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Fourier transform infrared as a powerful technique for the identification and characterization of filamentous fungi and yeasts

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Abstract

Fourier transform infrared is considered a powerful technique for characterizing chemical compositions of complex probes such as microorganisms. It has successfully been applied to fungal identification. In this paper, the current state of identification and characterization of filamentous fungi and yeasts by Fourier transform infrared is reviewed.

Keywords: FT-IR; Filamentous fungi; Yeasts; Microbial identification

1. Introduction

Fungi, which include filamentous fungi and yeasts, are very important organisms. They are employed in the production of pharmaceuticals, enzymes, organic acids and food, and some of them are associated with several diseases affecting humans and other animals [1,5,24,37,38,43,45,47]. The earliest system for fungal species classification relied on morphological characters, mainly those of reproductive structures. However, this method of classification presents critical limitations such as sterility of fungal cultures that have not developed reproductive structures, or morphological similarity among members of different species. The incorporation of biochemical and molecular characters (e.g. isozymes, nucleotide sequences) into fungal taxonomy has helped to solve such problems, at least in some cases [36]. Rapid and reliable physiological tests are available only for a limited number of taxa. Sequence data are also available only for certain taxa; moreover, application of molecular methods to routine requests is relatively expensive [9]. However, these conventional methods present delays in identification as well as limits in discriminating closely related species.

Recent genotypic approaches to the rapid identification of microorganisms are beginning to be used. Although widely accepted, these techniques present some technical limits due to protocol complexities, reagent costs, choices of specific primers for each species, and sensitivity to mutations, and have not been routinely used up to now [7]. The ideal method for replacing these labor-intensive processes would involve minimum sample preparation, direct analyses of samples (i.e. they would not require reagents), rapidity, automation and, (at least relatively) low cost. Furthermore, with recent developments in analytical instrumentation, some spectroscopic or spectrometry techniques provide a wealth of qualitative and quantitative information about a given sample. The spectroscopic or spectrometry spectrum of any compound is known to give a unique ‘‘fingerprint’’ [2,6,40,44].

Fourier transform infrared spectroscopy (FT-IR) is a powerful technique for characterizing the chemical composition of very complex probes such as microorganisms. This technique has been successfully applied in various fields of quality control and for the identification of filamentous fungi and yeasts [9,13,22,27,31,39,46]. Microbiologic FT-IR typing is fast, effective and reagent-free. Moreover, it is applicable to all microorganisms and requires a small quantity of biomass

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[8]. Here we present a review of the use of FT-IR as a technique for identification and characterization of filamentous fungi and yeasts.

2. FT-IR

FT-IR is an old and powerful technique for identifying types of chemical bonds in a molecule. One of the strengths of FT-IR spectroscopy is its ability, as an analytical technique, to obtain spectra from a very wide range of different compounds. The infrared region of the electromagnetic spectrum extends from the visible to the microwave (Fig. 1). Infrared radiation originates from a thermal emission from a hot source. It is conventionally specified by the "wave number", i.e. the number of waves per centimeter (denoted by "ν" and expressed by the unit cm^{-1}), extending from 10,000 to 10 cm^{-1} [3]. Moreover, infrared radiation is divided into near (NIR, $\nu = 10{,}000-4{,}000\ cm^{-1}$), middle (MIR, $\nu = 4{,}000-200\ cm^{-1}$) and far (FIR, $\nu = 200-10\ cm^{-1}$) infrared (Fig. 1).

At present, FT-IR instruments are now digitalized, which makes them faster and more sensitive than the older ones [14,19]. They can be applied to the analysis of solids, liquids and gasses. The basis of FT-IR is absorption of the infrared light by several molecules in a sample. Thus, the FT-IR technique involves subjecting an infrared beam energy that is emitted from a glowing ember source: thus the beam crosses a chamber for controlling the amount of radiated energy on the sample. The IR beam enters the interferometer where "spectral encoding" takes place; then, the resulting interferogram signal exits the interferometer. In addition, infrared beam energy enters the sample compartment where it is transmitted through or reflected away from the sample surface depending on the type of analysis being accomplished. Finally, the beam passes to the detector for final measurement and the measured signal is digitalized and sent back to the computer where Fourier transformation takes place, as shown in Fig. 2. The final infrared spectrum is then presented to the user [3,18].

