* [C] *ATCC Staphylococcus bacteria.*

The functional groups determining a cluster of the data are the C-O stretch of carbohydrate, amide I and amide II region of the protein, C=O stretch and aliphatic C-H stretch of lipid that cluster the *Trichosporon* strains into a single cluster away from the bacteria and other yeast. However, the spectra could not resolve the differences at the species level in un-derived spectra. Since IR spectra of complex biological systems are due to the overlapping spectral features of multiple components, an attempt was made to improve the resolution by second derivative analysis where band intensity is inversely proportional to the square of the original bands' half-width. Hierarchical cluster analysis followed by PCA was applied to the second derivative vector normalized ATR- FTIR spectra over the carbohydrate (1200-900 cm-1), protein window (1800-1500cm-1), and lipid window (3000-2200 cm-1). In the spectral window 900 –1800 cm^{-1}, several clusters could be distinguished, by inspecting the dendrogram; the following observations can be made when the heterogeneity threshold was fixed at 50% (D50), 4 groups can be distinguished in each group and different species can be delineated. In group-I *T. loubieri* clustered (**A)** whereas group-II clustered *T. inkin* **(B)**, group-III shows a large cluster of *T. asahii*(**C)**. In the last group-IV cluster **D** &cluster E both species *C. dermatis* and *T. asteroides* come together. There exist some misclassifications in the case of *T. asteroides* which were later checked with DNA sequencing results. So, the spectral window 1200-900 cm^{-1} was selected to create the final dendrogram to resolve misclassification as shown in **Fig.4**. As a result, 5 distinct clusters of all 5 species were used in this study. It can be concluded that using second derivative spectra of 1200-900 cm-1 carbohydrate window (100%, 75/75) is more conclusive than 1800-900 cm-1 mix carbohydrate & protein window (99%,74/75) to classify *Trichosporon* isolates up to their species-

level along with successful differentiation of *T. asteroids* with *C. dermatis* which was not classified using 1800-900cm-1 window. PCA score plot of the first and second component of ATR-FTIR spectra shown in **Fig.4** which displays a distinct clustering of five different species of *Trichosporon* and the explained variance by PC1, PC2& PC3is 61.81%, 19.69% & 10.13%.

