Potential of FTIR spectroscopy in chemical characterization of *Termitomyces* Pellets

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**ABSTRACT**

Potential of Fourier-Transform Infrared (FTIR) spectroscopy was assessed and Hierarchical Cluster Analysis (HCA) was applied over entire range of spectra for determining characteristic chemical compositional similarity of 11 different *Termitomyces* Heim strains using 20 days old pelletized dry biomass produced under submerged shaken condition at 28±1°C. Four dominant spectral windows showing C-H, O-H stretching region, amide I, amide II and polysaccharide regions were identified displaying characteristic variable bands across species at 3400-3200 cm\(^{-1}\), 2900-2850 cm\(^{-1}\), 2350-2215 cm\(^{-1}\), 1750 cm\(^{-1}\), 1585-1547 cm\(^{-1}\), 1375-1315 cm\(^{-1}\) and 900-725 cm\(^{-1}\). The HCA dendrogram showed formation of two major clusters based on their presumptive chemical similarity.

1. **INTRODUCTION**

The identification of fungi by traditional phenotypic methods and molecular methods requires special laboratory skills and expertise, besides being time consuming and expensive [1]. Fourier-transform infrared (FTIR) spectroscopy has been known to be a very promising method to characterize biological samples by their chemical composition and provides qualitative and quantitative estimates of lipids, polysaccharides, nucleic acids, proteins [2, 3]. FTIR spectrum is considered as a global “molecular fingerprint” which can be used for characterization, differentiation and identification of microorganisms [1] and has been widely applied for identification of bacteria [3-5], yeast, filamentous fungi [6, 7] and also some mushrooms [8-10]. Various fungal genera have been identified by using dry spores, fruit bodies or cultural biomass as source material for FTIR. These include *Aspergillus* [2], *Mucor* [11], *Penicillium* [2, 12], *Memnoniella*, *Fusarium* [12] and dermatophytes [13] and certain wood fungi [14], food spoilage fungi [15] and mushrooms genera such as *Agaricus*, *Amanita*, *Lactarius*, *Macrolepiota* and *Pleurotus* [8]. Fungal cell wall structure and chemical composition is found to be diverse from species to species and thus characterization of fungi by their cell walls is generally thought to be very difficult [16]. Chitin, a polymer of the acetylated amino N-Acetylgalactosamine in which the subunits are linked by β (1→4)-a glycosidic bond is a major chemical component of fungal cell walls. Other main fungal wall constituents are lipids, glycoproteins, cellulose, β glucan, mannan, chitosan and other polysaccharides [16, 17]. *Termitomyces* Heim is most popular and highly priced edible mushroom genus in Africa and Asia. *Termitomyces* species are known to have high nutritive value [18, 19] and also contain novel neurotogenic cerebrosides [20, 21]. However domestication of *Termitomyces* has failed due to its complex mutualistic nature with fungus growing termites Macrotermintae [22] thus, requiring approaches like submerged fermentation. In order to standardize *Termitomyces* submerged fermentation process there is need to characterize its pellet biomass for future validation of its purity and chemical signature. *Termitomyces* pellets are known to represent 3D heterogeneity [23]. Also *Termitomyces clypeatus* pelletized biomass has also been used for metal biosorption of Chromium from waste water [24-27] thus indicating need to understand its chemical profiling across different species. Consistent with previous attempts to use FTIR spectroscopic technique for characterization of viable fungal biomass this work was aimed at its application to determine presence or absence of distinct chemical signatures from submerged growth conditions, aiding in chemical characterization of different *Termitomyces* species.

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2. MATERIALS AND METHODS

2.1 Fungal Strains and Cultivation Conditions

In all 11 pure mycelial cultures of *Termitomyces* were isolated from sterile context tissues of fresh fruit bodies of six species. The purity of these cultures was checked macro and microscopically [28-34]. These were *T. albuminusus* (TAL1, TAL2), *T. striatus* (TSTR), *T. aurantiacus* (TAUR), *T. heimii* (THE2), *T. globulus* (TGLO), and *T. clypeatus* (TCL1, TCL2, TCL3, TCL4, TCL5). These cultures were maintained on Malt Extract Agar (20 g L\(^{-1}\) Agar bacteriological grade; 20 g L\(^{-1}\) Malt extract powder, HiMedia) in slants at 28°C in Goa University Fungus Culture Collection (WFCC Reg. no. 946).

