Application of Attenuated Total Reflection/Fourier Transform Infrared Spectroscopy in the Screening of Strains Producing Bioactive Molecules: A Metabolomics Approach

A Z Boumehira1,2,3, O Arous2, E A Elsayed4,5*, H Hacene2, N Yezli3, D Sukmawati6, H A El-Enshasy7,8

1University of Algiers, Faculty of Sciences, Algiers, Algeria
2University of Sciences and Technology Houari Boumediene, Algiers 16111, Algeria
3Centre de Recherche Scientifique et Technique en Analyses Physico Chimiques, Bou-Ismail, Tipaza 42004, Algeria
4Bioproducts Research Chair, Zoology Department, College of Science, King Saud University, Riyadh 11451, Kingdom of Saudi Arabia
5Natural and Microbial Products Department, National Research Centre, Dokki, Cairo 12311, Egypt
6Department of Biology, Faculty of Mathematics and Natural Sciences, Universiti Negeri Jakarta, Jakarta, Indonesia
7Institute of Bioprocess Development, Universiti Teknologi Malaysia (UTM), Johor 81310, Malaysia
8City of Scientific Research and Technology Applications, New Burg Al Arab, Alexandria 21934, Egypt

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The Recent developments in infrared spectroscopy have made it a method of choice for metabolomics study as it provides accurate analysis with low operating costs due to availability of spectra processing software and chemometrics analysis. The aim of this work is to demonstrate the high potential application of Attenuated Total Reflection/Fourier Transform Infrared Spectroscopy (ATR/FTIR) in rapid screening of microbial strains producing bioactive molecules. In this work, 101 strains were isolated from soil samples collected from the Algerian Sahara desert. ATR/FTIR spectroscopy was applied to eliminate replicate strains. After initial screening, the obtained spectra were divided into two regions. The first, in region between 4000-2250 cm⁻¹, contain C-H bond stretching vibrations of methyl and methylene of fatty acid membranes, which is very helpful in discriminating Gram positive from Gram negative bacteria. The second region, between 1800-900 cm⁻¹ (amides I and II), 1800-1500 cm⁻¹ (peptides and proteins), and 960 cm⁻¹ (nucleic acids) were observed. Applying this method as first step in screening enabled us to reduce the sample number by 71.28%. Combining this technique with antibacterial test of antagonism, further reduction in strain number by 87.12% was obtained. Accordingly, total screening costs as well as time for bioactive metabolite screening program were reduced.

Keywords: ATR/FTIR Spectroscopy, Bioactive metabolites, Metabolome, Rapid screening

Introduction

Great deal of research has been motivated to overcome problems of developing antimicrobial-resistant1-4. Increasing efforts are carried out to discover new antibiotics. However the number of anti-infective drugs introduced into the market has not allowed us to overcome this scourge, due to high cost demands for the discovery of new therapeutic molecules, and cumbersome drug registration process5-7. However, microorganisms remain the most important source of bioactive molecules for many research laboratories8-10. For better management of these programs, new techniques are usually required to reduce the cost and time for bioactive metabolites discovery11. Accordingly, screening programs use metabolomics studies for selecting bacterial isolates producing bioactive molecules12. These include the application of sophisticated platforms, such as Liquid Chromatography-Mass Spectrometry (LC/MS), Gas Chromatography-Mass Spectrometry (GC/MS) and Nuclear Magnetic Resonance (NMR). The biological activity screening also has been improved, especially after the introduction of High Throughput Screening (HTS) platform13. These platforms still suffer from the high cost of installation/maintenance, and the requirement of certain professional and technical skills, which are considered the main limitations which affect the speed of the exploration of natural resources in developing countries. In the present work, we applied a metabolomics approach, which is used for exploiting current developments in the field of the Attenuated Total Reflection/Fourier Transform Infrared Spectroscopy (ATR/FTIR), for rapid

Author for Correspondence
E-mail: ealsayed@ksu.edu.sa
screening of bioactive molecule producing strains. This technology is characterized by its low-cost, simple operating procedures, accuracy, and availability of software, which facilitate the treatment of recorded spectra and chemometrics analysis. This study combines ATR/FTIR Spectroscopy with a bacterial antagonism test for developing a simple, low cost and fast procedure, allowing rapid screening of microbial strains producing antibacterial molecules.

Materials and methods

Study location

Strains used in this study were isolated from saline soil samples (Sebkha), collected near to Timadinine (26.698356 N 0.108620 E); at Adrar province in Algeria, during the summer of 2012, as part of the research project No. 284/SA/2012, for Centre de RechercheScientifiqueen Analyses Physico-Chimiques-C.R.A.P.C. Algeria.

