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Classification of Bacteria Using FT-IR

D. Lefier, INRA-URTAL-Poligny, France,
B. Beccard, Thermo Fisher Scientific, France,
M. Bradley, Thermo Fisher Scientific, Madison, WI

The classification of bacteria is generally based on the morphology and biochemical reactions of the bacteria. However, these measurements are time consuming, and require training and expertise. Infrared (IR) spectroscopic measurements of bacteria followed by a formal chemometrics analysis could offer advantages of speed and consistency. Such an analysis was first tried in the late 1950s, with some success, but with limitations imposed both by the instrumentation and by the post-processing tools available (1).

The IR analysis of bacteria underwent resurgence in the 1980s with the advent of Fourier transform infrared (FT-IR) instruments. The higher signal to noise and generally better performance, coupled with significant advances in computing power, made the application to bacterial analysis feasible. Hopkinson et al., and Naumann et al. were pioneers (1988) in using FT-IR for identification and differentiation of microorganisms (2,3). Recent work has shown the method is effective with pathogenic and nonpathogenic bacteria.

The food industry, especially groups like cheese producers, has a high interest in bacterial screening. The basic concern is that the correct strain of bacteria, and no other, is present in a food product. Europe is leading a push for tighter regulations that would require food producers to increase the sampling frequency. The potential speed and consistency advantages of FT-IR analyses are thus of great interest.

The standard infrared methodology is based on transmission of light through a bacteria sample dried onto a ZnSe window. Regardless of sample preparation methods, only about 50 samples per day can be processed. However, Thermo Scientific offers a powerful set of high-throughput screening (HTS) hardware and software tools which could greatly accelerate this rate, helping meet the needs of the increased sample load. The throughput improvement is largely realized through automation of the data collection and analysis.

The hardware utilizes a Micro Well Plate Reader stage inserted into a Nicolet™ 6700 FT-IR spectrometer, as seen in Figure 1. The sample plates and reader stage are based on the industry standard 96-well plate footprint, which allows the user to mount many samples on a single IR-transmission plate. With proper loading, multiple strains can be tested simultaneously, and replicate measurements can be made automatically to mitigate reproducibility concerns.

The Array Automation™ software allows a high level of control over the experiment. Users can choose the standard 96-well format or define customized well-plate formats for data collection. Data collection parameters can be entered for the whole plate, or can be tailored for each well. Array Automation interfaces directly with Thermo Scientific's



Figure 1: Micro Well Plate Reader installed in a Nicolet 6700 FT-IR Spectrometer.

OMNIC™ Spectroscopy software for collection and processing, and to the TQ Analyst™ chemometrics software for analysis. A plate can be loaded, and the data collection and analysis will then proceed without further user intervention.

Experimental

The experiments were performed by Mr. Le Fier at the French National Institute for Agricultural Research (INRA, URTAL, Poligny). The spectra were collected using a Nicolet 6700 FT-IR spectrometer equipped with a KBr beam-splitter. The Micro Well Plate accessory has an embedded DTGS detector, for data collection via transmission. The sample plate itself was made from a rectangle of silicon subdivided into cells by a PTFE mask. The experiment set up and data collection was driven by the Array Automation add-in to the OMNIC spectroscopy software. Processing via a discriminant analysis using TQ Analyst within Array Automation allowed the differences in the spectra to be brought out consistently.

Propionic, mesophilic and thermophilic bacterial samples were grown on Petri dishes. Samples of the bacteria were transferred to the silicon plates in two ways. A time-tested but protracted procedure involving removal of bacteria from the Petri dish was first used, as diagrammed in Figure 2. The second procedure involved drying bacteria, re-suspending them in water, and then drying this suspension directly onto the silicon plate. This required considerably less time and effort.