2.2 Preparation of Fungal Pellets

Ten plugs of 5 mm diameter each excised from old colonies growing on Czapek Dox Agar plates containing 5 g L\(^{-1}\) Sucrose, as carbon source for 6 days were transferred to flasks in triplicates which contained 100 ml of Czapek Dox Solution with 5 g L\(^{-1}\) Sucrose and incubated at 28±1°C, pH 5.5, dark for 7 days at 150 rpm. The fungal biomass was obtained by centrifugation at 5000 rpm for 20 min in sterile centrifuge tubes and washed thrice with sterile distilled water. The biomass was resuspended into 100 ml sterile distilled water in Erlenmeyer flasks containing 100 sterile glass beads of 3.5-4.5 mm diameter and kept for maceration on orbital shaker at 300 rpm for 20 min.

The fragmented mycelial suspensions, 1% v/v, from biomass was added to flask with 100 ml Czapek Dox liquid medium with pH 5.5 and incubated on shaker at 28±1°C in dark at 150 rpm for 20 days.

2.3 FTIR Analysis

The pelletized biomass was washed with sterile distilled water and dried in an oven maintained at 75°C for 48 h. Dried composite of whole pelletized biomass was macerated using mortar and pestle. Samples of 1 mg were mixed with 100 mg of spectroscopic grade KBr, HiMedia. The FTIR spectra were determined between 4000 and 400 cm\(^{-1}\) using a Shimadzu IR Prestige 21 with the following parameters: Spectral resolution 4 cm\(^{-1}\), 40 scans min\(^{-1}\), encoding interval 1 cm\(^{-1}\), Happ-Genzel apodization and scanning speed 2.8 mm s\(^{-1}\).

2.4 Multivariate Statistical Analysis

These spectra were analyzed for signal processing procedure by smoothing on spectral second derivatives using Savitzky-Golay method with 9 points of window using Origin version 8 (OriginLab Corporation) graphing and analysis software [35] and the multivariate statistical analyses were performed using Hierarchical Cluster Analysis (HCA) using SYSTAT software version 13 (Systat Software Inc, Chicago, IL).

Cluster analysis easily classifies data into groups which helps to show similarities and is widely used for rapid differentiation and classification of spectral data of microorganisms [3].

3. RESULTS AND DISCUSSION

This is first report on application of FTIR for genus *Termitomyces* for following species *T. albuminusus, T. striatus, T. aurantiacus, T. heimii, T. globulus* and *T. clypeatus*. Most of the work reported in literature is done with natural fruitbodies and solid state cultural mat but rarely on pellet biomass produced by liquid submerged fermentation. Thus there are differences in metabolic cultures grown on solid and liquid cultures. Fig. 1 shows composite representation of FTIR spectra for 11 different *Termitomyces* strains belonging to six different species. Chemically significant regions of FTIR called spectral windows were identified which included fatty acid region dominated by C-H (3450–2850 cm\(^{-1}\)); amide region dominated by C=O amide I and N-H amide II bands of proteins and peptides (1800–1500 cm\(^{-1}\)); Mixed region (1500–1200 cm\(^{-1}\)); polysaccharides region (1200–900 cm\(^{-1}\)); true finger printing region (900 to 700 cm\(^{-1}\)) [24, 36-40]. Detailed FTIR bands assignments of characteristic infrared bands across *Termitomyces* species are shown in Table 1.

![FTIR spectra of *Termitomyces* species with some characteristic dominant spectral windows.](image)

**Table 1**: Assignment of Infrared absorption bands for *Termitomyces* species [24, 37-40].

<table>
<thead>
<tr>
<th>Frequency (cm(^{-1}))</th>
<th>Band Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>3400-3200</td>
<td>O-H stretching vibration of hydroxyl groups, Amine N-H stretching</td>
</tr>
<tr>
<td>2900-2850</td>
<td>CH(_3), CH(_2) stretching</td>
</tr>
<tr>
<td>2350-2215</td>
<td>C=C alkyne stretching</td>
</tr>
<tr>
<td>1750</td>
<td>C=O carbonyl stretching of esters</td>
</tr>
<tr>
<td>1658-1625</td>
<td>Amide I, chitin</td>
</tr>
<tr>
<td>1582, 1550, 1547</td>
<td>N-H bending -Amide II, chitosan</td>
</tr>
<tr>
<td>1450-1425</td>
<td>O-H bending polysaccharide</td>
</tr>
<tr>
<td>1375-1315</td>
<td>O-H bending polysaccharide, Amide III</td>
</tr>
<tr>
<td>1250-1025</td>
<td>C-O bond, β (1→3) glucan, cell wall polysaccharide</td>
</tr>
<tr>
<td>900</td>
<td>β (1→6) glucan, Finger print region</td>
</tr>
<tr>
<td>810-725</td>
<td>-N-H wag, Finger print region</td>
</tr>
</tbody>
</table>

