

Diffuse reflectance infrared fourier transform spectroscopic (DRIFTS) investigation of *E.coli*, *Staphylococcus aureus* and *Candida albicans*.

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The present study consists of a refined method for obtaining Diffuse Reflectance Infrared Fourier Transform Spectroscopic (DRIFTS) data for biological samples in the mid infrared region (4000 - 600 cm^{-1}). The biological cells used in the study included identified clinical strains of *E. coli* (Gram negative bacterium), *Staphylococcus aureus* (Gram positive bacterium) and *Candida albicans* (yeast). The method for obtaining DRIFTS data is described herein, which may be useful for studying the biochemical composition of microbial cells as well as for species-specific identification. The raw spectrum for each culture was treated using various algorithms (Kubelka Munk algorithm and Savitzky-Golay algorithm) and converted into its second derivative (2D). Hierarchical cluster analysis of 2D data, using Ward's algorithm produced dendrogram, which was distinct for each strain under study. Principal component analysis provided clusters of groups used in the study.

[Keywords: Fourier transform spectroscopy; biochemical composition; biological samples; *E. coli*; *C. albicans*; *S. aureus*]

Introduction

The use of infrared spectroscopy in identifying various functional groups in organic compound is known for more than six decades. However, using this technique to identify biological samples has developed very recently. Naumann and Co-workers were the first to employ FTIR spectroscopy in the mid IR range of dried films of bacteria for their identification^{1,4}. Numerous studies have since been reported on the identification of medically relevant microorganisms by vibrational spectroscopy⁵, discrimination of bacterial species using the same principle⁶, and characterization of various microorganisms using FTIR technique combined with multivariate statistical analysis or chemometrics⁷⁻¹⁵

Infrared spectroscopy, also called vibrational spectroscopy, measures bending and stretching vibrations of molecules having specific functional groups that are excited by an infrared beam. These vibrations produce bands in well defined regions, which are characteristic for specific class of compounds. Microorganisms too have specific biochemical composition and are thus known to produce unique spectra called fingerprint spectra over the mid-infrared region (4000-600 cm^{-1})⁹. Hence, microorganisms have molecular fingerprinting

capabilities¹⁶. Since microorganisms produce unique spectra in the mid infrared region, this may also be advantageous in identifying microorganisms. Hence, DRIFTS spectrum may be adequately applied both in studying the molecular composition as well as in the identification of biological samples.

In most of the previously reported FTIR studies, vibrational spectra were obtained from dried smears of cultures on glass slides and from dried films on ZnSe optical plates. An alternative method for the analysis of bacterial cells using diffuse reflectance-absorbance mode was developed by Goodacre *et al.*, (1996)¹⁷. They used 10 × 10 cm aluminium plate and FTIR analysis was initially performed on a plate without sample to provide a reference reading for each well. The FTIR spectrometer was equipped with a mercury-cadmium-telluride (MCT) detector, which was cooled with liquid nitrogen. We too use diffuse reflectance FTIR but with modifications as described in detail in later sections.

Statistical treatment of FTIR spectral data allows discrimination between genera, species and strains¹⁸. This is another reason why more and more researchers are interested in FTIR for the characterization of microbial cells^{7,11,19-24}. In this study, DRIFTS data were categorized by multivariate

analysis using PALSTAT²⁵. Hierarchical cluster analysis was applied to DRIFTS data and using Ward's algorithm distinct dendrogram was obtained for different spectral windows for the three strains *Candida albicans*, *E. coli* and *Staphylococcus aureus*.

The objectives of this study were to evaluate the feasibility of DRIFTS application in microbiology. This study includes details of the methods involved in (1) sample preparation (2) treatment of raw spectrum using various algorithm and (3) finally application of cluster analysis to DRIFTS data for distinguishing cultures from one another on the basis of its biochemical composition.

