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Laser induced breakdown spectroscopy for the discrimination of *Candida* strains

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ABSTRACT

The present study reports the evaluation of Laser Induced Breakdown Spectroscopy (LIBS) and Neural Networks (NN) for the discrimination of different strains of various species of *Candida*. This genus of yeast was selected due to its medical relevance as it is commonly found in cases of fungal infection in humans. Twenty one strains belonging to seven species of *Candida* were included in the study. Scanning Electron Microscopy with Energy-Dispersive X-ray Spectroscopy (SEM-EDS) was employed as a complementary technique to provide information about elemental composition of *Candida* cells. The use of LIBS spectra in combination with optimized NN models provided reliable discrimination among the distinct *Candida* strains with a high spectral correlation index for the samples analyzed, without any false positive or false negative. Therefore, this study indicates that LIBS-NN based methodology has the potential to be used as fast fungal identification or even diagnostic method.

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1. Introduction

Biomedical applications of Laser Induced Breakdown Spectroscopy (LIBS) have been extensively explored since the last decade. Among these studies the analysis of microorganisms especially bacteria with particular relevance to infectious diseases in humans have made a greater contribution [1–5]. The increased interest in this area has been due to the advantages offered by LIBS in providing a speedy and cost effective analysis as well as to the use of modern chemometric techniques to deal with the large amount of data produced by LIBS. Whereas most of the studies have focused on the identification and discrimination of bacterial samples [2,3,6,7–9], not much has been done towards the analysis of fungi samples by LIBS. Keeping in view the medical importance of fungi, the *Candida genus* has been selected for this study. *Candida* species are ubiquitous fungi and most common human fungal pathogens [10]. *Candida* is an opportunist pathogen but can cause mucocutaneous and disseminated infections [11]. Species of *Candida* can be part of the endogenous microbiota of digestive and urogenital tract [12,13] but have also been found frequently in the patients with cancer [14]. However, they are also found to be a leading cause of nosocomial infections, which are associated with a great deal of morbidity and mortality resulting in increased hospitalization period and treatment costs. Recent studies have reported an increase in these fungal infections [15,16]. In fact, *Candida* is the fourth most commonly recovered organism from blood cultures of hospitalized patients, with an estimated mortality rate of 38–75% [17]. Out of approximately 200 species of the genus *Candida*, about 20 species are implicated in clinical infections. Among them, *Candida albicans*, *Candida glabrata, Candida tropicalis, Candida parapsilosis and Candida krusei* are found more frequently than others. Some species such as *Candida guillermondii, Candida kefyr and Candida dubliniensis*, although are less frequent, also cause serious infections [12,15,16,18].

The taxonomic identification of a fungal sample is conventionally done by methods based on the analysis of macro and microscopic morphology and physiology. A common method relies on the analysis of the ability to develop germ tubes, which is a characteristic feature of *Candida albicans* that allows its differentiation from the non-*albicans* species. However, 5% of *C. albicans* isolates do not produce germ tubes, while some C. *tropicalis* isolates exhibit germ tube formation. Other identification procedures include biochemical-based assays, like carbohydrate fermentation and assimilation, serological and molecular analyses such as ELISA and PCR tests, chromogenic studies and fluorogenic assays [10].

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Moreover, sophisticated spectroscopic techniques like Fourier transform infrared spectroscopy (FTIR), Raman and matrix-assisted laser desorption/ionization time of flight/Mass spectrometry (MALDI-TOF/MS) have also been employed to identify *Candida isolates* [11,17,19,20].

Conventional methods are time consuming and may take up to 96 h for a complete identification. Main drawbacks of most of the molecular methods are the high cost of the analysis and the requirement of complex equipment [10,21]. Thus, there is a clear need for rapid and cost-effective novel approaches to be used in clinical setting for the diagnosis of fungal diseases.

Having LIBS focused more towards bacterial identification, the utility of this technique for the analysis of fungal strains remains to be established. Therefore, the motivation of this study was to evaluate the potential of LIBS technique in achieving discrimination between different *Candida* strains based on the information given about the LIBS elemental composition. Furthermore, Neural Network algorithms were applied to the analysis of LIBS data in order to design the classification models to identify and discriminate the *Candida* strains. In order to reveal the cellular structure, three-dimensional visualization [22] and chemical elemental composition of *Candida*, analysis by scanning electron microscopy (SEM) and Electro Dispersion X-Ray Spectroscopy (EDS) was also performed.