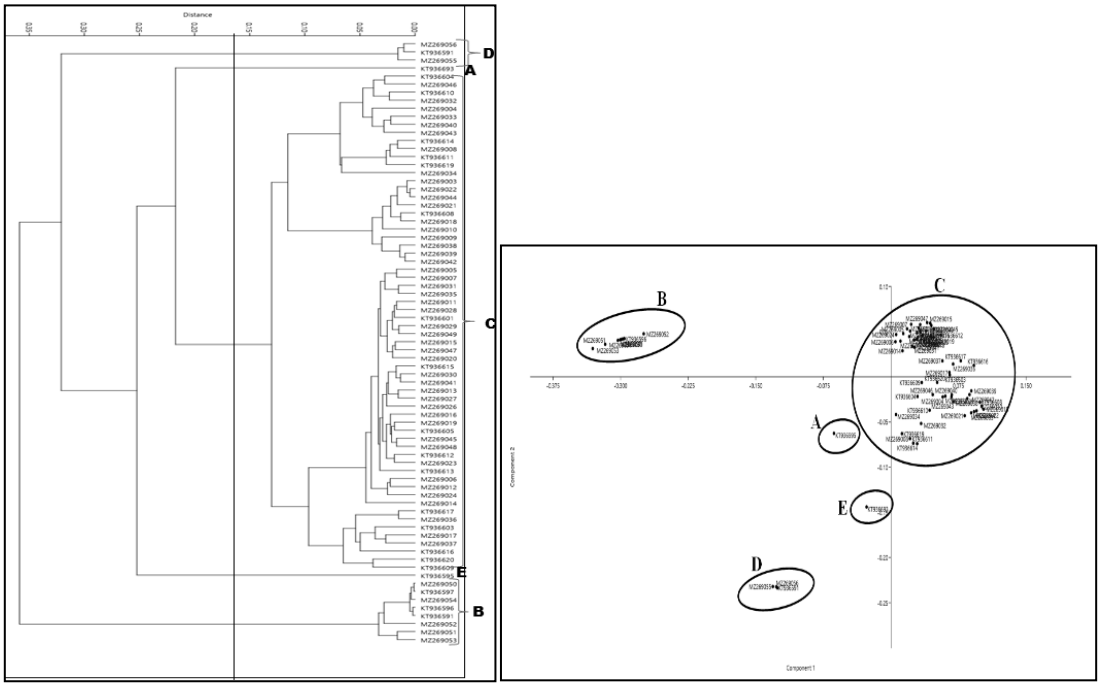

Figure 4: Hierarchical clustering analysis (HCA) & Principal component analysis (PC1 & PC2)- Score plot of smoothened vector normalized second derivative spectra of *Trichosporon* yeast over the range of 1200cm^{-1}- 900cm^{-1} showing 5 mains clusters: [A] *A. loubieri* [B] *T. inkin* [C] *T. asahii* [D] *C. dermatis*& [E] *T. asteroids*

Clustering data using statistical tools showed that the polysaccharide signatures (1200–900 cm^{-1}) were critical markers for species discrimination in this study. As ATR-FTIR was able to reproduce the species level variation comparable to sequencing results, shows the capability of the technique for differentiation of organisms up to this extent

Identification with a spectral library using designed Process

As already mentioned earlier, protein specific signatures 1800-900 cm-1 &carbohydrate specific signatures 1200-900cm-1 are significant region which were than used for R & T calculation on the un-derived/ second derivative spectral data from section 2 to provide routine fast identification of *Trichosporon species.*

Genus level identification (900-1800 cm^{-1})

As per the process defined in this study reference spectra (MZ269036) were selected on the basis of its minimum of D value and the threshold value (T= 0.0724) shows the classification on the test group as shown in **Table.4**&**Table.5**. All the test isolates were identified as *Trichosporon* species, confirming that they belong to same genus and extracted out other two genera.

Table 4: Reference and threshold calculation for genus level identification using Euclidean distance matrix of spectral library isolates (un-derived, vector normalized spectral absorbance values in cm-1).

Euclidean distance in between corresponding isolates(ATR-FTIR spectral library n=70+ 3 outliers)	1	2	3	4	.....	70	Outlier 1 (*S. aureus*)	Outlier 2 (*C. albicans*)	Outlier 3 (*C. krusei*)
1	0.0000	0.0200	0.0636	0.0625	.....	0.0734	0.2072	0.1264	0.1461
2	0.0200	0.0000	0.0708	0.0576	.....	0.0547	0.2245	0.1224	0.1498
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
70	0.0734	0.0547	0.1084	0.0644	.....	0.0000	0.2782	0.1191	0.1604
D(sum square)	**0.1148**	**0.0979**	**0.3996**	**0.3242**	.....	**0.3279**	**3.5477**	**1.0871**	**1.5756**
Minimum value of sum-square (D) is "RFERENCE SPECTRA (R)"									
Maximum value of Euclidean distance (d)in Reference spectra to spectra of other isolate is "THRESHOLD VALUE (T)"		*0.0724*							

Table 5 Validation of spectral library for genus level identification using Reference and threshold with test group (n=5) isolates. Euclidean distance calculation of Reference (*Trichosporon* genus) to test spectra along with outliers from different genus (*Staphylococcus, Candida*) (un-derived, vector normalized spectral absorbance values in cm-1).

Wave-length (cm-1) Protein & Carbohydrate window	Reference spectra-Genus *Trichosporon* (R)	TEST 1	TEST 2	TEST3	TEST 4	TEST 5	Outlier 1 (*S.aureus*)	Outlier2 (*C.albicans*)	Outlier 3 (*C.krusei*)
1800	0.0006	0.0008	0.0005	0.0018	0.0001	0.0001	0.0016	0.0012	0.0017
1799.5	0.0006	0.0008	0.0005	0.0018	0.0001	0.0001	0.0016	0.0012	0.0017
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
900.5	0.0007	0.0010	0.0007	0.0016	0.0001	0.0001	0.0016	0.0012	0.0017
900	0.0008	0.0010	0.0007	0.0016	0.0001	0.0001	0.0016	0.0012	0.0017
Euclidean distance(d) from Reference spectra	**0.0000**	***0.0294***	***0.0351***	***0.0567***	***0.0711***	***0.0709***	0.2245	0.1224	0.1498
Identification using Threshold value (T)= 0.07241	R	*Trichosporon*	*Trichosporon*	*Trichosporon*	*Trichosporon*	*Trichosporon*	NI	NI	NI[a]