FT-IR spectra of pure compounds are generally so unique that they look like molecular "fingerprints". While organic compounds have very rich detailed spectra, inorganic compounds are usually much simpler. For most common materials, the spectrum of an unknown compound can be identified by comparison with a library of known compounds. The chemical structure and three-dimensional orientation of the molecules are responsible for generating different IR absorption. Furthermore, when the chemical bond absorbs the infrared light, it vibrates in varying ways depending on its own nature. Reflecting the different types of bonds, a number of events can occur [29–32,34]. The emitted infrared light can have two different behaviors: on one hand, this light may be absorbed by the sample molecules which consume a number of emitted energy infrared lights: on the other hand, it may produce a wave that is "out of synch" with the light that has not passed through the sample.

The capacity to express a specific "fingerprint" enables FT-IR spectroscopy to be used for identification of unknown microbial strains using spectral data libraries [29]. The FT-IR technique has great sensitivity, the microbial sample preparation is simple, no reagent is needed and data acquisition is faster than with other physico-chemical techniques [12]. It has been used as a powerful tool for differentiating fungi [9,31,48]. IR spectra are highly specific for each strain and species, representing the total cell chemical composition such as lipids, proteins, nucleic acids and polysaccharides [23].

By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. The sum of vibrational spectra for macromolecule content of cells (nucleic acids, proteins, lipids, polysaccharides, etc.) can produce an infrared absorption spectrum that is like a molecular "fingerprint" for that biological material. By itself, this spectrum can be diagnostically used in typing or identification applications [8] through clustering. Furthermore, FT-IR can be utilized to qualify some components of an unknown mixture [26]. It can reveal small variations stemming from cultural parameters (culture time, medium composition and pH, temperature, water content, etc.) or culture storage conditions. Standardization of culture conditions, sample preparation and spectral acquisition parameters (number of scans and spectral resolution) is a critical point for achieving reproducibility of spectral data acquisition [8].

3. Filamentous fungi

Filamentous fungi play an important ecological role in nature as decomposers. They have been employed in the production of pharmaceuticals, enzymes, organic acids and food, and some of them are useful in numerous other areas [16,28,35,38]. The primary technique for identifying fungi is based on morphology. Physiological tests and molecular biology can also be employed in later stages [40]. However, a rapid, reliable and automated identification system for routine analyses of fungi has economic significance. In this perspective, Fischer et al. [9] created a simple and sophisticated method for preparation of samples and identification of airborne fungi by FT-IR spectroscopy. The method was performed to reproducibly differentiate *Aspergillus* from *Penicillium* species at the generic, species and strain level. Both filamentous fungi species were analyzed by the classical FT-IR spectroscopy technique, where conidia samples were applied to an infrared transparent sample plate after further purification. Results obtained showed that such an analytical method can serve as a basis for the development of a database for species identification and strain characterization of microfungi. Furthermore, this method can be used to improve and facilitate risk assessment in case of bioaerosol exposure.

FT-IR microscopy and imaging were the techniques used by Naumann et al. [31] to evaluate their potential for localizing and identifying fungi in wood. Two wood-rotting fungi *Trametes versicolor* and *Schizophyllum commune* in experimentally infected beech wood blocks were evaluated. The analyses were recorded using FT-IR microscopy combined with a focal plane array detector and image analysis. Cluster analysis between FT-IR spectra of fungal mycelium grown over a wood surface and inside vessel lumina demonstrated the