Strains isolation and purification

For each sample, 10 grams of soil were suspended in 100 ml of sterile saline solution. Then decimal dilutions with distilled water (10^-1, 10^-2, 10^-3 and 10^-4) were prepared. 0.1 ml of each dilution was used to inoculate ISP2 agar medium, containing different concentrations of NaCl: 0%, 2%, 15% and 30%. All inoculated dishes were incubated at 30°C up to 8 days. The grown colonies were further purified in order to obtain pure cultures. The selection of colonies is based on the macroscopic appearance of the colonies (by the use of Stereomicroscope, Zoom 2000™, Leica Microsystems, Germany), the primary selection parameters are: color, shape, diameter, surface, opacity and the presence of mycelium. Figure 1 provides an overview of the approach used in this study.

Sample preparation and ATR/FTIR spectroscopy measurements

Fourier Transform Infrared Spectroscopy (FTIR) coupled with Attenuated Total Reflection (ATR) is a new method applied for in vitro and ex vivo experimental procedures, and can be used for monitoring biological processes. In our study, ATR infrared spectra, obtained from an accumulation of 128 interferograms at a resolution of 2 cm^-1, were recorded with ALPHA FT-IR Spectrometer (Bruker Optics Inc., MA, USA) and monitored by a Opus 6.5 software. A hemispherical Ge ATR crystal was used with polarized radiation at a 65° incident angle. Prior to all ATR/FTIR measurements, a standard experimental procedure concerning: first, comparison between strains was after the use of the same culture media, and adjusted to pH 7; second, the same cultivation time; third, same spectral registration parameters; and fourth, the strain must be pure. For spectral measurements one full platinum loop of microbial cells was deposit in the object holder of spectrometer. Typically, three replicate samples were measured for each bacterial colony.

In vitro antibacterial test

For antimicrobial determination, two standard strains were used in this study: Micrococcus luteus ATCC 10420 and Staphylococcus aureus ATCC 25923. These strains were cultured in Mueller Hinton Broth at 37°C for 20 h, then transferred onto Petri dishes containing Mueller Hinton Agar (70191, FlukaBioChemika, Switzerland). The agar discs (6 mm diameter) of the isolated strains, with unique metabolome profile in MIR-ATR-FTIR, are deposited on the surface of the Mueller Hinton Agar plates seeded with the indicator strain. Negative and positive control discs, un-inoculated culture medium and Vancomycine 30 μg, respectively, were also included in the test. Incubation was at 37°C for 24 h. The antimicrobial activity was determined in terms of the inhibition zone (in mm) measured by Read biotic viewer (International Pbi S.p.A. Italy). A zone of inhibition equal or more than the diameter of the cylinder (6mm) was considered indicative of antimicrobial effect.

