Attenuated Total Reflectance-Fourier-Transform Infrared Microspectroscopy a Rapid Method for Microbial Strain Characterization

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ABSTRACT

Fourier-Transform Infrared (FTIR) spectroscopy and BHI 50CH were used to identify bacteria of the Lactobacillus (L.) at the species level. A previously developed method for measuring FTIR spectra and a strategy for their analysis provided the basis for selecting the FTIR spectra of reference bacterial strains and created a spectral library. The research was launched in which the spectra collected in the above library were used for developing a spectral reference for known reference bacterial strains and the practical value of the generated library was verified based on the results of identification of four bacterial strains viz. L. plantarum, L. casei, L. lactis and L. fermentum of known taxonomy as well as identification of 15 bacterial strains isolated from rumen extracts and identified on the basis of their taxonomy and biochemical tests. The application of prepared lactic acid bacteria reference library for analysis of advanced analysis of FTIR was provided an accurate identification of 90% of bacterial strains of the genus Lactic acid bacteria identified by FTIR-microspectroscopy.

Keywords: Lactic Acid Bacteria, FTIR Spectra, Rumen Extract, Bacterial Isolation

1. INTRODUCTION

The latest methods for microorganism differentiation and identification are oriented towards simplicity, reducing the time needed to perform analysis towards high specificity and quality of microbial identification results. One of the methods that meet these criteria is Fourier-Transform Infrared (FTIR) spectroscopy. Studies conducted by (Dziuba et al., 2006; 2007; Mossoba et al., 2003; Nauman, 2000; Chohan et al., 2010) showed that FTIR spectra can be treated as specific to particular bacterial strains. They reflect the distinguishing features of all bacterial cell components, such as fatty acids, membrane and intracellular proteins, polysaccharides and nucleic acids. The differences between FTIR spectra of microorganisms are difficult to observe, thus referring to creative reference library and proper statistical methods are required for accurate and acceptable identification of isolated microorganisms (Dziuba, 2007; Samelis et al., 2010).

However, results obtained by FTIR are acceptable and dependent on the interpretation of spectrum through similarities with spectrum of known strains (Johnsen and Nielsen, 1999; Timmins et al., 1998; Huleihel et al., 2009). The results of the identification of bacterial spectra obtained by the above methods are comparable to those obtained with the use of other techniques such as biochemical identification method (API 50 CHL). Apart from classical methods, FTIR method is often applied for microorganism identification (Kirschner et al., 2001; Udelhoven et al., 2000; Dziuba et al., 2006; 2007; Sameli et al., 2011). Among numerous reports on microorganism differentiation, classification and identification using FTIR spectroscopy (Weinrichter et al., 2001; Igisu et al., 2009) a few only deal with lactic acid bacteria. However, the FTIR spectral characteristics of lactic acid bacteria was based exclusively on spectral differentiation index to describe the relationships between FTIR spectra and bacteria as molecular systems in a way that would permit their proper microbial
identification. Generally FTIR/FTIR-ATR acquired spectra of bacteria are composed of hundreds or even thousands of overlapping bands that cannot be separated. It follows that their analysis requires the application of pattern recognition techniques, analyzing spectra as fingerprints. One of the most advanced and promising methods of this group is to create a reference library with organisms of concern, therefore, the objective of this study was to use FTIR/FTIR-ATR to create proper reference library and to differentiate microbial strains with particular attention to lactic acid bacteria as strains with Probiotic features used for several health attributes.

2. MATERIALS AND METHODS

2.1. Bacterial Strains and Growth Conditions

*L. plantarum* (ATCC 8014), *L. casei* (ATCC 303), *Lactobacillus lactis* (ATCC 7830) and *L. fermentum* (ATCC 9338) strains used in the study were purchased from international collections ATCC (Microbiologics, Medimark, Europe, France), microbial strain collection from the Department of Food Science, University of Manitoba, Canada (UoM) and strains isolated from rumen extracts of sheep, goat, camel and cattle that collected from different slaughterhouses located in northern and southern part of Jordan. API 50CHL and API 50 CHL medium were purchased from Biomerieux (BioMérieux, Durham, USA). Bacterial strains were cultured anaerobically in solid MRS agar media for 48±2h under optimum conditions at 30°C.