[a] NI = Not identified

Species level identification (1200-900 cm^{-1})

The reference spectra for *T. asahii* (MZ269036) &*T. inkin* (MZ269054) was selected using the mentioned process. The threshold values for both the species were (T(*asahii*)=0.1137), (T(*inkin*)= 0.0601) were calculated in same way as shown in **Table.4**. The test group was processed along with the reference spectra of both the species one by one and with the help of threshold values all the isolates could be identified at the species level as shown in **Table.6**. The test group isolates were confirmed by IGS-1 region sequencing. This further explained that carbohydrate

signature itself has sufficient resolution to discriminate between *Trichosporon* at the species-level.

Table 6: Validation of spectral library for species level identification using Reference and threshold with test group (n=9) isolates. Euclidean distance calculation of Reference (*T. asahii & T. inkin species*) to test spectra (second derivative, vector normalized spectral absorbance values in cm^{-1}).

Wave-length (cm-1)	Refrence spectra-1 *Trichosporon asahii* (R1)	Refrence spectra-2 *Trichosporon inkin* (R2)	TEST 1	TEST 2	TEST3	TEST 4	TEST 5	TEST6	TEST 7	TEST 8	TEST 9
1200	-0.00584	-0.00317	-0.02555	-0.02436	-0.02645	-0.00093	-0.01381	-0.01000	-0.00307	-0.00312	-0.00444
1199.5	-0.00382	-0.00083	-0.02028	-0.01917	-0.02115	-0.00023	-0.01041	-0.00684	-0.00073	-0.00078	-0.00271
1199	-0.00167	0.00155	-0.01562	-0.01462	-0.01644	0.00044	-0.00731	-0.00357	0.00166	0.00161	-0.00087
1198.5	0.00056	0.00402	-0.01243	-0.01157	-0.01314	0.00102	-0.00524	-0.00026	0.00412	0.00407	0.00103
.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
901.5	0.00203	0.00289	-0.00236	-0.00212	-0.00257	0.00298	0.00009	0.00275	0.00294	0.00291	0.00243
901	0.00061	0.00248	-0.00295	-0.00284	-0.00302	0.00178	-0.00092	0.00134	0.00252	0.00250	0.00110
900.5	-0.00080	0.00202	-0.00292	-0.00303	-0.00276	0.00094	-0.00163	-0.00012	0.00204	0.00203	-0.00024
900	-0.00218	0.00152	-0.00215	-0.00246	-0.00180	0.00048	-0.00168	-0.00159	0.00152	0.00152	-0.00157
Euclidean distance(d) from Reference spectra-1	**R1**		0.33721	0.33674	0.34070	0.21712	0.27151	**0.09650**	0.36684	0.37031	0.05640
Identification using Threshold value of R1 (T)= 0.11371	→		NI	NI	NI	NI	NI	***T. asahii***	NI	NI	***T. asahii***
Euclidean distance(d) from Reference spectra-2	**R2**		0.35279	0.35350	0.35236	0.35011	0.35086	0.34583	**0.00809**	**0.00404**	0.40006
Identification using Threshold value of R2 (T)= 0.03233	→		NI	NI	NI	NI	NI	NI	***T. inkin***	***T. inkin***	NI

NI= Not Identified

The results clearly indicate the efficiency of this process for genus/ species level identification with the use of ATR-FTIR spectral libraries of defined pre-processed spectral data.

Discussion

Phenotypic examination and PCR were the most acceptable methods for identification of yeasts. However, phenotypic methods are not fast and reliable enough, and PCR is expensive. Recently, for rapid & eco-friendly cost effective diagnosis of bacteria and yeasts, use of FTIR spectroscopy has significantly increased[1,3,17]. To our knowledge, no prior study has evaluated ATR-FTIR spectroscopy for a large dataset of rare clinical yeast, *Trichosporon* and related genera. In this study the ATR-FTIR spectra exhibited well defined spectral regions that correspond to the vibration of a chemical constituent of the yeast cell.