FTIR spectral bands of pellet biomass were found to be contributing characters of three physiological different regions. Central pellet zone (chemical zone-I) consisting of less oxygen tension, limited energy metabolism leading to autolysis, cell wall degeneration, vacuolation and also secondary metabolite
production [41, 42]. Intermediate pellet zone (chemical zone–II) is with less or partial degeneration of chitin microfibrils, cell wall glycoproteins and beginning of some secondary metabolite production. Barberel and Walker [41] have modified the zones of hypothetical fungal pellet from Trinci [43]. According to Barry and Williams [42] and Bizukojc and Ladakowicz [44] the illustrations of pellet morphology indicates the densely stained central pellet zone as inactive layer. Pirt [45] claimed that when the pellet diameter exceeds certain value, the growth is limited to a certain thickness at peripheral zone and it depends on diffusion coefficient and nutrient availability. Outer pellet zone (chemical zone–III) known as active layer consists of high oxygen availability and nutrient uptake indicating fresh cell wall material [42], de novo chitin, chitosan biosynthesis, no secondary metabolite production [46] and healthy cytoplasm. The vacuolated cells are metabolically different than the actively growing apical and subapical cells and they are known for the production of secondary metabolites [47]. Thus it is assumed that the spectral chemotypes of Termitomyces pellets could be directly proportional to the contributions from all these three pellet zones.

![Diagram](image)

**Fig. 2**: The dendrogram indicates the difference between the species of Termitomyces.

Fig. 2 shows the dendrogram calculated by cluster analysis, where the separation shows clear heterogeneity distances among Termitomyces species forming two cluster groups that occur about the same horizontal distance of 0.053. TGLO forms outlier to the two sister clade resolved at distance 0.018. Different species of Termitomyces showed heterogeneity below 0.06 (Table 2). The distance measures were comparably lower in TAU, TAL1, TAL2, TCL1, TCL2, TCL4, TCL5 and TSTR indicating less heterogeneity than THE2, TAUR and TGLO. The FTIR spectral chemometrics of different Termitomyces species showed sub grouping with other species may be due to heterogeneity of chemical components in different pellet zones. Grouping various strains to form clusters representing species or genera based on taxonomical classification is not always satisfactory by using physiological and morphological characters [48, 49]. The chemical heterogeneity between these species may be affected by the geographic origin, morphology, physiology of culture and ability for metabolite production. The FTIR spectral cluster analysis helped in grouping chemically related species together indicating strains could be grouped based on their comparably higher similarity in metabolite production [2]. According to previous reports by Kummerle et al., [50] and Lecellier et al., [1] HCA of spectra from different species of the same genus and also strains from the same species generally did not cluster together. Thus we can conclude that the use of FTIR spectroscopy for taxonomic purposes is limited but it does not prevent it from being a powerful identification system based on spectral chemotypes [50].

### Table 2: Distance Metric - Pearson Correlation Coefficient Ward Minimum Variance Method.

<table>
<thead>
<tr>
<th>Cluster Joining</th>
<th>At Distance No. of Members</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAU</td>
<td>TAL2</td>
</tr>
<tr>
<td>TCL5</td>
<td>TCL2</td>
</tr>
<tr>
<td>TCL1</td>
<td>TAL1</td>
</tr>
<tr>
<td>TCL4</td>
<td>TSTR</td>
</tr>
<tr>
<td>TCL3</td>
<td>TCL1</td>
</tr>
<tr>
<td>TCL4</td>
<td>THE2</td>
</tr>
<tr>
<td>TAUR</td>
<td>TCL4</td>
</tr>
<tr>
<td>TCL3</td>
<td>TAUR</td>
</tr>
<tr>
<td>TCL5</td>
<td>TCL3</td>
</tr>
<tr>
<td>TGLO</td>
<td>TCL5</td>
</tr>
</tbody>
</table>

## 4. CONCLUSION

The FTIR spectroscopic technique coupled with chemometric methods found principal advantage in understanding chemotypic similarity between different Termitomyces species in 3-D pelletized form but may not be helpful to classify strains based on their taxonomic ranking.

## 5. ACKNOWLEDGEMENTS

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## Conflict of Interests: There are no conflicts of interest.

## 6. REFERENCES


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