Materials and Methods

Strains and growth conditions

Gram negative bacterium (*E. coli*), Gram positive bacterium (*Staphylococcus aureus*) and yeast (*Candida albicans*) were generous gifts from Goa Medical College, Goa which provided the reference material for obtaining DRIFT spectrum. Bacterial cultures were individually grown on nutrient agar plates (composition: 10 g peptic digest of animal tissue, 10 g beef extract, 5 g sodium chloride, and 12 g of agar dissolved in 1 liter distilled water) while yeast culture was grown on potato dextrose agar (composition: 200 g potato infusion, 20 g dextrose and 30 g agar dissolved in 1 liter distilled water) purchased from HiMedia, Mumbai. The strains were grown for 24 h at $28 \pm 2^\circ\text{C}$. Strains were streaked onto the respective agar plates using four-quadrant streak pattern.

Sample preparation and DRIFT spectroscopic analysis

A single colony of culture cells in replicates of 5, were carefully removed from the fourth quadrant using a platinum loop. The cells were transferred into sterile glass vials and dried in an incubator at 45°C for 30 min. Special care was taken to free samples completely from moisture by vacuum treatment using a Jouan lyophilizer (RC10-22, Heto DRYWINNER, France). Microbial cells weighing 0.9-1.3 mg dry weight was thoroughly mixed with 2-2.5 mg of KBr in a smooth agate mortar to obtain DRIFT spectrum. The quality of the spectrum depends on the intimacy of mixing and the reduction of any suspended particles.

Measuring method

The diffuse reflectance (DR) measuring instrument ORS-8000 is one of the accessories (Fig. 1) of Shimadzu Fourier Transform Infrared

spectrophotometer, FTIR-8000 Series (FTIR-8201 PC, Kyoto, Japan). To measure the diffuse reflectance spectrum of samples, initially, finely powdered potassium bromide (KBr) acting as control was placed in the DR cup (having an internal diameter of 2 mm) and mounted on the DR mount compartment to make the background measurement. This was followed by placing test sample (comprising of transparent cells, well homogenized with KBr) in the sample compartment in a similar manner as described above, for spectral acquisition over wavenumber range $4000 - 600 \text{ cm}^{-1}$. In FTIR spectroscopy, light reflects on the plane mirrors M_1 and M_2 and the off-axis ellipsoidal mirror M_3 . The ellipsoidal mirror with the magnification of 1/5 creates an image of approximate 2 mm on the sample surface. The light scattered on the sample surface is collected on the off axis ellipsoidal mirror (M_4) and then reflected on the plane mirrors M_5 and then M_6 (Fig. 2). The intensity of light is measured and then fourier-transferred to the detector to produce a plot of intensity versus wavenumber on the screen.

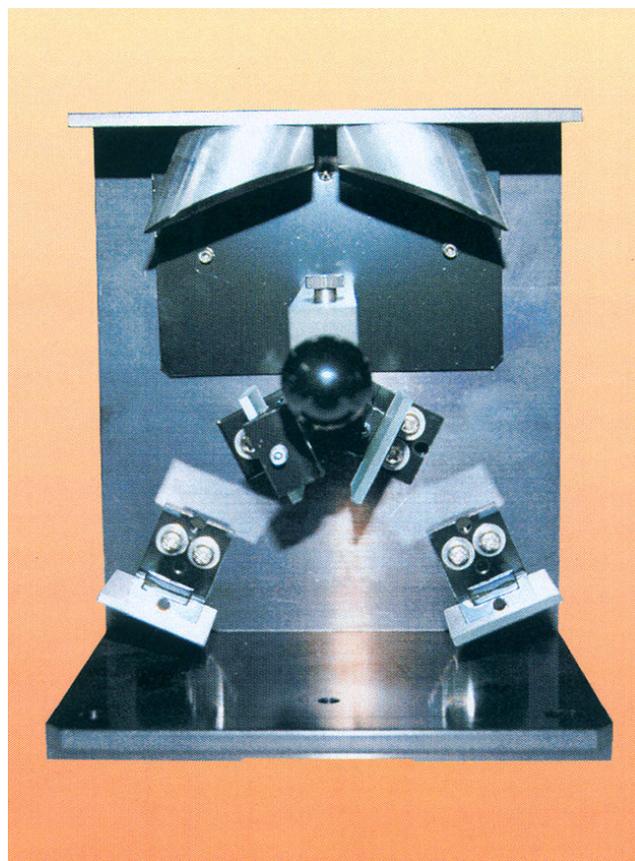


Fig. 1—Show the Diffuse Reflectance (DR) attachment used for the measurement of DRIFT spectrum