The spectra were collected using 100 scans at 4 cm^{-1} resolution. Cell A1 was reserved for background (see Figure 8) collection, and the remainder of column A was left

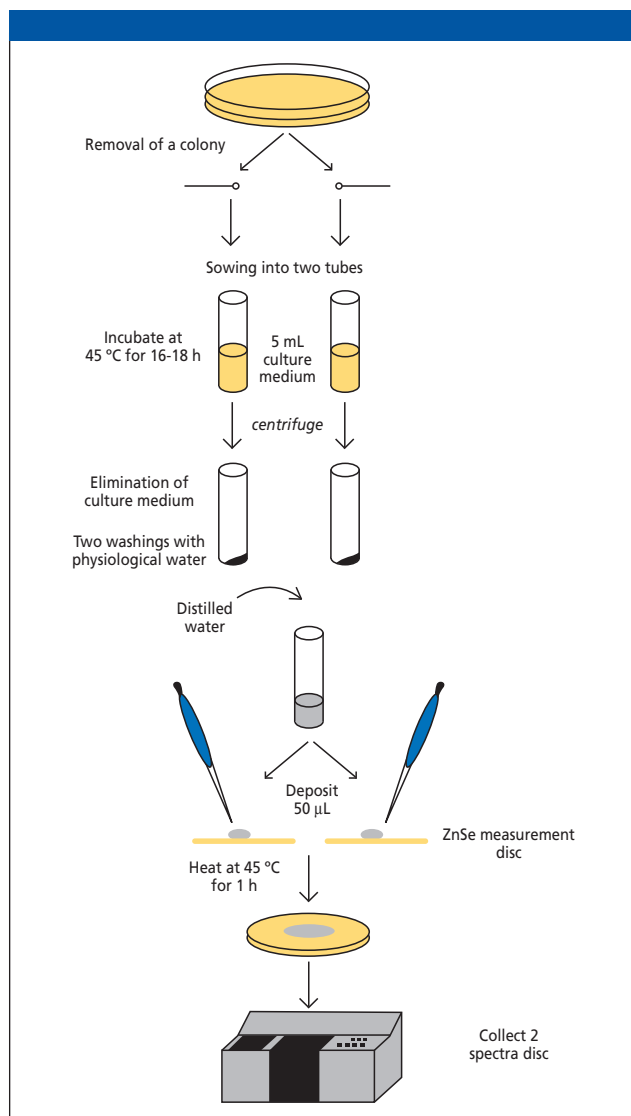


Figure 2: Standard process for preparing bacterial samples for FT-IR analysis.

empty. Eight replicates of a bacterium to be tested were introduced into each column, so the column numbers delineated different materials. Data collection for a full 89 wells (96 minus the 7 empty ones in column A) required approximately 134 minutes, or about 90 seconds per well. Video images of each well were also captured.

The FT-IR spectra for known examples of each class of bacteria were collected. These were used to generate a discrimination method based on assignment of spectra to classes. As with any biological sample, there is considerable variation within the class, so region selection and spectral processing are critical. The analysis defines the characteristics of each class mathematically. Unknowns are then assigned a Mahalanobis distance, representing how like or unlike the unknown is to each class. An assignment is made to the closest class, with reporting of nearest neighbor classes for comparison. The results are then color-coded for visualization.

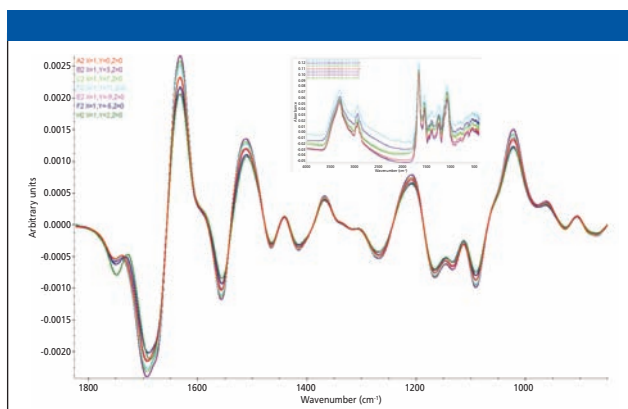


Figure 3: First derivative FT-IR spectra collected from a column of a well plate (same bacterial samples, 8 replicates). Inset shows raw spectra.

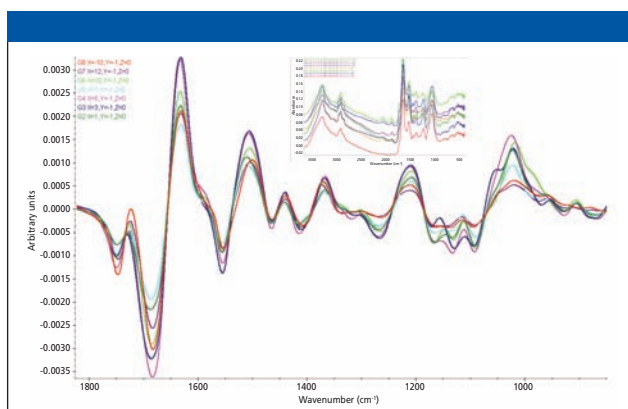


Figure 4: First derivative FT-IR spectra collected from a row of a well plate (same bacterial samples, 8 replicates). Inset shows raw spectra.