2.2. Sample Preparation, Measurement and Evaluation

Reference and isolated microorganism cultures were grown on agar plate for 24 h and the cell material is harvested from the agar plate and suspended in sterilized physiological saline. A 120-µL of the bacterial suspension was loaded onto special reusable micro plates in 96 well formats and dried for 30 min at 50°C. FTIR measurements were performed in transmission mode. All spectra were recorded between 4000 and 600 cm\(^{-1}\) with a Tensor 27 FTIR spectrometer, equipped with HTS-XT accessory for rapid automation of the analysis (Bruker Optics GmbH, Germany). Spectral resolution was set at 4 cm\(^{-1}\), sampling 256 scans per sample. After drying, the plate was inserted into Bruker Optics’ microplate reader HTS-XT and scanned between 500 to 4000 cm\(^{-1}\). The measurements were repeated for four replicates. The acquired spectrum were evaluated using OPUS 5.5 software and compared with previously created spectrum similarities of different isolated microorganism for lactic acid bacteria of known strains and with those of ATTC.

Three samples of two independent cultures (a total of six samples) were prepared for each strain. The experiment was conducted in three replications. A biochemical tests using API 50CHL were also performed to compare FTIR with biochemical bacterial identification.

2.3. FTIR/ATR-FTIR for Microbial Identification

Microbial identification of food product is essential to verify the safety of commercially distributed food commodities for human consumption as well as to determine how effective of food processing in killing food spoilage or food-borne pathogens. ATR/FTIR micro spectroscopy capable of identifying microorganism through infrared spectrum of the biomolecules found specific for every microbial strain found within bacterial cells with fast, reliable, cheap compared with known other molecular or biochemical with customizable generated libraries. Attenuated Total Reflectance (ATR) Fourier transform infrared spectra were collected with a bench-top spectrometer (Tensor 27, Bruker Optics GmbH, Ettlingen, Germany). Spectra were recorded in a range from 850-4000 cm\(^{-1}\) at a resolution of 4 cm\(^{-1}\) and with an aperture of 6 mm. Data storage, spectra processing, substance comparison and the quantitative analysis of the spectra were done with the software OPUS 6.5 (Bruker Optics, GmbH, Ettlingen, Germany).

2.4. Database Library Creation

The quality of the databases has a fundamental importance for the reliability of the identification. After all, each spectrum of an unknown microorganism is compared against all spectra from a created library for the known strains. A database library has to contain all relevant species, as well as all spectral variances of different strains from a single species. Food relevant microorganisms from reference stocks are not the only strains in the library, but the data of isolates from different production sites are also included. Prior to building up libraries, all microorganisms in the database were identified through reliable reference techniques.

2.5. Data Analysis

The recorded FTIR spectra together with the results from API 50 CHL reference analysis were analyzed using partial Least-Squares (LSD) regression using SAS version 9. Spectrum during preparation of the calibration
models were visually checked for principal component
(Weinrichter et al., 2001). The spectral data were divided
into three sets (learning, validation and testing) to
include the spectra of all strains analysed. The validation
set consisted of FTIR spectra of 4 reference strains. The
testing set included 4 spectra of reference strains of
known taxonomy and 7 spectra of bacteria isolated from
the rumen extract. For the identification of
_Lactobacillus_ strains at the species level, the output
classifier consisted of ten neurons with assigned classes.
The bacterial spectra were stored in a generated library
for _L. casei, L. delbrueckii_. Subspecies _lactis; L.
_fermentum_ and _L. plantarum_. Neurons organized in the
layers, were connected in a way specific to a given type
of artificial neural network.

3. RESULTS

The spectral differentiation of bacteria of the genus
_lactic acid bacteria_ of the isolated or ATCC collections
strains are presented in **Fig. 1 and 2** It is obvious that
reference bacterial spectrum used for the identification of
lactic acid bacteria in the present study were useful and
gives and accurate results. One of the advantages of
Bacterial Reference Spectrum (BRS) is their capability
to generate knowledge to new strains with previously
unseen bacterial strains, not fed into the reference
library. At the same time BRS are able to memorize the
acquired knowledge, which can be used at any moment
without the need to feed the information again.