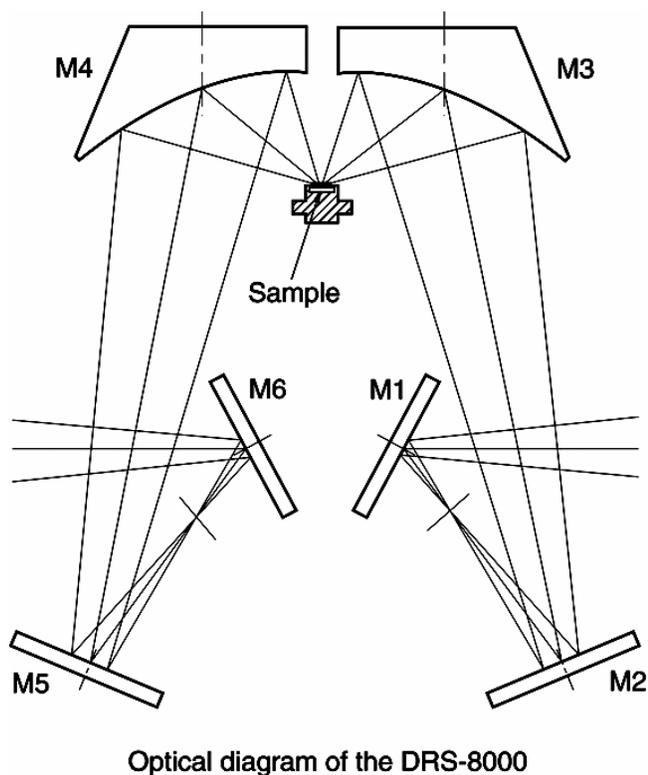


Fig. 2—Show the optical diagram of the diffuse reflectance measuring instrument

Typically, 20 scans were signal-averaged for a single spectrum. Each spectrum was displayed in terms of absorbance as calculated from the reflectance-absorbance spectra using the Hyper-IR software. It controls the interferometer, measures and converts spectra, and processes data. To minimize the difficulties arising from unavoidable baseline shifts, baseline correction was applied. Normalization produces a spectrum in which maximum value of absorbance becomes 2 and minimum value 0. To improve the resolution of complex bands, the digitized original spectra were smoothed on noisy spectrum using Kubelka Munk algorithm and converted into its second derivative (2D) using the Savitzky-Golay algorithm²⁶ using 21-point smoothing. The bands obtained were studied for the biochemical composition by comparing the values reported by Naumann (1998)²⁷ as shown in Table 1.

Cluster analysis

The acquired DRIFTS data was imported into the statistics program PALSTAT. Multivariate statistical analysis of replicate samples of each strain was employed for cluster analysis of the total complex

spectrum. The Euclidean distance was used to produce unique dendrogram clusters for the three stains (*C. albicans*, *E. coli*, and *S. aureus*) under study. Principal component analysis of the same spectra was used to get graphical representations of similarities/differences in terms of clusters of objects.

Results and Discussion

Strains *E. coli* and *S. aureus* were grown on solid Nutrient Agar (NA) medium while the yeast, *Candida albicans* was grown on solid Potato dextrose agar (PDA) medium at $28 \pm 2^\circ\text{C}$ and harvested after 24 h. All the three strains in replicates of 5 were individually dried and homogenized with KBr to obtain Diffuse Reflectance Infrared Fourier Transform (DRIFT) spectral profile. The raw DRIFT spectrum for the strains *C. albicans* (Fig. 3A I), *E. coli* (3B I) and *S. aureus* (3C I) is shown in Fig. 3. The spectrum of *S. aureus* was comparable with those reported by Naumann *et al.*, (1991 b). Microbial strains showed similar basic DRIFT spectral pattern (Fig. 3 A, B, & C). The interpretation of such a complex DRIFT spectra of microbial cells is difficult because of the diverse array of chemical compounds present which can result in band overlapping. Also, because, a specific band in the spectra could be a composite of many bands containing information on more than one type of molecular vibration²⁸.