In this study, the main observed features were characteristic vibrations occurred due to the peptide bond of proteins (Amide I in the 1700–1600 cm-1 and Amide II in the 1600–1480 cm-1), nucleic acids PO2 (1300-1180 cm-1) and carbohydrates (1200-900 cm-1) bands. The carbohydrate signatures were the most characteristic feature recorded for the species level clustering[18]. This can be explained by the fact that the members of *Trichosporon* genus express glucuronoxylomannan (GXM) in their cell walls. GXM is a 1,3-linked mannan backbone attached to short side chains of 1,4-linked mannose and 1,2-linked xylose residues by substituting the 2 or 4 portion of the 1,3-linked mannose residues of the main group[1,19].

For classification & identification, the spectral features were mined from the raw IR spectra with sequential preprocessing steps viz. Resolution enhancement by derivation, normalization, spectral window

selection, feature selection and pattern recognition methods (cluster analysis and artificial neural network (ANN)[20], partial least square regression (PLSR)[21] or partial least square discriminant analysis (PLSDA)) which is in line with other reported studies. In data preprocessing steps, second derivative Savitzky–Golay smoothing/derivative, filtering, normalization & pre-selection of spectral windows carrying specific spectral information was performed which is consistent with other literatures[14,16,22,23]. Exploratory data analysis has revealed hidden patterns in complex spectral data by reducing the information to a more logical form and showing whether there are patterns or trends in the data. Exploratory algorithms applied, such as HCA and PCA, were involved to reduce large complex data sets into a series of optimized and interpretable views. In HCA, dendrograms showed distinct clusters of 5 different species of genus *Trichosporon* and related genera considering the spectral window of protein & carbohydrate functional group[14,16,22]. The subsequent confirmation of clustering was done with the PCA of the un-derived & derived spectra to reorganize information in a data set of samples. The results showed differences existing between the spectral profiles of study isolates at genus & species level. Five distinct clusters were recognized, which reliably discriminate between the five different species of *Trichosporon*, as established by molecular genetic techniques (Sequencing PCR). This confirms that the PCA is well suited to explore important information from ATR-FTIR spectra for identification in corroboration with others[24-26].

The key factor for obtaining successful identifications highly depends on the choice of appropriate and powerful techniques for data analysis. Supervised and unsupervised methods can be employed but with certain limitations as experienced in this study which are consistent with literature[27]. Although unsupervised methods like HCA or PCA are used to differentiate or checking reproducibility of measurements and the overall separation of groups (with and without a priori knowledge)[28-30], in general, differentiation and identification are challenging in the case of HCA for analyzing complex data sets because it is computationally demanding and a virtual clustering approach is used. In this process, two spectra with the shortest inter-spectral distance are calculated and merged to form a new cluster; the distances between the new cluster and the other spectra are then examined until the final clustering is completed. Furthermore, it cannot provide an objective criterion of best partitioning because the number of classes must be decided by the user, whether the partition achieved will be helpful or not, and the best HCA linkage algorithm must be identified by observation and experimentation[31,32].Whereas PCA is highly sensitive to small changes in number of variables used[33]. Supervised techniques are recommended for identification, but they necessitate prior knowledge of huge and thorough reference databases. As a result, very high accuracy is usually achieved at the expense of robustness[31,34]. However, in this study the number of strains collected was too small to allow a separation into supervised models.

In light of the aforementioned limitations, a novel mathematical operation (Reference & Threshold (R&T)) is proposed here. This is all about calculating the reference spectra and threshold value using pre-processed spectroscopic data. It has been demonstrated here that using calculated reference spectra and thresholds for each species, it is possible to classify and identify unknown spectra with accuracy and reliability. Using this method & so formed spectral library can help in rapid and accurate identification of the clinical strains of *Trichosporon* and related genera

