RAPID CLASSIFICATION OF BACTERIA USING LIBS IN MULTI-PULSE LASER REGIME AND NEURAL NETWORKS PROCESSING

CLASIFICACIÓN RÁPIDA DE BACTERIAS USANDO LIBS CON UN LÁSER EN REGIMEN MULTI-PULSO Y PROCESAMIENTO CON REDES NEURONALES

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Recently, efforts have been increased for the rapid identification of microbiological samples to improve health diagnosis. Existing methods are time consuming and require pre-treatment of the sample. For these reasons, the Laser Induced Breakdown Spectroscopy (LIBS) technique has been proposed as an alternative method to reduce bacterial identification times. In this work, spectra were obtained from two types of bacteria: *Escherichia coli* and *Staphylococcus aureus*, their elemental identification, as well as the use of an artificial neural network for classification among them. As a source of excitation, a compact laser was used in the Q Multi-pulse switch, which favors the intensity of the plasma emission and facilitates detection. The results presented show a clear identification between the two types of bacteria.

Recientemente, han incrementado los esfuerzos para disminuir el tiempo de identificación de muestras biológicas y así mejorar los diagnósticos clínicos. Los métodos existentes requieren pretratamiento de las muestras y tiempos extendidos. Por esta razón, la Espectroscopia de Plasma Inducido por Laser (LIBS) se ha propuesto como un método alternativo para reducir el tiempo de identificación de una bacteria. En este trabajo, se obtuvieron espectros de dos especies de bacteria: *Escherichia coli* y *Staphylococcus aureus*. Estos fueron identificados elementalmente y clasificados con el uso de una red neuronal artificial. Como fuente de excitación se usó un láser compacto con Q switch multi-pulso, que favorece la intensidad de la emisión del plasma y facilita la detección. Los resultados muestran una identificación clara entre los dos tipos de bacteria.

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I. INTRODUCTION

In recent years there is growing concern about the significant increase in the appearance of bacteria resistant to many antimicrobial therapies [1]. One of the reasons for this increase is the widespread and indiscriminate use of antibiotics to treat infections [2]. A nosocomial infection (HI) is one that is acquired in a hospital or another health service [3]. Among the bacteria producing HI are *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium* [4]. Infections caused by *S. aureus* present a challenge for clinical entities, particularly for serious conditions such as bacteremia, severe pneumonia, and skin infections. The *S. aureus* bacterium is the second cause in HI [5].

There are numerous other emerging and existing technologies that purport to provide a real-time medical diagnosis of pathogens, such as polymerase chain reaction, micro array assemblies, fluorescence *in situ* hybridization, and other fluorescence techniques to name a few. These techniques all possess their own unique limitations [6].

An alternative method for the identification of bacteria more quickly is through the use of Laser-Induced Breakdown Spectroscopy (LIBS) and the use of neural networks for their proper classification [7, 8].

Some of the advantages of the LIBS technique are the ability to perform fast measurements *in situ* of several elements simultaneously [9], requiring little or no previous preparation of the samples. This has enabled numerous applications of the LIBS technique in different areas: cultural property [7], metallurgy [10,11], security [12], forensic science [13], biomedicine [14], pharmaceutical industry [15], foods [16, 17], environment [18, 19], archeology [20] and has even been used in the exploration of the surface of the Moon and Mars [21, 22]. Also, the atmospheric conditions and extreme temperature of the planets like Mars or Venus are revealed as suitable for LIBS analysis [23].

Since 2003 LIBS has been used to identify and discriminate between a variety of microorganisms based on the emissions of inorganic trace elements contained in microorganisms [24]. One of the most significant advantages of this technique applied in this area is that it can identify all bacterial pathogens including strains of the same species [25].

There are currently several approaches to microbiology analysis with LIBS [26], and there is no standard protocol recommended by the scientific community. Different types of lasers have been used, although the fundamental frequency (1064 nm) of Nd: YAG lasers of nanoseconds are the most common [27–32]. Other harmonics have also been used in the

near infrared using Ti: sapphire femtosecond lasers [33]. The motivation of these studies is to evaluate the ability of LIBS to provide rapid identification, when compared with traditional methods of bioanalysis, by benefiting from the possibility of combining with chemometric methods to increase the performance of the technique.