Hence, mathematical transformations of DRIFTS data were required for qualitative analysis. Firstly, the measured raw spectra were corrected and smoothed by applying the Kubelka Munk algorithm as evident in Fig. 3; A II, B II & C II respectively. Further, the original spectrum for each strain was transformed to second derivative spectra (2D) using the Savitzky-Golay algorithm (Fig 3; A III, B III & C III). All the above statistical treatment helps to discriminate the samples used in the study and to compare the results of our study with those previously reported. After mathematical transformation of spectra (Fig. 3 II & III), the biological samples showed marked differences in the vibrational spectra especially in the information-rich region of $4000\text{-}600\text{ cm}^{-1}$. The DRIFTS bands have been tentatively assigned to chemical functional groups present in whole cells and macromolecules on the basis of interpretations made by earlier researchers^{7,10,27} (Helm *et al.*, 1991; Van der Mei *et al.*, 1993; Naumann 1998). Thus, Fourier-transform infrared (FTIR) spectroscopy allows analysis of

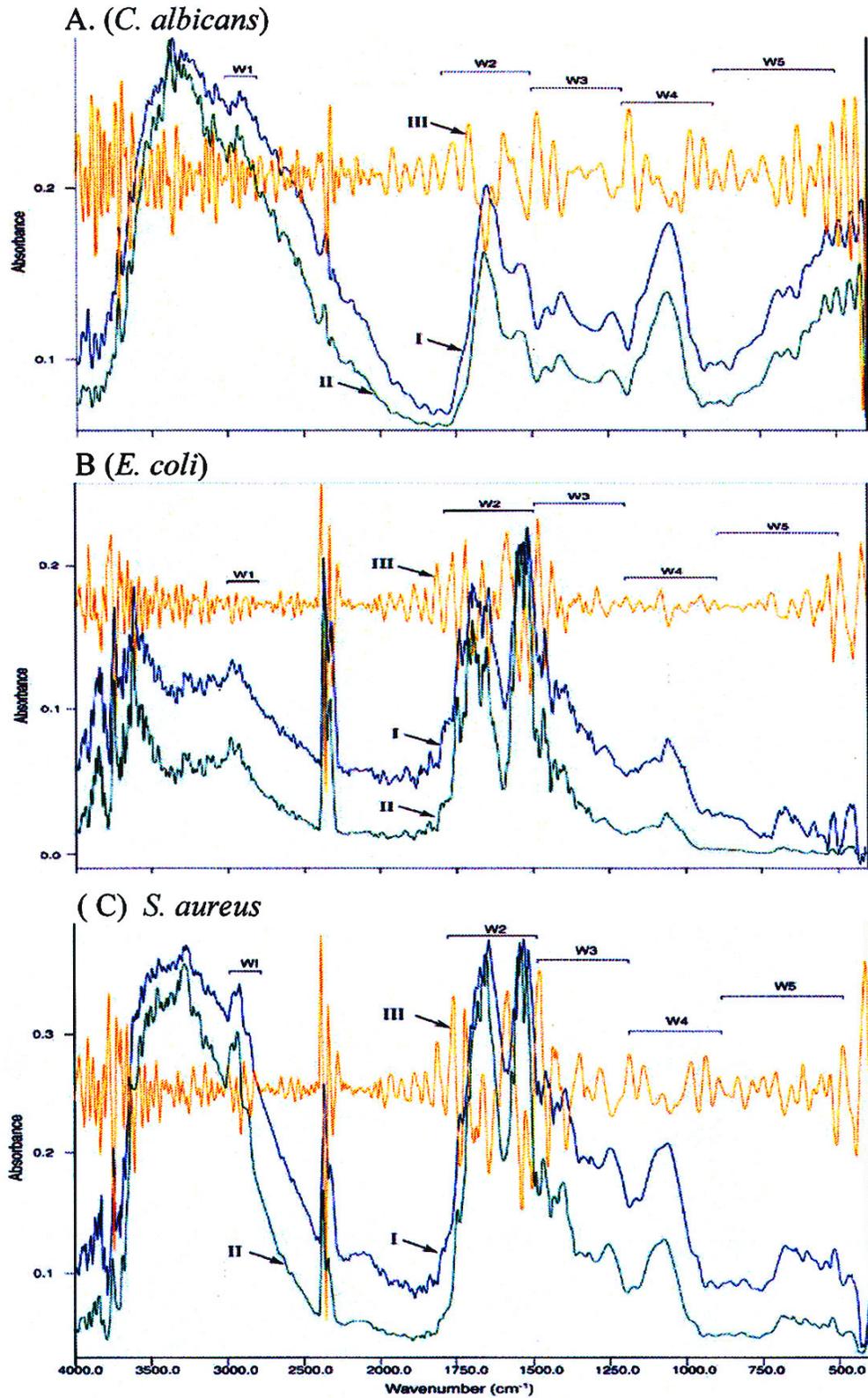


Fig. 3—Representative original mid-infrared absorption spectra (I), Kubelka Munk (II) and second derivative (III) for A) *Candida albicans* B) *E. coli* and C) *Staphylococcus aureus*

biochemical composition through the interaction between the infrared radiation and the sample.

The spectrum is divided into several spectral windows as assigned by Naumann (2000)²⁹, and used later by several researchers for interpreting FTIR spectrum of biological samples. The absorption between 3000 to 2800 cm^{-1} is defined as the fatty acid region. A band absorbing at 2920 cm^{-1} was detected for both *E. coli* and *Staphylococcus aureus*, which is due to the C-H asymmetric stretching of CH_2 methylene in fatty acids. Another absorbance at 2858 cm^{-1} in *S. aureus* is due to the C-H symmetric stretching of CH_2 methylene in fatty acids. On the other hand in the case of the strain *Candida albicans*, absorption bands were evident at 2931 cm^{-1} and 2894 cm^{-1} which is due to C-H asymmetric stretching of CH_2 methylene and due to C-H stretching of C-H methine respectively.

The region between 1800 and 1500 cm^{-1} , is dominated by the amide region of proteins and peptides. Amide I and amide II bands are two major bands of the protein infrared spectrum. The amide I band arising between 1700 and 1600 cm^{-1} is mainly associated with the C=O stretching vibrations as is evident for *E. coli* (1745 cm^{-1}), *S. aureus* (1746 cm^{-1}) and *C. albicans* (1740 cm^{-1}). Amide II bands were witnessed between 1600 and 1500 cm^{-1} . Absorption band at 1542 cm^{-1} was observed for *E. coli*, and at 1543 cm^{-1} for *S. aureus*. These amide II bands arose mainly due to N-H bending vibration and from C-N stretching vibrations. An absorption band at 1515 cm^{-1} typical of tyrosine was evident for *C. albicans*.

The region between 1500 and 1200 cm^{-1} , is a mixed region containing vibrations of fatty acids, proteins, and polysaccharide. The absorption between 1200 and 900 cm^{-1} is characteristic for carbohydrates. Amino sugars of peptidoglycan and phospholipids (cell wall component) absorb strongly in this region. The region between 1300 and 1200 cm^{-1} is assigned to asymmetric stretching of phosphate nucleic acid group. The so-called fingerprint region ranges from 900 to 600 cm^{-1} . This region contains bands, which are most characteristic at the species level as evident for all the strains. Phosphates, found in nucleic acids and phospholipids, absorb at 1250 cm^{-1} as seen for both bacteria *E. coli* and *S. aureus*. The band absorbing at 1060 cm^{-1} (seen in both the bacterial strains) was assigned to the COOH stretch.