Results

The three strains of bacteria examined represent real case studies. As an example, propionic bacteria are present in the manufacture of Swiss-type cheese. In the first stages of manufacturing, lactic acid bacteria are used to convert lactose to lactate. During aging, propionic bacteria convert lactate to propionic acid, acetic acid and carbon dioxide. The CO₂ bubbles become the holes in the cheese, while the propionic acid gives the cheese the nutty flavor. Thermophilic bacteria thrive at elevated temperatures, while mesophilic bacteria are those functioning optimally around normal (25 °C to 40 °C) temperatures; both are important in cheese production and food spoilage.

Figure 3 shows a set of spectra obtained from one column (one class of bacteria), along with the first derivatives (which eliminates the baseline variance). The spectra and derivatives are clearly quite similar. Contrast this with Figure 4, which shows the data and derivatives across one row (seven different materials). The variance in the latter plot is clear, especially in the derivative. These comparisons are the basis for the discrimination analysis (DA).

An extensive set of spectra from well-defined bacterial colonies was used to build a database for the different bacteria. The results of the DA are shown in Figure 5. The axes

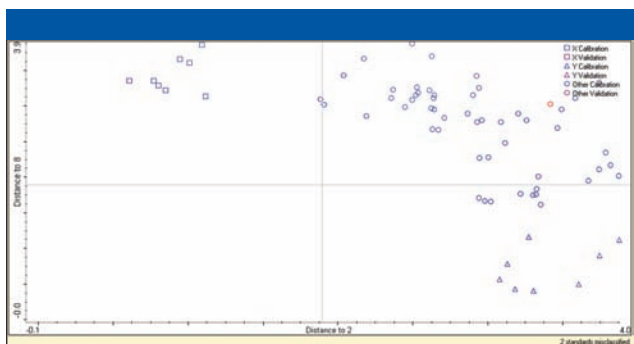


Figure 5: Class map showing distances from classes. See text for details.

Index	Spectrum Title	Actual Class	Calculated Class	Distance	Next Class	Next Distance	Distance to 2	Distance to 3
1	Mon Sep 18 15:25:54 2006 (GMT+02:0) 11	11	11	1.0639	10	1.7540	2.6714	1.9407
2	Mon Sep 18 15:25:54 2006 (GMT+02:0) 1	1	1	0.7946	6	2.6910	4.4939	3.6670
3	Mon Sep 18 15:25:54 2006 (GMT+02:0) 2	2	2	1.0084	4	2.8896	1.0084	3.6116
4	Mon Sep 18 15:25:54 2006 (GMT+02:0) 3	3	3	1.5081	6	2.0739	3.1337	4.5363
5	Mon Sep 18 15:25:54 2006 (GMT+02:0) 4	4	4	0.7157	5	1.9344	2.1744	3.6845
6	Mon Sep 18 15:25:54 2006 (GMT+02:0) 5	5	5	0.6564	4	1.0008	2.5259	3.1894
7	Mon Sep 18 15:25:54 2006 (GMT+02:0) 6	6	6	0.7322	7	1.4414	2.9276	2.7791
8	Mon Sep 18 15:25:54 2006 (GMT+02:0) 7	7	7	1.0023	9	1.6508	2.9237	3.4268
9	Mon Sep 18 15:25:54 2006 (GMT+02:0) 8	8	8	0.8998	9	2.4725	3.7469	0.8998
10	Mon Sep 18 15:25:54 2006 (GMT+02:0) 9	9	9	1.2289	11	2.6721	4.2073	2.9530
11	Mon Sep 18 15:25:54 2006 (GMT+02:0) 10	10	10	1.4339	7	2.3692	3.4631	2.6900
12	Mon Sep 18 15:25:54 2006 (GMT+02:0) 11	11	11	1.5521	9	2.3162	4.7370	2.3712
13	Mon Sep 18 15:25:54 2006 (GMT+02:0) 1	1	1	2.0782	6	3.4830	4.4366	4.5105
14	Mon Sep 18 15:25:54 2006 (GMT+02:0) 2	2	2	1.0919	4	2.8178	1.0919	3.8629
15	Mon Sep 18 15:25:54 2006 (GMT+02:0) 3	3	3	0.7482	4	1.9079	2.9626	4.7212

Figure 6: Results of calibration. The class assignments were given, and the calculated assignment is compared.

represent degree of correlation; similar spectra are grouped in clusters. Ideally, the clusters will be tight and have only one class of bacteria in them, and there would be considerable variance from other clusters. Two of the standards (from a set of 80 samples) were misclassified. A subset of the tabular results is presented in Figure 6. Calibrations were obtained for bacterial samples made with the two different preparation schemes.