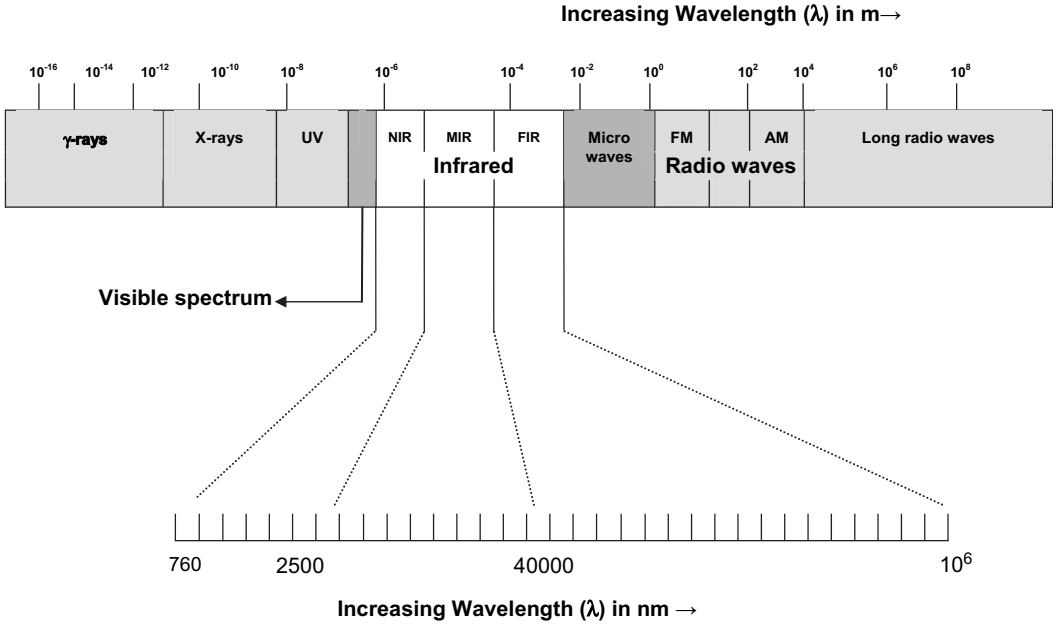

Fig. 1. Schematic representation of the electromagnetic spectrum.

potential of FT-IR for discriminating between fungal species in decaying wood. Thus, this work clearly showed the power of FT-IR for characterizing wood-rotting fungi and detecting their relative distribution within wood even at very low concentrations.

Several filamentous fungi display typical infrared spectra which significantly differ from spectra of substrate grains such as corn [13]. On this basis, photoacoustic spectroscopy (PAS) and diffuse reflectance spectroscopy (DRS), coupled with FT-IR spectroscopy, can be used to differentiate between safe and fungal-infected grains [15]. These techniques that provide information about the mid-infrared absorption spectra of solids were chosen by Greene et al. [15] as trial system for corn contaminated with *Fusarium moniliforme* and *Aspergillus flavus*. Present results indicate that FT-IR-PAS and -DRS data from corn infected with these two hazardous fungi were dramatically different from those of uninfected corn. Both techniques showed high sensitivity for distinguishing fungal contamination in the corn. However, FT-IR-PAS was classified as more sensitive for detecting such fungal contamination. In addition, FT-IR-PAS offers other advantages over FT-IR-DRS: it requires no sample preparation and it is a spectroscopic technique that can analyze an intact kernel. Unfortunately, from a practical point of view, in the present study, the authors were able to analyze only one intact kernel at a time using FT-IR-PAS.

Zotti et al. [48] used FT-IR spectroscopy equipped with attenuated total reflectance (ATR) as a tool for characterizing the paper surface from different paper substrates and for verifying the presence of fungi in 9 samples of biodeteriorated 18th century etchings. Analyses were directly performed with each single paper sample, resulting in 15 isolated fungal entities, three of them never before observed on paper materials. Specifically, 14 species were filamentous fungi, while one yeast form (*Aureobasidium pullulans*) was identified. Of the filamentous fungal genera, the best represented by far was