Result and Discussion

Microbiologists from the end of 19th-century showed that soil contains some microorganisms that can inhibit pathogenic strains. Therefore, in this study, we isolated microorganisms from saline soil samples, collected from the Algerian Sahara desert. The culture medium used is ISP2, a rich medium (g/l: Malt extract 10g, Yeast extract 4g and Glucose 4g), frequently used for isolation and study of strains of the genus Streptomyces, genus known for the production of antibiotics. In this work, we isolated 101 strains from the soil samples. The isolated strains were purified on solid medium. After initial macro- and microscopic study, the strains were transferred to the spectroscopic study, as shown Figure 1. The classification of microorganisms was traditionally based on morphological characterization at the species level. The development of molecular biology has allowed more accurate identification, based on the similarity in DNA sequence, allowing identifying and classifying microorganisms; however the process is
long and tedious to implement. Infrared spectroscopy can be considered as a good alternative to differentiate and identify microorganisms. The use of infrared spectroscopy in microbiology is not new. In 1952, Stevenson and Bolduan have shown that this technique could be used for the identification of bacteria. It is especially the introduction of Fourier transform spectroscopy and the development of computer science which give interest to this technique. Furthermore, the works of Naumann's group, have given a better use of this technique in the field of microbial identification. Afterwards, the use of this technique became increasingly present in microbiology laboratories. This is explained by the fact that infrared spectroscopy allowed the visualization of structural peculiarities of different
Microorganisms, unicellular entities, are formed from structural components such as proteins, carbohydrates, lipids, nucleic acids and minerals. These various components of the cell contribute to the specificity of the spectral information and thus allow characterizing a bacterium in comparison to another. However, as in the characterization of chemicals, it is necessary to standardize the recording conditions of the spectrum, it is also necessary to define and to follow a microorganism preparation procedure in the analysis, in order to minimize the impact of culture conditions on morphological and physiological characteristics. Time and temperature of incubation of the strains, pH and the nature of culture medium used are parameters which may cause significant modifications to the recorded spectrum. In this work, firstly, we divided the spectra into two regions. The first region is from the 4000-2250 cm\(^{-1}\). This region is specific for the C-H bond stretching vibrations of methyl and methylene of fatty acid membranes. This region is of particular interest in the discrimination between Gram positive and Gram negative bacteria. This is related to the physico-chemical composition of the membranes since the wall of Gram negative bacteria is richer in lipids. The cell wall of Gram positive bacteria is generally composed of peptidoglycan and teichoic acid, which represent near to 50% of the weight of this wall, while the cell wall of Gram negative bacteria contains lipoprotein and glycolipids. The second region is between 1800 and 900 cm\(^{-1}\). In this region we can find the peaks of primary and secondary amides, peptides and proteins (1800 and 1500 cm\(^{-1}\)). In addition, the part between 1500 and 1200 cm\(^{-1}\) is dominated by the vibration of the double bond P=O of phospholipids. Low visible absorption band at 1230 cm\(^{-1}\) is representative of the hydroxyl group of the polysaccharides. Peaks between 1200 and 900 cm\(^{-1}\) are dominated by stretching vibrations C-O-C and C-O-P bonds of oligosaccharides and polysaccharides, respectively, whereas 960 cm\(^{-1}\) are the absorbance wavelength for nucleic acids. Figure 2 and 3 show samples of spectra recorded for the distinction in three strains, Z1, Z3 and Z5, which are phenotypically close. These strains were originally isolated on a medium ISP2 supplemented by 15% NaCl. Major peaks of Z1 and Z5 relative to the control (Culture medium) were those recorded at 2959 cm\(^{-1}\), 2926 cm\(^{-1}\), 2876 cm\(^{-1}\), 2855 cm\(^{-1}\), 1745 cm\(^{-1}\), 1556 cm\(^{-1}\), 1458 cm\(^{-1}\), 1420 cm\(^{-1}\), 1314 cm\(^{-1}\), 1285 cm\(^{-1}\), 1249 cm\(^{-1}\), 1026 cm\(^{-1}\), 902 cm\(^{-1}\), 807 cm\(^{-1}\), 743 cm\(^{-1}\). When studying the peaks of the strains Z1 and Z5 compared to Z3, we observed the presence of the peak at 1400 cm\(^{-1}\) for Z1 and Z5, which is absent in Z3 sample. It was also found that the strain Z3 has peaks at 1694 cm\(^{-1}\), 1420 cm\(^{-1}\), 1379 cm\(^{-1}\), 1285 cm\(^{-1}\) and 807 cm\(^{-1}\), while we do not find these peaks in samples of the strains Z1 and Z5. Therefore, we can conclude that the strains Z1 and Z5 have the same metabolic profile, while Z3 has a different profile. From 101 initially isolated strains, we were able with this approach to identify 29 classes of strains with distinct metabolome profile, and thus we eliminated duplicated strains, which represented 71.28% of the total number of isolated strains (72 out of 101 strains). This can be attributed to the fact that metabolomic analysis by attenuated Total Reflection/Fourier Transform Infrared Spectroscopy (ATR/FTIR), allows the visualization of all the functional group present in the samples, which are responsible for biological activity and particularly, the antibacterial activity. One strain of each metabolomic class was selected for biological screening assay (antibacterial susceptibility test). M. luteus and S. aureus are frequently chosen as indicator strains in anti-infective molecules research programs. M. luteus is more sensitive, and different strains of this species were used for this purpose, such as: M. luteus ATCC 4698, M. luteus ATCC 10420, M. luteus ATCC 272 and M. luteus ATCC 9341, this last strain was reclassified as Kocuria rhizophila ATCC 9341\(^{16-19}\). Staphylococcus aureus, Gram-positive pathogens, are a critical component in bloodstream infection, food borne infections and nosocomial diseases\(^{20}\). Therefore, it is frequently used as an indicator strain in antibacterial susceptibility tests, and three strains are especially recommended: S. aureus ATCC 25923, S. aureus ATCC 29213 and S. aureus ATCC 43300. In this study, we used two indicator strains: M. luteus ATCC 10420 and S. aureus ATCC 25923. The results showed clearly that 13 strains exhibited antibacterial activity against M. luteus ATCC 10420 and/or S. aureus ATCC 25923 with interesting metabolic profile (29 out of 101 strains). This approach allows rapid screening of bioactive metabolites screening program and thus the potential producer strains undergo for further molecular identification, biological activity, extraction and
characterization of active compounds. In this study, we have reduced the number of studied strains with a level of 87.12%, which means a similar cost savings percentage, by reducing the number of tests to be performed and working time in the laboratory (13 out of total 101 strains).

**Conclusion**

The development achieved in terms of Attenuated Total Reflection/Fourier Transform Infrared Spectroscopy (ATR / FTIR), contributed in the development of approaches easily adapted to the routine laboratory for developing countries. It allowed the reduction of time and costs of research projects in the field of screening new therapeutic molecules produced by microorganisms.
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