2. Material and methods

2.1. Samples preparation

All samples were used with no further preparation than that described herein. *Candida* strains were grown on YPD plates (10 g/L of yeast extract, 20 g/L of peptone, 20 g/L of dextrose and 20 g/L of agar) for 48 h at 30 °C. Then, cells were resuspended in sterile water and 70 μL of this suspension spread on the surface of a small petri dish (5 cm in diameter) containing selective Sabouraud Dextrose Agar medium (5 g/L of pancreatic digest of casein, 5 g/L of peptic digest of animal tissue, 40 g/L of dextrose, 15 g/L of agar and 0.05 g/L of chloramphenicol to inhibit the growth of bacteria) for 48 h at 30 °C. Table 1 shows the *Candida* strains analyzed. Strains were kindly provided by Ana Alastruey-Izquierdo from the Instituto de Salud Carlos III, Spain (CNM-strains) and by Elvira Marín from Dept. Microbiología II, Universidad Complutense, Spain.

Table 1
Candida strains used in this study.

Candida spp.	Strain	Sample ID
C. parapsilosis	*ATCC CP22019*	*CpA*
	CNM-CL9608	*CpB*
	CNM-CL9607	*CpC*
C. dubliniensis	*CBS 7987*	*CdA*
	CNM-CL9531	*CdB*
	CNM-CL9472	*CdC*
C. krusei	*ATCC 6258*	*CkA*
	CNM-CL9526	*CkB*
	CNM-CL9524	*CkC*
C. glabrata	*CBS138*	*CglA*
	CNM-CL9600	*CglB*
	CNM-CL9555	*CglC*
C. guilliermondii	*CNM-CL9533*	*CgA*
	CNM-CL9603	*CgB*
	CNM-CL9602	*CgC*
C. tropicalis	*CNM-CL9537*	*CtA*
	CNM-CL9690	*CtB*
	CNM-CL9470	*CtC*
C. albicans	*CNM-CL9534*	*CaA*
	CNM-CL9535	*CaB*
	CNM-CL9536	*CaC*

2.2. Experimental setup

The LIBS technique and methodology used have been previously described [23]. The more relevant parameters are discussed here. Fig. 1 shows a schematic view of the experimental setup. The experimental configuration used for the LIBS measurements included Q-switched Nd: YAG laser (Quantel, Brio model) at 1064 nm, with a pulse duration of 4 ns full width at half maximum (FWHM), 4 mm beam diameter and 0.6 mrad divergence. The laser beam was focused onto the sample surface with a 125 mm focal-distance lens. The diameter of the spot on sample surface was ≈150 μm. The laser fluence was set at 20 J/cm^2 per pulse and the repetition rate was set to 1 Hz. Collection of light was done by a 4 mm-aperture fiber optic, (with a 1000 μm core diameter and 0.22 numerical aperture), coupled with a 7 mm focus fused silica collimator placed at 45° with respect to the surface normal, and at a distance of 5 cm from the sample. The optical fiber was coupled to the entrance of the spectrometer. The spectrometer system was a user-configured miniature single-fiber system (USB4000, Ocean Optics, Dunedin, FL, U.S.A., grating 600 l/mm, spectral resolution 1 nm, 10 μm entrance slit) with a gated CCD detector. The spectral range covered was from 200 to 900 nm. The detector integration time was fixed at 100 ms, obtaining whole spectral information for ions, atoms and molecules. A delay of 2 μs was set to trigger the detector using a digital delay generator (Stanford model DG645) in order to prevent the detection of bremsstrahlung. The spectrometer was computer-controlled using an interface developed with Matlab (Mathworks, 2014b). Scanning Electron Microscopy (SEM) analyses was carried out by usinf a microscope (Hitachi S-3000N). Energy – Dispersive X-Ray Spectroscopy (EDS) analyses were performed with an attached EDS analyser (Oxford Instruments INCAx-sight).

2.3. LIBS measurements

A single spectrum was obtained for each laser shot, recording 150 spectra for each petri dish. From the recorded set of spectra, 100 were used to create the training library while the second set of 50 spectra composed the test library. Each library contains the intensity at different wavelengths in rows and the spectra in columns. Thus, our training library has 3648 rows (one for each wavelength) and 100 columns or spectra for each sample. The remaining 50 spectra were used to construct the test library in similar way. The pre-treatment of data was done by normalizing the spectra using the intensity emission line Hα (i.e. 656 nm) in order to avoid data variations.

2.4. NN analysis

Statistical analysis of LIBS spectral data was performed by artificial intelligence algorithms i.e. NN in order to develop the classification models. The neural networks consists of a large number of neurons (processing units) densely interconnected to each other and arranged in layers (input, hidden and output) receiving information from all of the neurons of the previous layer. NN can be trained using the given input data to recognize and categorize complex patterns [24]. The theoretical fundamentals, topology and architecture of NN have been previously described in detail in the following cited studies [1,9,25] and only the more relevant aspects are given here. Custom-made Neural Networks software based on (Mathworks, 2014b) was specifically developed to design the classification models in order to perform the discrimination between the *Candida* strains using the LIBS data. The

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