The calibrated DA was then used to identify the bacteria present on new 96-well plates. The Array Automation software allowed this processing to occur either during collection or post-collection, with color coding for the results. Figures 7 and 8 show the results of the analysis for plates prepared using both methods. Color consistency down a column is a measure of the reproducibility of the results, and small differences appear as slight color changes. Clearly, the columns show a high degree of agreement, and the rows show variation.

Conclusions

The Micro Well Plate Reader combined with Array Automation allows the processing of a well plate in around two hours. This would allow upward of 350 samples to be analyzed in a typical workday, compared to the 50 per day presently possible. TQ Analyst's discriminant analysis of the data removes user-bias from the analysis, enabling rapid and consistent results to be obtained. Coupled with the reliability of the Nicolet 6700 FT-IR spectrometer, this provides a tool of great power to anticipate and comply with regulations in the food industry.

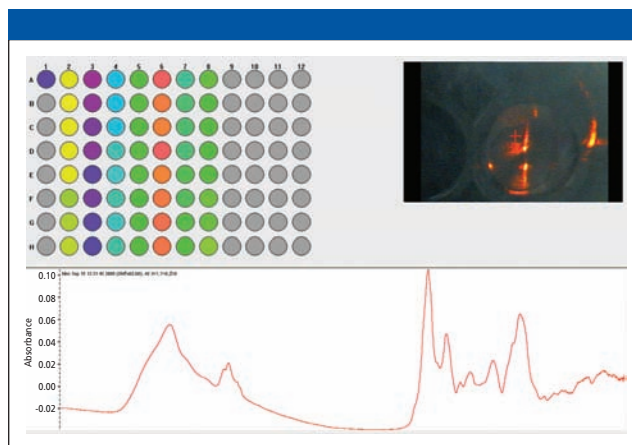


Figure 7: Colorized map of 96-well sample prepared using the standard method (Figure 2) after application of the TQ Analyst discriminant analysis. The visual image shows one well, and the spectrum shown is from the sample in this well.

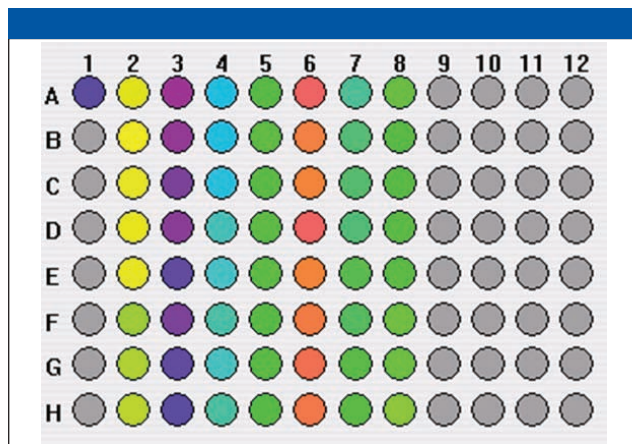


Figure 8: Colorized map of 96-well sample prepared using the shortened method (showing only the well plate analysis).

References

- (1) J.D.S.Goulden and M.E.J. Sharpe, *Gen. Microbiol.* **19**, 76–78 (1958).
- (2) J.H. Hopkinson, J.E. Newbery, D.M. Spencer, and J.F. Spencer, *Biotechnol. and Appl. Biochem.* **10**, 118–123 (1988).
- (3) D. Naumann, D. Helm, H. Labischinski, and P. Giesbrecht, *Modern Techniques for Rapid Microbiological Analysis*, W. Nelson, Ed., ISBN 3-527-28022-7, pp 43–96.

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81 Wyman Street, Waltham, MA
Tel. (781) 622-1000, Fax (781) 622-1207
www.thermofisher.com