Artificial Neural Networks (ANN) works are those in which only a partial regression analysis is used for difference of least squares or classifiers. In some cases the correlation of their results was of 85 % [25, 34, 35]. Other studies [30, 31] have shown that ANNs have promising potential for classifying and predicting bacterial samples at the genus level with a high degree of accuracy. In the present paper, we show the classification of two types of bacteria: *Escherichia coli* and *Staphylococcus aureus*, through the use of a LIBS system with a compact multi-pulse and portable laser [36]. The artificial neural network used is easily programmable, with six input neurons and one output neuron, which facilitates the understanding of the results.

II. METHODOLOGY

II.1. Preparation of samples

Samples of *Escherichia coli* and *Staphylococcus aureus* are second growth ATCC reference strains. These were cultivated with the streak technique and left to incubate for 24 hours. The samples were separated from the growth agar with a sterilized handle into a glass holder and allowed to dry for 15 minutes, this resulted in a rounded speck of around 5 mm thick. This was to remove the bacterium from the medium and thus ensure ablation of only bacterial sample, which was solid to avoid the effect of spattering caused by the agar and its possible spectral interference during the process of ablation.

II.2. Experimental Installation

The LIBS installation consisted of a Nd:YAG laser emitting in multipulse mode with Q: Passive switch, emitted with a wavelength of 1064 nm (Bralax, Mexico).The laser beam was directly impacting on the sample being focused with a lens with 5 cm of focal length. It is important to take into account the dimensions of this laser, 14 x 6 x 4 cm, which makes it an extremely compact and inexpensive plasma excitation medium, since the price of the device is less than $5000. The samples were placed so that their surface coincided with the focus of the lens. The impact of laser pulse on the surface ablates a certain amount of material and generates a plasma, the emission of which is transmitted to an Ocean Optics USB4000 spectrometer using a P600-1-SR optical fiber. The spectrometer has an optical resolution of 0.3 nm with a spectral range of 200-900 nm. Its reading begins upon receiving the signal from a photodetector model PDA10A (Thorlabs Inc., USA) which in turn sends the electrical signal once it detects the emission of the laser pulse. The spectrometer information is transmitted and processed on a computer using Spectra-Suite software to obtain spectra, which were stored in plain text format. Fig. 1 shows the diagram of the experimental installation.

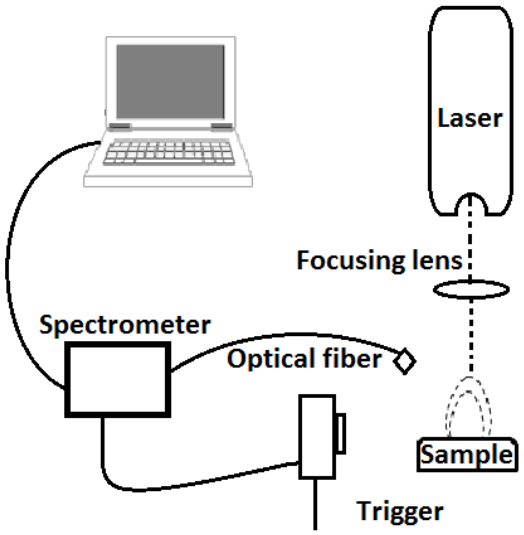

Figure 1. LIBS setup.

The final experimental conditions were: laser energy 450 mJ; Spectrum capture delay set by 0.5 μs spectrometer software; Distance between the optical fiber and the sample of 1.7 cm; Focal length of 6.5 cm and time between shots of 1 minute.

The emission of the laser consists of a sequence of 6 pulses with a separation between them of 40 to 60 μs, which generates a new material extraction at each pulse. Due to the separation between pulses, the captured emission is not associated to a plasma re-excitation, but to the contribution of several processes of ablation and emission, independent of each other, the sum of which contributes to the improvement of the signal of Intensity-noise [34].

II.3. Obtaining and processing of spectra

From a total of 6 different samples (3 per bacterium) in the above conditions, a total of 106 spectra were captured (53 per sample); 50 for training and three for use in the neural network.

For the chemometric analysis, spectral range was reduced from 300 nm to 800 nm because no relevant information was found outside this range. Then, an internal normalization was made taking the line 656.3 nm as the standard since it is found fin both samples. This normalization was done to reduce fluctuations in the spectral intensities between shots.