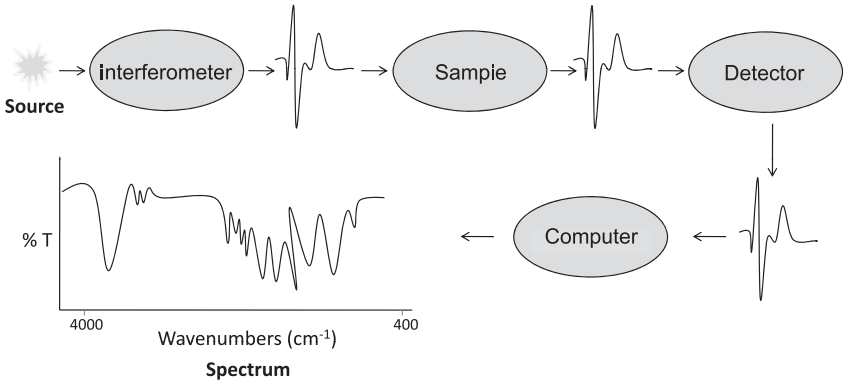

Fig. 2. Step-by-step IR spectrum acquisition.

Penicillium, with five different species. The authors consider FT-IR analysis to be a fruitful technique for identification of paper composition, for monitoring centenary papers and for indicating the best means of conservation so as to avoid fungal attack.

In the characterization process of filamentous fungi, the best technique for combination with FT-IR spectroscopy should be chosen. As described above, different techniques are available. Namely, microscopy, PAS, DRS, ATR, etc. The use of FT-IR spectroscopy not combined with other techniques is a very simple method. It is performed on solid samples in KBr pellets [34] or on an infrared transparent ZnSe plate [9]. However, in order to analyze different materials, the choice of the best technique should be performed as a function of the material to be analyzed. For this propose, the most widespread technique seems to be microscopy.

FT-IR spectroscopy, when combined with microscopy, appears to be a precise tool for localization and identification of fungi in materials such as wood. In this case, FT-IR microscopy improves chemical data quality, since FT-IR analysis gains local resolution when diagnosing the fungi in situ. Furthermore, FT-IR microscopy has the potential for monitoring chemical changes in the wood simultaneously with localization of the fungi [31].

The material to be analyzed by FT-IR microscopy should be sectioned into thin layers. This seems to be a disadvantage when compared to FT-IR-PAS. This technique is capable of evaluating whole materials such as kernels of corn. The majority of the signal arises from the surface of the kernel and this leads to a greater sensitivity for detecting fungal infections, presumably due to the pattern of fungal growth. Furthermore, other than drying, no sample preparation is necessary [15]. On the other hand, when compared to FT-IR-DRS, FT-IR-PAS presents a signal-to-noise ratio that is potentially much higher. It is not surprising that FT-IRs are poorly designed for PAS applications [15]. However, the fact that there is no need for sample preparation, along with intact kernel analysis, make FT-IR-PAS more advantageous than FT-IR-DRS and microscopy.

Among the techniques presented above, FT-IR-ATR is a technique that offers further possibility for directly investigating the chemical composition of smooth surfaces of various materials. The advantage of this technique over other techniques is its capacity to investigate complex material on the interface of ATR crystals. Furthermore, this technique is not destructive and can be performed in situ and in real time. It is largely employed for discrimination of microorganisms through a very simple sample preparation [4,33,48].

FT-IR-ATR can be used for the observation of complex material such as biofilms forming directly on the interface of ATR crystals [41]. This method is suitable for different studies such as analysis of fungal biodeterioration of historic paper [48] and fundamental biofilm research [41]. In the latter case, it can monitor biofilm formation in an ultrapure or drinking water system, for example. Furthermore, FT-IR-ATR also enables analytical discrimination between microorganisms, inorganic material or other foulants [41]. The versatility makes FT-IR-ATR an advantageous technique compared to other presented techniques. However, the choice of the best and most advantageous technique is not based in a single parameter. It should be performed taking into account the characteristic of the sample to be analyzed.