in routine laboratory. Overall in this study it was very encouraging that 100% correct identification was achieved at both genus level and species level, respectively, which is consistent with other literatures[35,36]. Our results show the promising candidature of FTIR spectroscopy in the rapid, cost effective, reliable identification of species of *Trichosporon* & related genera. Similarly, Sandt *et* al.,[37] that FT-IR spectroscopy is potent enough to identify *C. albicans* with a high sensitivity. FTIR micro-spectroscopy was reported to identify micro-colonies of *Candida* species[38]. Pebutowa *et* al.,[39] demonstrated the feasibility of the ATR-FTIR technique for intra-species comparison of three *Candida* species with three sample preparations methods. Some researchers worked on large group of yeast isolates but very less number (n= 4 out of 128 veterinary & non clinical), (n= 10 out of 263 clinical) of *Trichosporon* strains were used[18,35]. FTIR competes strongly with other high-throughput techniques as a spectroscopic technique. Mass-based techniques such as matrix-assisted laser desorption/ionization time of light (MALDI-TOF MS) are potential alternative in term of accurate identification although the mass spectrometers are still very expensive and require costly reagents and specialized maintenance compared to infrared spectrometers[40-42]. All these problems greatly increase the cost and time of the analysis, comparing ATR-FTIR technique in which no reagents are required, and it is a single-step protocol employed for the analysis of clinical isolates makes this technology both superior and more cost-effective than any currently available identification platform, with the added advantage of requiring no chemical processing and hence eco-friendly. The new distance calculation method achieves easy identification and better generalization ability.

This study has confirmed that ATR-FTIR spectroscopy in combination with suitable mathematical computing methods especially R & T calculation as used in this work is a promising diagnostic tool. is a promising diagnostic tool suitable for large-scale screening for *Trichosporon* spp and other yeasts, because of its high differentiability, reliability, simplicity, the avoidance of chemicals (i.e. costs and environmental impact), rapidity (less than 1 minute) as compared to conventional/genetic molecular and MALDI-TOF mass spectrometry[24,25,43].

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Reference:

1. Colombo AL, Padovan ACB, Chaves GM. Current Knowledge of Trichosporon spp. and Trichosporonosis. Clinical Microbiology Reviews. 2011 Oct 1;24(4):682–700.
2. Iturrieta-González IA, Padovan ACB, Bizerra FC, Hahn RC, Colombo AL. Multiple Species of Trichosporon Produce Biofilms Highly Resistant to