II.4. Identification of spectral lines

Frequently, in LIBS the measurements are obtained by an averaged set of spectra produced by several laser pulses. The average of consecutive spectra will produce weak values in the signal-to-noise ratio. The spectra of each sample were averaged over a single spectrum with the following ratio

$$I = \frac{I_1 + I_2 + \cdots + I_{n-1} + I_n}{n}, \tag{1}$$

where I is the average intensity associated with a wavelength, I_n is the intensity associated with a wavelength of a single shot spectrum and n the number of spectra obtained.

The identification of spectral lines was performed with reference to the National Institute of Standards and Technology [35] and United States Army Research Laboratory databases [36].

II.5. Artificial Neural Network

A major component analysis was performed to see that despite the internal differences between the spectra types, there were no similarities with the opposite bacteria. The average spectra were subtracted so that the differences were noticeable and thus to be considered as input neurons in the neural network.

The neural network was programmed in Matlab r2010b. The network contains: six neurons in its entrance layer; 50 neurons in the hidden layer, which allow the creation of weights and thresholds for a better development of the training; only one neuron in its exit layer, in order to give results of type 0 and 1.

II.6. Principal Component Analysis

PCA is a technique that finds combinations of many variables, called principal components; these components are accountable for most of the variance, thus finding trends within the data. The results from PCA are usually showed with a graph that relates variation in the samples, called score plot [37].

II.7. RESULTS AND DISCUSSION

Fig. 2 is the average spectrum of the 50 spectra corresponding to the *S. aureus* sample. Both elemental peaks and recombination bands are observed. Some of the characteristic peaks are Ca (393.36 nm, 396.84 nm), H (486.13 nm, 656.3 nm), N (499.9 nm), Li (610.54 nm, 670.79 nm), K (766.49 nm, 769.89 nm), Nm (589.59 nm) and O (777.2 nm).

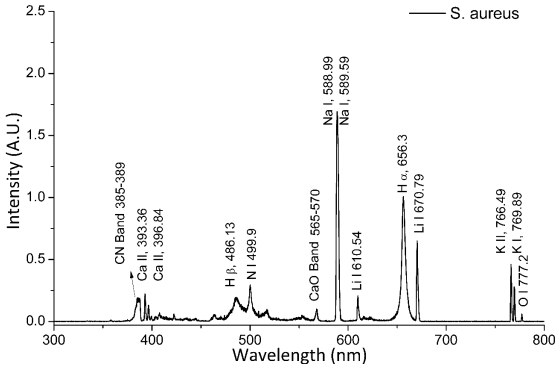

Figure 2. Emission spectrum of *Staphylococcus aureus*.

It is well known that mineral salts are the source of anions and cations for bacteria. The following cations, in particular, are needed in relatively large amounts: K^+, Mg^{++}, Ca^{++}, Fe^{++}, so its presence in the spectra is not uncommon.

The molecular bands found were: CN (385-389 nm) and CaO (565-570 nm). The molecular bands of CN are indicators of the presence of free amino acid radicals in the structure of the biological material, and the atomic intensities are related to the abundance in these structures.

Fig. 3 is the averaged spectrum of the 50 spectra corresponding to the *E. coli* sample, and here also both elemental peaks and molecular bands are observed. Among the characteristic peaks are Ca (393.36 nm, 396.84 nm, 422.67 nm), H (486.13nm, 656.3 nm), N (499.9 nm), K (766.49 nm, 769.89 nm), Na (588.99 nm, 589.59 nm) and O (777.2 nm). In the cytoplasmic membrane large, dense and refringent clusters of insoluble calcium salts can be found. The detected mineral elements are potentially usable as spectral markers because they are essential in this type of bacteria.

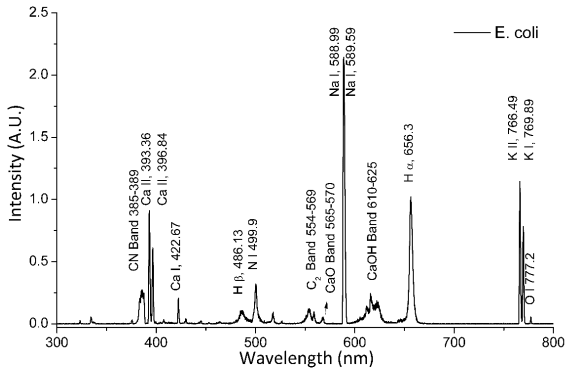

Figure 3. Emission spectrum of *Escherichia coli*.

