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Rapid classification of heavy metal-exposed freshwater bacteria by infrared spectroscopy coupled with chemometrics using supervised method

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ABSTRACT

Rapid, cost-effective, sensitive and accurate methodologies to classify bacteria are still in the process of development. The major drawbacks of standard microbiological, molecular and immunological techniques call for the possible usage of infrared (IR) spectroscopy based supervised chemometric techniques. Previous applications of IR based chemometric methods have demonstrated outstanding findings in the classification of bacteria. Therefore, we have exploited an IR spectroscopy based chemometrics using supervised method namely Soft Independent Modeling of Class Analogy (SIMCA) technique for the first time to classify heavy metal-exposed bacteria to be used in the selection of suitable bacteria to evaluate their potential for environmental cleanup applications. Herein, we present the powerful differentiation and classification of laboratory strains (*Escherichia coli* and *Staphylococcus aureus*) and environmental isolates (*Gordonia* sp. and *Microbacterium oxydans*) of bacteria exposed to growth inhibitory concentrations of silver (Ag), cadmium (Cd) and lead (Pb). Our results demonstrated that SIMCA was able to differentiate all heavy metal-exposed and control groups from each other with 95% confidence level. Correct identification of randomly chosen test samples in their corresponding groups and high model distances between the classes were also achieved. We report, for the first time, the success of IR spectroscopy coupled with supervised chemometric technique SIMCA in classification of different bacteria under a given treatment.

1. Introduction

Various industrial activities spoil the quality of natural freshwaters by releasing different heavy metals [1]. Overwhelming metal contamination poses great danger to public health since they generally cannot be transformed into the safe forms in nature [2]. To prevent these dangers, the contaminants should be removed efficiently. In many studies, bacteria species have been employed for the decontamination of environmental metal pollutants since in their natural habitat, they adapt themselves to survive and grow under the toxic effects of pollutants including silver (Ag), cadmium (Cd) and lead (Pb) [3]. However, until now none of these studies recommends selection strategies for bacteria to be used in general bioremediation applications [4]. The major drawback for biological decontamination is the efficient and accurate selection, identification and characterization of the appropriate microorganisms among numerous species or strains. In addition, low decontamination capacity of some microorganisms due to the difficulties during the adaptation is another disadvantage [2]. A rapid and an efficient selection of specific microbial strains exhibiting strong heavy metal adaptation ability appear to be the key point for the removal and/or degradation of target contaminants from the ecosystem [5]. Therefore, approaches for screening, characterization and classification of the bacteria are essential. To achieve these processes, the bulk chemical data obtained from analyses of microorganisms should be described and interpreted in a qualitative and quantitative manner [6]. To identify, discriminate and classify bacteria, traditional culture-based, brand-new molecular (PCR based) and immunological techniques have been employed as standard procedures in microbiology [7–9]. However, these techniques do not provide quick and routine applications especially for the fieldwork. In this context, advances in spectroscopy-based fast, easy to use and low-cost techniques provide an opportunity [7,9]. Infrared (IR) spectroscopy elucidates chemical properties of the biomolecules in detail resulting in establishing chemical fingerprint. It is a rapid, versatile, reliable, accurate and automated technique. Low cost and reduced sample preparation steps make this technique a very convenient tool for analytical measurements. Abundant data can be

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obtained from the specimen through the interpretation of its IR spectrum. Furthermore, definite wavenumber detections, superior signal-to-noise ratios and multiplex advantage, put IR spectroscopy in a favorable position among other analytical techniques [10].

As indicated by Naumann in 2006, it is possible to detect, enumerate, classify and identify microorganisms in one single IR apparatus and interpret the outcomes of the experiment in 24 h followed by their isolation and growth [11]. The technique is exceptionally specific to differentiate the strains, species and genus compared to common identification methods. In addition, it is used in process controls in biotechnological operations and to diagnose microbial accumulations in different industrial facilities [11]. Furthermore, IR spectroscopy generates qualitative and quantitative information about the composition, function and structure of the molecules which is unique for the studied system [12,13]. Due to these advantages, this technique has been extensively used in the determination of bacterial components [14–17]. Moreover, it is a fast, sensitive, easy to use, nondestructive, relatively inexpensive and comprehensive method [18]. Along the same line, we have previously determined gross biomolecular alterations happening in cobalt-acclimated as well as Cd and Pb-adapted bacteria by taking advantage of qualitative and quantitative molecular discrimination power of IR spectroscopy that measures the vibrations of atoms, enabling the identification of functional groups of molecules [15,16].