Hence, the variation in the IR spectrum of biological strains is due to the microheterogenous

composition as a whole and is based on complex vibrational modes. The main IR-spectroscopic differences are to be expected in the characteristic spectral pattern in the fingerprint region (900-600 cm^{-1}). The majority of computerized rapid microbiology systems use algorithms to make decisions about the data generated. An algorithm can be described as a formula, or series of logical statements that can be used to make decisions regarding data.

Data analysis and investigation of clustering was accomplished using Ward's algorithm on the second derivative spectrum. In this method, a Euclidean distance measured produced unique dendrogram for each strain under study. Tree dendrogram for 3 variables using single linkage Euclidean distances is shown in Fig. 4, for *E. coli*, *S. aureus* and *C. albicans*. Principal component analysis, analysed by hierarchical clustering is represented in Fig. 5. Three distinct clusters were evident for the three strains. Thus, it was possible to distinguish the cultures using cluster analysis.

The method for obtaining DRIFTS data from biological samples have been standardized in the present study. In this method, the IR beam penetrates the cell structure and therefore produces a fingerprint characteristic of the total cell component rather than the outer surface alone. The raw spectrum obtained was smoothed using Kubelka Munk algorithm and further converted into the second derivative using Savitzky-Golay algorithm. Such a conversion allows for comparison of data with available data. Finally DRIFTS data helped to understand the biochemical composition of microbial cells. This when coupled with cluster analysis helped to differentiate between the cultures under study. The advantage of using

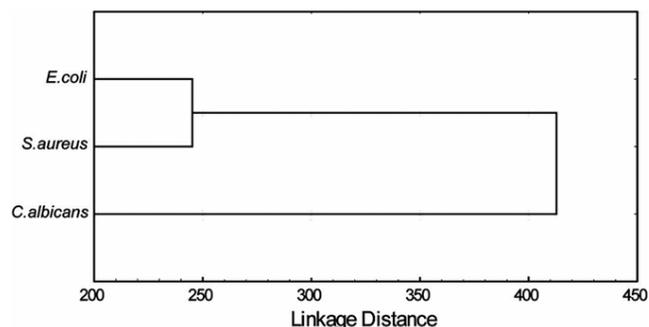


Fig. 4—Dendrogram of a hierarchical cluster analysis performed on 2D spectral data of *E. coli*, *S. aureus*, and *C. albicans*, considering the spectral ranges 3000-2800, 1800-1500, 1500-1200, 1200-900 and 900-600 cm^{-1} .

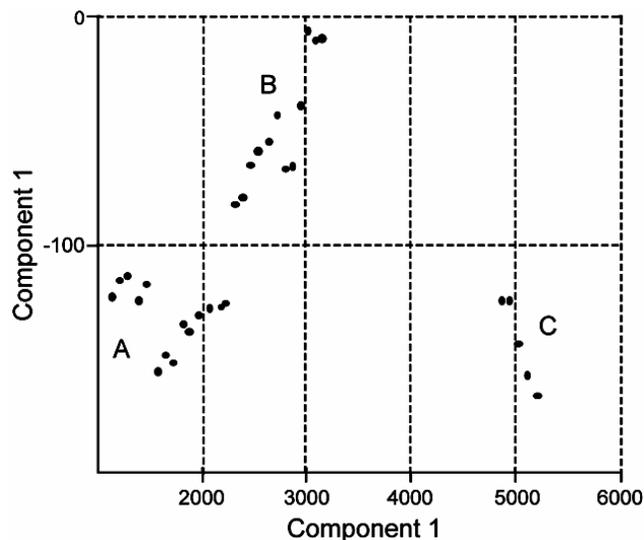


Fig. 5—Differentiation of microbial colonies, A (*E. Coli*), B (*C. albicans*) and C (*S. aureus*) using principal component analysis, analysed by hierarchical clustering. The points represent 2D spectrum of each isolate. For projection of data in factor space the factorial components 1 and 2 were used.

DRIFT spectroscopy is the rapidity with which the spectra can be obtained from small amount of biological samples.

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