4. Yeasts

Yeasts are unicellular fungi. They play key roles in modern biotechnology and some of them can act as pathogens. Traditional identification of yeasts has been achieved by applying morphological and physiological tests which determine enzyme production profiles and growth characteristics [46]. However, as described for the filamentous fungi above, a rapid, reliable and automated identification system for routine analyses of yeasts is economically significant [22]. In this way, FT-IR appears to be a promising method able to identify yeasts. It presents reliable and rapid results in which identification is limited only by the quality of the reference spectrum library, which can be improved steadily by adding further yeast isolates to the database [22,33,39,44]. Furthermore, FT-IR spectroscopy has been proven to be very simple to use and highly sensitive to small changes in the composition of cells, and it is now possible identify yeasts routinely by FT-IR [22].

Wenning et al. [46] developed a standardized procedure for cultivation and sample preparation for identification of food-borne yeasts using FT-IR microspectroscopy as the tool of analysis. To investigate the potential of identification by this technique, the authors generated two model spectral libraries, one using FT-IR microspectroscopy and another using FT-IR macrospectroscopy. These libraries comprised the average spectra of 45 yeast strains representing 9 genera and 13 species. The study performed by FT-IR microspectroscopy identified 67% of the strains correctly at the species level, and the study performed by FT-IR macrospectroscopy identified 65% at the same level. However, concerning the number of false or unidentified spectra, the authors found a larger difference between the two methods used. While FT-IR microspectroscopy misidentified 31% of the strains, FT-IR macrospectroscopy misidentified 22% of the spectra. The authors point to the composition of the database as a crucial factor in improving the reliability of these results. To overcome difficulties due to intraspecific genetic variabilities, it is suggested that more strains of the same species be included in the library. This is an important approach, since new and diverse strains will close gaps between strains contained in the database.

The cultivation and sample preparation procedures of FT-IR macro- and microspectroscopy differ. While measurement by FT-IR macrospectroscopy takes two days, requiring an additional purification streak and the choice of a single colony to perform the analysis, analysis by FT-IR microspectroscopy uses only a single step: After dilution of the sample, organisms are grown to microcolonies and directly transferred from the agar plate to the IR-transparent ZnSe carrier.

Wenning et al. [46] concluded that identification by FT-IR microspectroscopy is equivalent to that achieved by FT-IR

macrospectroscopy. However, in the first case, the time-consuming isolation of the organisms prior to identification is not necessary. Furthermore, that method also provides a rapid tool for analyzing mixed populations. Finally, the high level of identification of 30 different yeast strains analyzed by the authors [46] demonstrated that the resolution power of FT-IR microspectroscopy is an advantage because it may also be used for yeast typing at the strain level.

Sandt et al. [39] used FT-IR spectroscopy as a new phenotypic approach to evaluate the potential of this physico-chemical technique for typing yeast strains belonging to the same species. This study was carried on with 79 strains of *Candida albicans* isolated over a 4-month period from nine patients hospitalized in two intensive care units. Strains were isolated from multiple anatomical sites with repeated sampling. FT-IR spectroscopy results were analyzed by hierarchical clustering performed with the second-derivative spectra. This identification showed nine groups, one per patient. Only one spectrum out of 79 was misclassified by the FT-IR spectroscopy method. All data were compared to randomly amplified polymorphic DNA (RAPD) results. Analyses demonstrated that amplification patterns of strains isolated from a given patient were identical and different patients had different profiles. Thus, authors [39] point to FT-IR spectroscopy as an excellent technique for clinical yeast identification. Furthermore, the authors conclude that when nosocomial candidiasis transmission is suspected and urgent information is needed, the FT-IR technique may be useful as a rapid identification tool for providing solid clues prior to confirmation by a genotypic method.

The study developed by Essendoubi et al. [7] using FT-IR microscopy for rapid and early identification of the most frequently encountered microcolonies of *Candida* species in human pathology revealed the excellent identification and discriminating potentialities of this technique. By exploiting the discriminating power in this study, 6 species (*C. albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei* and *Candida kefyr*) were identified. All biological material come from 57 clinical *Candida* strains collection isolated from hospitalized patients. Data obtained clearly categorize FT-IR microscopy as a sound and successful clinical approach because, when compared to conventional and molecular techniques, it is both time- and cost-effective, as well as showing good identification and discriminating potential and an effective automated high-throughput routine system. Essendoubi et al. [7] concluded that FT-IR microscopy constitutes a promising new approach that can be improved in terms of reliability and that will allow a considerable gain in time during microorganism identification and characterization.