Since the IR spectra of microorganisms contain complex chemical fingerprint information, their analyses and interpretation require chemometric tools. This is necessary to establish an adequate generality in the accurate and rapid classification of the microbes. In other words, proper and reliable data mining assays should be selected for the efficient classification and discrimination of microorganisms [19]. Chemometric methods use multivariate analysis tools and mathematical modeling to the analytical data [20]. The meaningful information can be gathered quickly and efficiently from the complex and large chemical data sets by using these techniques [21]. Although the previous studies showed the applicability of IR based unsupervised and supervised methods for the discrimination of bacteria [11,15,22–25], supervised ones have not been applied yet to select and differentiate the heavy metal-exposed bacteria. In our previous work, although, we have used unsupervised hierarchical cluster analysis (HCA) and principal component analysis (PCA) for differentiation of the Cd and Pb-exposed bacteria, the development of powerful supervised chemometric models with SIMCA and their thorough testing are necessary to classify the heavy metal-exposed bacterial classes properly with high assurance. Therefore, in the current study, for the first time, we aimed to develop an accurate and rapid differentiation and most importantly classification method for Ag, Cd and Pb-exposed laboratory strains and bacterial isolates from a freshwater environment. In this study, bacterial strains such as *Escherichia coli*, *Staphylococcus aureus*, *Gordonia* sp. FS18 and *Microbacterium oxydans* FS45 [26] were tested because of their capability to survive and grow at growth inhibitory concentrations of Ag, Cd and Pb. In this context, we first performed unsupervised chemometric tool- PCA, which is a mandatory step, and then, we performed SIMCA on IR spectral data of the bacteria.

The identification and classification of heavy metal-exposed bacteria would help to determine the most appropriate strains with sufficient biosorption or transformation capacities and eliminate the inapplicable strains in accurate, fast and productive manner. Thereupon, our approach would contribute for the establishment of sustainable, green and worthwhile biogeotechnological operations to rehabilitate the contaminated soil and water.

2. Material and Methods

2.1. Bacterial Growth Conditions

Laboratory (*Escherichia coli* ATCC 8739 (*E. coli*) and *Staphylococcus aureus* ATCC 6538 (*S. aureus*)) strains and environmental (freshwater) bacterial isolates (*Gordonia* sp. FS18 and *Microbacterium oxydans* FS45 (*M. oxydans*)) were grown respectively at 37 °C and 28 °C under aerobic conditions, in an orbital shaker at 200 rpm. The freshwater bacteria were previously isolated from Lake Mogan and identified via both microbiological and genetic (16sRNA) techniques by our group [26]. Nutrient broth (5 g peptone from meat and 3 g meat extract per liter, *Merck*, US) and nutrient agar (5 g pancreatic digest of gelatin, 3 g beef extract, and 15 g agar per liter, *Becton Dickinson*, US) were used as culture media. Cadmium chloride and silver nitrate (*Sigma*, US) stock solutions were prepared by dissolving in dH_2O and lead nitrate was dissolved in 1:10 diluted nitric acid (in dH_2O). For the sterilization of the stock solutions, 0.22 μm filters (*Pall*, US) were used. The standard metal solutions were added to the growth media after they were autoclaved and cooled to 45–50 °C. All bacteriological procedures were carried out under sterile conditions, in a laminar flow hood (*Esco*, US).

2.2. Sample Preparation for Attenuated Total Reflectance (ATR)-Fourier Transform Infrared (FTIR) Spectroscopy Measurements

The bacterial concentrations were adjusted as 0.5 OD at 600 nm using sterilized distilled water (*UV-2600/2700*, *Shimadzu*, JP). The bacteria were collected by centrifugation at 10,000 g (*Sigma 1–14 Microfuge*, *SciQuip*, UK) for 10 min. After the supernatant was removed, the pellets were gently resuspended in sterilized distilled water. The sample preparation steps were performed according to the established nondestructive routine laboratory procedures for obtaining the IR spectra of intact bacterial cells [11,13,15,27].

2.3. ATR-FTIR Spectroscopic Measurements and Data Preprocessing

Spectrum 100, FTIR spectrometer (*PerkinElmer*, US) equipped with a Universal ATR accessory, was used to collect the IR spectra of control and heavy metal-exposed bacteria. The spectrum of air was used as a reference. Each sample (5 μl) was placed on a diamond/ZnSe crystal plate (*PerkinElmer*, US) and mildly purged and dried under inert nitrogen (N_2) gas flux for 2 min. Purging samples with noninvasive N_2 was applied in order to remove the overlapping free water bands from the samples (while keeping the bound water in the system) as a common procedure in ATR-FTIR studies. The samples were scanned over the spectral range 4000 to 650 cm^{-1}, at room temperature, 100 times per sample and with a resolution of 4 cm^{-1}. For each bacteria group, 15 independent spectra were collected in triplicate and average spectra of these triplicates were used in all data analyses. Spectrum 100 software (*PerkinElmer*, US) was used for data collection and analyses, which included averaging of spectra within samples, smoothing and baseline corrections. In data preprocessing, the second derivative and vector-normalized IR spectra were obtained using *Opus 5.5* software (*Bruker*, US). Vector normalization is carried out in the following way: spectra are first mean-centered, i.e. the average value of the absorbances is calculated for the spectral region indicated. This value is then subtracted from the spectrum. Then, the spectra are scaled such that the sum squared deviation over the indicated wavelengths equals one.

2.4. Chemometric Methods

2.4.1. PCA

PCA is an extensively employed versatile unsupervised pattern-recognition method [28] for the differentiation of microorganisms using large and complex IR spectral data [29]. PCA illustrates the items through the variables generated after the linear integrations of authentic variables. These linear integrations termed principal components are estimated along the course of most extreme change and opposite to one another. In spite of the fact that PCA grants an outlined representation of the information set, its fundamental utility is to show structure in the information [30]. The peculiar scores obtained for each information set can be utilized to group the information in the PC-based coordinate