4. DISCUSSION

The process of bacterial identification involved
bacterial strains assigned classes had been divided into
three sets of validation and testing spectral techniques.
The spectra from the validation served to control the
identification process and to evaluate the validity of
differentiation process. The capability to generalize the
knowledge acquired during the tests was tested on the
basis of the spectra contained in the testing. A total of
100 spectra were used during the development of
artificial reference library for lactic acid bacterial
identification at the species level. The acquired set was
composed of 100 FTIR spectra, while the validation
lactic acid bacterial groups were 10 FTIR spectra of 4
reference strains. The testing set included 20 spectra of
reference strains, 4 spectra of bacteria of known
taxonomy and 8 spectra of bacteria isolated from
collected animal rumen extracts. The numbers of spectra
of each lactic acid bacterial strains of reference strains
and from bacterial species isolated from the rumen
extracts were similar to be in balance in their
demonstration along with sensitivity analysis to predict
the proper spectral strains with specific variables based
on the polysaccharide spectrum. The greatest extent of
identifying bacterial strains with FTIR compared with
biochemical test kit (API CHL 50). The selected
variables provided the basis for creating another
neural network. The trained network enabled to obtain
fully correct results. The correctness of identification of
_Lactobacillus_ strains at the species level was verified
according to a two-stage procedure. At the first stage
artificial neural networks were tested based on the
spectra of 4 reference strains. At the second stage the
spectra of 4 strains of known taxonomy and 10 isolated
strains, identified with biochemical tests (API CHL 50),
were used. All reference spectral libraries were generated
on FTIR spectra of the tested lactic acid bacteria.
Similarly as in the case of bacteria identification at the
genus level (Dziuba et al., 2007; Naumann et al., 1991;
Sameli _et al_., 2011), the optimum reference bacterial
library were selected as a consequence of searching for
the most relevant lactic acid bacterial parameters and
spectral reference similar to the isolated bacteria. The
RBS presented in **Fig. 1 and 2** correctly identified all
reference strains of testing set I, whereas bacteria of
testing set II were correctly identified in 89 to 93%
of cases. The best results were achieved using reference
bacterial spectra for a combination of the spectral ranges.
The reference bacterial correctly identified all reference
strains and all strains of known taxonomy. The affiliation
of the following four strains was not determined
unambiguously: Lb. _casei, Lb. fermentum, Lb. plantarum_
and Lb. _delbrueckii_ subspecies _lactis_, respectively. It was
found that differentiation of _Lactobacillus_ strains at the
species level based on analysis of individual spectral
regions and the entire spectrum was limited. The present
results confirmed the correctness of conclusions drawn
from a comparison of bacterial spectra determined on
the basis of the differentiation index, but also proved the
existence of such combinations of spectral ranges
which permitted the most reliable identification of
bacterial strains. The best results of differentiation of
bacteria of the genus _Lactobacillus_ at the species level
were obtained for combinations of the ranges of the first
derivatives of spectra for the polysaccharide region, the
fingerprint region and the mixed region. Curk et al.
(1994); Weinrichter et al. (2001) and Sameli _et al_. (2011)
also achieved the best results for the above combinations.
However, these authors did not use artificial neural networks and we demonstrated previously (Dziuba, 2007; Mariey et al., 2001; Rodriguez-Saona et al., 2001) that the FTIR spectral characteristics of bacteria based exclusively on the differentiation index D and cluster analysis are not sufficient to describe the relationships between FTIR spectra and bacteria as molecular systems in a way that would permit their proper identification.

The application ATR-FTIR of a reference library and created reference strains spectrum enabled correct identification of 93% of bacterial strains of the used Lactic acid bacteria. Moreover, the results could be probably improved if the number of strains was increased, preferably including the so-called typical strains. A promising solution is to develop multilevel artificial neural networks, forming a single structure. The networks organized in this way would enable identifying microorganisms at various taxonomic levels.

![FTIR absorbance spectra in the region from 1,800 to 900 cm⁻¹ for intact Reference lactic acid bacteria gram-positive rods grown on MRS agar at 37°C for 24 h. Pure ATTC strains from L. casei and L. plantarum. The bands indicated with arrows correspond to the main absorption bands assigned of the lactic acid bacteria.](image)
Fig. 2. FTIR absorbance spectra in the region from 1,800 to 900 cm\(^{-1}\) for intact Reference lactic acid bacteria gram-positive rods grown on MRS agar at 37°C for 24 h. (a) Isolated strains from rumen extract of Sheep, Cattle, Camel, and Goat. The bands

5. CONCLUSION

- The measurement region of FTIR at wavelength between 1200 cm\(^{-1}\) to 900 cm\(^{-1}\) for bacterial strains polysaccharide with the specific region for differentiation between 900 cm\(^{-1}\) - 700 cm\(^{-1}\) and regions 1500 cm\(^{-1}\) to 1200 cm\(^{-1}\) of the bacterial acquired were found most appropriate spectra for FTIR characterizing strains of lactic acid bacteria
- The application FTIR reference bacterial strains spectrum library is considered as method of choice in using FTIR for correct identification of lactic acid bacterial strains with > 90 confidence limit
• ATR-FTIR/FTIR spectroscopy, combined with reference library was found to be suitable, simple, fast and accurate technique in microbial identification of lactic acid and could be used in identification of food borne pathogens and food spoilage bacteria at strain level and seems to be very promising

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