Yeasts not only affect human and animal health, but are also responsible for food and drink spoilage. On the other hand, these microorganisms provide humans with biotechnologically produced food and drink such as wine, bread, beer and fermented milk products, and some species are of medical importance [22] as discussed above. The same FT-IR technique for yeast involved in clinical problems can be employed in the food and beverage industry, where pure microbial cultures are of critical importance for product quality and consistency.

With the goal of finding a rapid differentiation technique between brewing yeast strains, Timmins et al. [44] analyzed the performance of two rapid spectroscopic approaches for whole-organism fingerprinting: pyrolysis mass spectrometry (PyMS) and FT-IR. In that study, 22 *Saccharomyces cerevisiae* strains were evaluated using these two phenotypic approaches. Phenetic classifications were found to be very similar to those previously obtained using genotypic studies of the same brewing yeasts. Both spectroscopic techniques were shown to be successful in discriminating yeasts, and both techniques were rapid; however, FT-IR was faster than PyMS (typically 2 min for PyMS and 10 s for FT-IR). Those authors reported these whole-organism fingerprinting methods to be satisfactory methods when used in brewery quality control laboratories.

FT-IR was also the technique of choice used by Kummerle et al. [22] to identify food-borne yeasts, where the analyzed strains were predominantly formed by fermentative yeasts. In this study, a reference spectrum library was congregated and supported on 332 defined yeast strains from international yeast collections. All strains were previously identified by conventional methods using physiological and morphological traits. The quality of identification was assessed using 722 other unknown isolated yeasts. These strains were not included in the reference spectrum library and were identified through both techniques: classical methods and by comparison of their FT-IR spectra with those from a reference spectrum library. The results obtained showed that more than 97% of isolate strains were correctly identified by FT-IR. Moreover, the authors emphasize the ease of handling, rapid identification (within 24 h when starting from a single colony) and high differentiation capacity of the FT-IR technique, thus showing this physico-chemical technique to be clearly superior to other routine methods for the identification of yeasts.

5. Biomarkers

Another area for FT-IR application is the detection of compounds which can indicate several infections or contaminations by filamentous fungi or yeasts [6,11,13,15,17]. These compounds are called biomarkers. At a microbial level, biomarkers can be a cellular component of the microorganism under investigation or they can be produced intra- or extracellularly as a biocompound. In case of disease, for example, a compound, in order to be a specific biomarker, must be more strongly based on a correlation with the disease than on a detailed understanding of its biochemical mechanisms [6].

FT-IR spectroscopy provides a high information spectra and the Lambert–Beer law is followed, making it possible to quantify the concentration of several components in a sample. It can also occur in a complex sample using multivariate data analysis, consequently avoiding time-consuming separation steps prior to measurement [10].

Jilkine et al. [19] developed a sensitive method for examining whole-cell biochemical composition in single cells of filamentous fungi using Synchrotron FT-IR microscopy as

a powerful analytical technique. This technique can be used to study fungal cell biology by fingerprinting varieties of carbohydrates, proteins and lipids of around 6 μm spatial resolution. In this study, the hypha and spore compositions of two fungi, *Neurospora* and *Rhizopus*, were compared. Results obtained clearly showed that the technique can provide spatially resolved biochemical information on fungal spore development, complementing information obtained from molecular genetic investigations.

Biochemical information was also the key used by Erukhimovitch et al. [6] to discriminate microorganism infections. The potentiality of FT-IR microscopy was evaluated as a detection tool to distinguish biochemical differences between bacteria and fungi. Results obtained showed representative specific spectral biomarkers for both microorganisms, due to specific proteins and lipids signs. Furthermore, these data proved detectable and significant spectral differences between bacterial and fungal samples. The representative biomarker peaks clearly appeared in the spectra of a mixture of both of them. This technique might thus be used for rapid discrimination between bacterial and fungal infections and contaminations.