In *Escherichia coli* case, the molecular bands found were: CN (385-389 nm), C_2 (554-569 nm), CaO (565-570 nm) and CaOH (610-625). In relation to the bands of C−C, we can assume that they are associated with organic compounds like starches, present in the structure of the bacterium.

The principal component analysis (Fig. 4) shows a clear separation of two groups, each encompassing one type of bacteria. This indicates that the results of the bacteria analyzed are spectrally different from each other. The most distant points of the main group are those samples that showed more internal differences in the RMSE analysis.

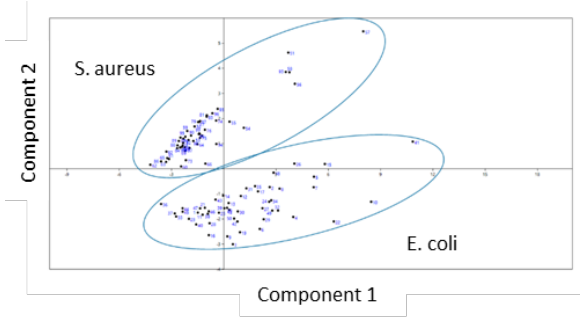

Figure 4. Principal component analysis.

The noticeable spectral differences are most clearly seen in Fig. 5, where the average spectrum of *E. coli* was subtracted from the average spectrum of *S. aureus*. Among the elemental differences, the Ca II peaks (393.36 and 396.84 nm), the predominant Na I intensity (588.99 nm) and the appearance of K I (766.49 and 769.89 nm) were observed by *E. coli* bacteria. On the part of the sample of *S. aureus*, only the line of Li I (610.54 nm) was considered as a remarkable difference. These differences were considered as input neurons.

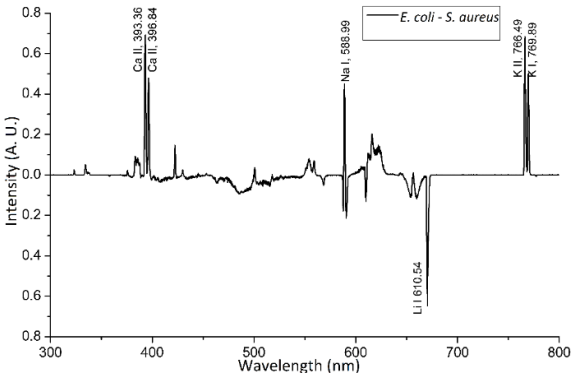

Figure 5. Spectral remains *E. coli - S. aureus*.

To train and validate the algorithm of the neural network, we used the 100 LIBS spectra obtained. In this training, the molecular bands were not included in order to verify that the elemental spectral information is enough to perform the discrimination. Of the spectra 70 % were used for the training, 15 % for the validation and 15 % for the internal test process. After completing the data processing in the network, we analyzed the extra spectra for checking the implementation of the network the results are shown in Table 1.

Table 1. Results of the application of the neural network.

Sample	Network Result	Result
1	1	*E. coli*
2	1	*E. coli*
3	1	*E. coli*
4	0	*S. aureus*
5	0	*S. aureus*
6	0	*S. aureus*

The classification according to the values entered in the pre-training network (1 for *E. coli* and 0 for *S. aureus*) is consistent with those obtained after the analysis of external samples. With this, it is verified that the unequivocal classification between two bacteria using a neural net of basic programming is possible.

III. CONCLUSIONS

The use of LIBS, the excitation of which is carried out using a multi-pulse emission laser, based on a low-cost passive Q: Switch, allows the identification of adequately identified spectra for different types of bacteria.

It is demonstrated that it is possible to use a neural network to obtain a correct classification of bacteria by LIBS using only spectral information from the elemental emission.

The combination of data processing using a neural network, coupled with the use of a low-cost multi-pulse laser system, can become a very useful tool for the rapid discrimination of bacteria under field conditions.

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