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Rapid discrimination of *Alicyclobacillus* strains in apple juice by Fourier transform infrared spectroscopy

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Abstract

Alicyclobacillus spp. are thermoacidophilic, spore-forming bacteria. Some of which cause spoilage in pasteurized and heat-treated apple juice products through the production of guaiacol. Fourier transform infrared (FT-IR) spectroscopy was used to discriminate between eight *Alicyclobacillus* strains (WAC, 81-2, Oly#21, 51-1, KF, 1016, 1101, and A-Gala A4) in apple juice. FT-IR vibrational combination bands reflected compositional differences in the cell membranes of *Alicyclobacillus* strains in the "fingerprint region" at wavenumbers between 1500 and 800 cm^{-1}. Distinctive segregation among spectral sample clusters of different *Alicyclobacillus* strains was observed using principal component analysis (PCA). Two closely related strains (1016 and 1101) of *Alicyclobacillus acidoterrestris* could be distinguished, suggesting that this method can be highly selective. Results of soft independent modeling of class analogy (SIMCA) demonstrated that guaiacol-producing and non-guaiacol producing *Alicyclobacillus* strains could be differentiated up to 89% of the time. This technique may provide a tool for fruit juice producers to detect *Alicyclobacillus* rapidly and to monitor and control guaiacol formation.

Keywords: *Alicyclobacillus*; Apple juice; FT-IR; Spectroscopy; PCA; SIMCA

1. Introduction

Alicyclobacillus spp. are thermophilic, acidophilic, non-pathogenic, spore-forming bacteria. Some species of *Alicyclobacillus* spp., such as *Alicyclobacillus acidoterrestris*, can cause commercially pasteurized

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apple juice to spoil (Lee et al., 2002; Matsubara et al., 2002). *A. acidoterrestris* was originally classified as *Bacillus acidoterrestris* in the 1980s and later reclassified into a new genus *Alicyclobacillus* in 1992 (Wisotzkey et al., 1992). *A. acidoterrestris* is an important spoilage organism of acidic foods because its spores are able to germinate and grow in highly acidic environments and produce guaiacol which causes "medicinal" or "antiseptic" off-flavors (Yamazaki et al., 1997). *A. acidoterrestris* grows slowly, but at levels of 10^5–10^6 CFU ml^{-1} in apple juices it produces enough guaiacol to cause off-flavor (Pettipher et al., 1997; Silva et al., 1999).

Alicyclobacillus spoilage of apple juice is difficult to detect by visible inspection because the spoiled juice usually looks normal, with light sediments and no gas production (Silva et al., 1999; Sinigaglia et al., 2003). Furthermore, not all *Alicyclobacillus* spp. produce guaiacol, making it more difficult to monitor product for potential guaiacol formation during storage and distribution. Currently, several procedures have been proposed for detecting *Alicyclobacillus*, including a microscopic method involving morphological characterization, olfactory evaluation (Pettipher and Osmundson, 2000), biochemical analysis, molecular methods such as PCR or 16S rRNA gene sequence analysis (Yamazaki et al., 1997; Murakami et al., 1998), and the combination of membrane filtration with an optimized recovery medium (Chang, 2003). However, these methods are time-consuming and laborious. Furthermore, the use of different isolation protocols can yield different results for the same sample. Therefore, it is crucial for juice manufacturers to have a rapid and accurate method to detect *Alicyclobacillus* in acidic food products, and to be able to determine if a specific strain will produce guaiacol.

Fourier transform infrared (FT-IR) spectroscopy is a potential method and is useful for the classification and identification of bacteria with minimal sample preparation (Naumann, 2000). FT-IR provides biochemical information on various components of the cell wall and cytoplasm, including proteins and peptides, polysaccharides, peptidoglycan (murein), nucleic acids, and phospholipids. In recent years, FT-IR has been widely used to discriminate, classify, and identify various microorganisms, such as yeast (Lucia et al., 2001), cyanobacteria (Kansiz et al., 1999), lactic acid bacteria (Oberreuter et al., 2000), *Bacillus* spp. (Lin et al., 1998), *Listeria* spp. (Lefier et al., 1997), and coryneform bacteria (Oberreuter et al., 2003), to monitor microbial spoilage of meat (Ellis et al., 2002), and to investigate microbial colony heterogeneity (Choo-Smith et al., 2001).

The aim of this study was to investigate the feasibility of using FT-IR with multivariate statistical methods to discriminate between *Alicyclobacillus* strains recovered from apple juice samples.