Triazoles and Amphotericin B. PLOS ONE. 2014 Oct 31;9(10):e109553.
3. Sugita T, Nakajima M, Ikeda R, Matsushima T, Shinoda T. Sequence Analysis of the Ribosomal DNA Intergenic Spacer 1 Regions of Trichosporon Species. Journal of Clinical Microbiology. 2002 May 1;40(5):1826–30.
4. Liu X-Z, Wang Q-M, Theelen B, Groenewald M, Bai F-Y, Boekhout T. Phylogeny of tremellomycetous yeasts and related dimorphic and filamentous basidiomycetes reconstructed from multiple gene sequence analyses. Stud Mycol. 2015 Jun;81:1–26.
5. Girmenia C, Pagano L, Martino B, D'Antonio D, Fanci R, Specchia G, et al. Invasive Infections Caused by Trichosporon Species and Geotrichum capitatum in Patients with Hematological Malignancies: a Retrospective Multicenter Study from Italy and Review of the Literature. Journal of Clinical Microbiology. 2005 Apr 1;43(4):1818–28.
6. Suzuki K, Nakase K, Kyo T, Kohara T, Sugawara Y, Shibazaki T, et al. Fatal Trichosporon fungemia in patients with hematologic malignancies. Eur J Haematol. 2010 May;84(5):441–7.
7. Raoult D, Fournier PE, Drancourt M. What does the future hold for clinical microbiology? Nat Rev Microbiol. 2004;2(2):151–9.
8. Maquelin K, Choo-Smith L-P, Endtz HP, Bruining HA, Puppels GJ. Rapid Identification of Candida Species by Confocal Raman Microspectroscopy. J Clin Microbiol. 2002 Feb;40(2):594–600.
9. O'hara CM. Manual and automated instrumentation for identification of Enterobacteriaceae and other aerobic gram-negative bacilli. Clin Microbiol Rev. 2005 Jan;18(1):147–62.
10. Pfaller MA. Molecular approaches to diagnosing and managing infectious diseases: practicality and costs. Emerg Infect Dis. 2001;7(2):312–8.
11. Lam LMT, Dufresne PJ, Longtin J, Sedman J, Ismail AA, Warnock DW. Reagent-Free Identification of Clinical Yeasts by Use of Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy. Journal of Clinical Microbiology. 2019;57(5):e01739-18.
12. Smith BC. Fundamentals of Fourier Transform Infrared Spectroscopy. [Internet]. 2nd ed. CRC Press.
13. Rastogi V, Honnavar P, Rudramurthy SM, Pamidi U, Ghosh A, Chakrabarti A. Molecular characterisation and antifungal susceptibility of clinical Trichosporon isolates in India. Mycoses. 2016 Aug;59(8):528–34.
14. Helm D, Labischinski H, Schallehn G, Naumann D. Classification and identification of bacteria by Fourier-transform infrared spectroscopy. J Gen Microbiol. 1991 Jan;137(1):69–79.
15. Choo-Smith L-P, Maquelin K, van Vreeswijk T, Bruining HA, Puppels GJ, Thi NAN, et al. Investigating Microbial (Micro)colony Heterogeneity by Vibrational Spectroscopy. Appl Environ Microbiol. 2001 Apr;67(4):1461–9.
16. Lasch P. Spectral pre-processing for biomedical vibrational spectroscopy and microspectroscopic imaging. In: Chemometrics and Intelligent Laboratory Systems. Robert Koch-Institut, Biologische Sicherheit; 2012.
17. Walsh TJ, Groll A, Hiemenz J, Fleming R, Roilides E, Anaissie E. Infections due to emerging and uncommon medically important fungal pathogens. Clin Microbiol Infect. 2004 Mar;10 Suppl 1:48–66.
18. Taha M, Hassan M, Essa S, Tartor Y. Use of Fourier transform infrared spectroscopy (FTIR) spectroscopy for rapid and accurate identification of Yeasts isolated from human and animals. International Journal of Veterinary Science and Medicine. 2013 Jun;1(1):15–20.
19. Mizobe T, Ando M, Yamasaki H, Onoue K, Misaki A. Purification and

characterization of the serotype-specific polysaccharide antigen of Trichosporon cutaneum serotype II: a disease-related antigen of Japanese summer-type hypersensitivity pneumonitis. Clinical & Experimental Allergy. 1995;25(3):265–72.
20. Udelhoven T, Naumann D, Schmitt J. Development of a Hierarchical Classification System with Artificial Neural Networks and FT-IR Spectra for the Identification of Bacteria. Appl Spectrosc. 2000 Oct;54(10):1471–9.
21. Oust A, Møretrø T, Kirschner C, Narvhus JA, Kohler A. FT-IR spectroscopy for identification of closely related lactobacilli. Journal of Microbiological Methods. 2004 Nov 1;59(2):149–62.
22. Helm D, Labischinski H, Naumann D. Elaboration of a procedure for identification of bacteria using Fourier-Transform IR spectral libraries: a stepwise correlation approach. Journal of Microbiological Methods. 1991 Nov 1;14(2):127–42.
23. Paulina Z, Lukasz L, Grzegorz C, Wiesław K. Fourier Transform Infrared Spectroscopy (FTIR) as a Tool for the Identification and Differentiation of Pathogenic Bacteria. Current Medicinal Chemistry. 2015 May 1;22(14):1710–8.
24. Pandey KK, Pitman AJ. FTIR studies of the changes in wood chemistry following decay by brown-rot and white-rot fungi. International Biodeterioration & Biodegradation. 2003 Oct;52(3):151–60.
25. Szeghalmi A, Kaminskyj S, Gough KM. A synchrotron FTIR microspectroscopy investigation of fungal hyphae grown under optimal and stressed conditions. Anal Bioanal Chem. 2007 Feb 19;387(5):1779–89.
26. Jilkine K, Gough KM, Julian R, Kaminskyj SGW. A sensitive method for examining whole-cell biochemical composition in single cells of filamentous fungi using synchrotron FTIR spectromicroscopy. Journal of Inorganic Biochemistry. 2008 Mar 1;102(3):540–6.
27. Lasch P, Naumann D. Infrared Spectroscopy in Microbiology. In: Encyclopedia of Analytical Chemistry [Internet]. American Cancer Society; 2015 [cited 2021 Sep 18]. p. 1–32.
28. Samelis J, Bleicher A, Delbès-Paus C, Kakouri A, Neuhaus K, Montel M-C. FTIR-based polyphasic identification of lactic acid bacteria isolated from traditional Greek Graviera cheese. Food Microbiology. 2011 Feb 1;28(1):76–83.
29. Tindall BJ, Brambilla E, Steffen M, Neumann R, Pukall R, Kroppenstedt RM, et al. Cultivatable microbial biodiversity: gnawing at the Gordian knot. Environmental Microbiology. 2000;2(3):310–8.
30. Santos MCD, Morais CLM, Lima KMG. ATR-FTIR spectroscopy for virus identification: A powerful alternative. Biomedical Spectroscopy and Imaging. 2020 Jan 1;9(3–4):103–18.
31. Wenning M, Scherer S. Identification of microorganisms by FTIR spectroscopy: perspectives and limitations of the method. Appl Microbiol Biotechnol. 2013 Aug;97(16):7111–20.
32. Lasch P, Naumann D. Infrared Spectroscopy in Microbiology. In: Encyclopedia of Analytical Chemistry [Internet]. American Cancer Society; 2015. p. 1–32.
33. Jolliffe IT, Cadima J. Principal component analysis: a review and recent developments. Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences. 2016 Apr 13;374(2065):20150202.
34. Vogt S, Löffler K, Dinkelacker AG, Bader B, Autenrieth IB, Peter S, et al. Fourier-Transform Infrared (FTIR) Spectroscopy for Typing of Clinical Enterobacter cloacae Complex Isolates. Frontiers in Microbiology. 2019; 10:2582.