Very important biomarkers produced by several filamentous fungi are mycotoxins [25,42]. FT-IR potentiality use for mycotoxin detection in cereals has been demonstrated in recent years [20,21,25]. Galvis-Sánchez et al. [11] developed a method to explore the capacity for FT-IR use associated with ATR to detect ochratoxin A (OTA) in dried vine fruits. The sensitivity of the technique used was evaluated at a range of concentrations between 2 and 50 $\mu g\,kg^{-1}$ OTA (The European Union established a maximum OTA limit of 10 $\mu g\,kg^{-1}$ for food containing dried vine fruits, EC n° 472/2002 of 12 March). Those authors concluded that the FT-IR-ATR method is well suited for detection of OTA contamination in dried vine fruits, and this mycotoxin can be detected and identified by infrared spectroscopy under an evaluated concentration range. Moreover, the method represents an improvement in terms of time of analysis and sample manipulation when compared with traditional methods for detection and quantification of OTA.

FT-IR-ATR was also the technique of choice used by Marder et al. [26] to investigate total mycotoxins in organic extracts. All extracts were obtained from four fungal strains of *Bipolaris sorokiniana*. Furthermore, these mycotoxins were obtained from biomass of related fungal strains cultured on potato dextrose agar. Isolated mycotoxins were analyzed in order identify the presence of characteristic bands of the mycotoxin structure. Results obtained showed that all isolated mycotoxins were successfully characterized with identification of the same structures for the four strains.

Adt et al. [2] analyzed structural differences occurring in blastospores and hyphae of *C. albicans* through FT-IR spectroscopy. Quantitative evaluation of differences between these fungal forms using curve fitting of the polysaccharide, protein and fatty acids regions was performed. The difference observed could be due to both changes in structure and the content of components of the cell wall such as β-glucans, mannoproteins and lipids, respectively. Thus, using FT-IR spectroscopy, it was possible to differentiate blastospores and hyphae taking their components as specific compounds of these fungal forms.

6. Conclusions

The identification of species is the main goal in mycological taxonomy. Information about each fungus (e.g. morphological description, physiological and biochemical traits, ecological roles, and societal risks or benefits) is the principal element in this process. Fungal identification can be a hard task with frequent revisions of the taxonomic schemes, making it a long and complex process. Furthermore, it is gradually becoming clearer that fungal identification and authentication require a polyphasic approach to generate quality data which are accurate and useful. FT-IR spectroscopy has demonstrated its powerful characteristic as a sound technique applied to identification, characterization and authentication of several filamentous fungi and yeast strains. The advantages of this new approach as a microbial authentication method are: (a) a simple sample preparation procedure, (b) a short time of analysis; and (c) reliability of the data. From a statistical point of view the reference spectrum library is crucial for accurate microbial characterization. It should be assembled based on well-characterized strains and species. When an unidentified isolate is measured under the same condition as those well-characterized isolates used on the reference spectra, it generates an FT-IR spectrum that is compared to spectra in the reference spectrum library. If the library contains an identical or a very similar spectrum, identification is possible. The success of the method is therefore directly dependent on the complexity of the reference spectrum library [22]. Identification is limited only by the quality of the reference spectrum library, which can be improved steadily by adding further microorganism isolates to the database. However, it is very important to keep in mind that FT-IR spectra are influenced by variation of plating methods, growth temperature, incubation time and even the drying method of the microorganism suspension located on the sample holder [22]. The standardized preparation procedure should be taken in account to achieve a high level of spectrum reproducibility that is crucial to avoid misidentification.

FT-IR has the potential for use as a current technique for rapid identification and characterization of filamentous fungi and yeasts in hospitals, health centers, laboratories of clinical analysis, food, feed, beverage and water industries, etc. FT-IR may also be an effective tool for rapid identification and quantification of biocompounds produced by filamentous fungi and yeasts in different fields as described above. Furthermore, FT-IR equipment is not expansive when compared to other physico-chemical equipment used in fungal strain characterization.

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