35. Lam LMT, Dufresne PJ, Longtin J, Sedman J, Ismail AA. Reagent-Free Identification of Clinical Yeasts by Use of Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy. Warnock DW, editor. J Clin Microbiol. 2019 Feb 20;57(5):e01739-18.
36. Wang Y-Y, Li J-Q, Liu H-G, Wang Y-Z. Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR) Combined with Chemometrics Methods for the Classification of Lingzhi Species. Molecules. 2019 Jan;24(12):2210.
37. Sandt C, Sockalingum GD, Aubert D, Lepan H, Lepouse C, Jaussaud M, et al. Use of Fourier-transform infrared spectroscopy for typing of Candida albicans strains isolated in intensive care units. J Clin Microbiol. 2003 Mar;41(3):954–9.
38. Essendoubi M, Toubas D, Bouzaggou M, Pinon J-M, Manfait M, Sockalingum GD. Rapid identification of Candida species by FT-IR microspectroscopy. Biochim Biophys Acta. 2005 Aug 5;1724(3):239–47.
39. Pebotuwa S, Kochan K, Peleg A, Wood BR, Heraud P. Influence of the Sample Preparation Method in Discriminating Candida spp. Using ATR-FTIR Spectroscopy. Molecules [Internet]. 2020;25(7). Available from: https://www.mdpi.com/1420-3049/25/7/1551.
40. Pinto A, Halliday C, Zahra M, Hal S van, Olma T, Maszewska K, et al. Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry Identification of Yeasts Is Contingent on Robust Reference Spectra. PLOS ONE. 2011 Oct 13;6(10):e25712.
41. Ghosh AK, Paul S, Sood P, Rudramurthy SM, Rajbanshi A, Jillwin TJ, et al. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry for the rapid identification of yeasts causing bloodstream infections. Clin Microbiol Infect. 2015 Apr;21(4):372–8.
42. Roberts AL, Alelew A, Iwen PC. Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry to differentiate between Candida albicans and Candida dubliniensis. Diagn Microbiol Infect Dis. 2016 May;85(1):73–6.
43. Heraud P, Caine S, Sanson G, Gleadow R, Wood BR, McNaughton D. Focal plane array infrared imaging: a new way to analyse leaf tissue: Methods. New Phytologist. 2007 Jan;173(1):216–25.