2. Materials and methods

2.1. Preparation of bacterial cultures

The bacterial strains used in this study were obtained from the culture collection of the Department of Food Science and Human Nutrition at Washington State University. The *Alicyclobacillus* strains were WAC, 81-2, Oly#21, 51-1, KF, 1016, 1101, and A-Gala A4. Among eight selected strains, 81-2, 51-1, KF, and 1016 are guaiacol-producing, while the other four strains are non-guaiacol producing (Chang, 2003). The strains from frozen spore stocks were activated by heat shocking at 80 °C for 10 min. The activated spores were streaked onto potato dextrose agar (PDA, pH 5.6; Becton, Dickinson, Cockeysville, MD, USA) and grown at 43 °C for 48 h. A representative colony was then picked and inoculated into 50 ml of pasteurized apple juice (pH 3.7; Treetop Inc., Selah, WA, USA) and incubated at 43 °C for 7 days. At this point, cell numbers were ~10^6–10^8 CFU ml^{-1}. Bacterial cells were enumerated using a standard spread plating method on PDA. The plates were incubated at 43 °C for 48 h.

Alicyclobacillus cells were harvested by centrifuging 50 ml of the apple juice for 15 min at 4000 rpm at room temperature using a Centra CL-2 Model 120 centrifuge (Thermo IEC, San Jose, CA, USA). The pellets were washed twice by resuspending in 9 ml 0.9% saline and centrifuging as before to remove juice components and bacterial metabolites. The final pellet was suspended in 3 ml of 0.9% saline solution and vortexed vigorously to obtain a homogeneous cell distribution. Then 500 μl of the bacterial/saline suspension was applied onto an aluminum oxide membrane filter of 0.2 μm pore size and 25 mm OD

(Anodisc; Whatman Inc., Clifton, NJ, USA). Also, 500 μl of apple juice was applied onto a filter as a control. The filters were then air dried under laminar flow at room temperature for 1 h to obtain a dry, homogeneous film of bacterial cells. The experiment was repeated in duplicate.

2.2. FT-IR spectroscopy

FT-IR spectra were collected using a Thermo Nicolet Avatar 360 FT-IR spectrometer (Thermo Electron Inc., San Jose, CA, USA). During measurement, the membrane filters coated with bacterial cells were placed in direct contact with an attenuated total reflection (ATR) zinc selenide (ZnSe) crystal. This arrangement is widely used to study the chemical composition of smooth surfaces such as biofilms and membranes in a relatively undisturbed state (Schmitt and Flemming, 1998). Twenty spectra were acquired at room temperature for each culture. The resolution was set at 4 cm^{-1} with each spectrum composed of an average of 36 separate scans.

2.3. Data analysis

Data analysis was conducted using OMNIC (Thermo Electron Inc.) and Delight version 3.2.1 (Textron Systems, Wilmington, MA, USA) software. Data pre-processing algorithms were employed with the spectra smoothed using a Gaussian function over 12 cm^{-1}. This was followed by a second derivative transformation with a gap value of 12 cm^{-1} (Lin et al., 2003). After data pre-processing, principal component analysis (PCA) and soft independent modeling of class analogy (SIMCA) were employed.

3. Results and discussion

Table 1 provides a list of absorption band assignments for various biochemical functional groups in the FT-IR region between 4000 and 700 cm^{-1}. These bands arise from major cellular components such as lipids, proteins, polysaccharides, and nucleic acids. The prominent absorption peaks around 3400 cm^{-1} are primarily from water. Typical FT-IR ATR absorbance spectra of the *Alicyclobacillus* strains are shown in Fig. 1.

Table 1
Absorption band assignments in the FT-IR region (4000–700 cm^{-1})

Wavenumber (cm^{-1})	Assignment
~3400	O–H stretch of water
~2960	Asymmetric stretch C–H of methyl groups
~2929	Asymmetric stretch C–H of methylene groups
~2875	Symmetric stretch C–H of methyl groups
~2850	Symmetric stretch C–H of methylene groups
~1740	C=O of ester functional groups of lipids, fatty acids
~1650	C=O stretching vibrations of amides of proteins, e.g. Amide I band
~1550	N–H bending of amides of proteins, e.g. Amide II band
~1455	Asymmetric deformation of CH_3 and CH_2 of proteins
~1400	Symmetric deformation of CH_3 and CH_2 of proteins, and symmetric stretch of C–O of COO^- groups
~1240	Asymmetric stretch of P=O of nucleic acids
~1080	Symmetric stretch of P=O of nucleic acids
1200–900	C–O–C of polysaccharides, stretching vibrations of the phosphate

Data from Zeroual et al. (1994), Schmitt and Flemming (1998), Kansiz et al. (1999) and Maquelin et al. (2002).

A unique characteristic of *Alicyclobacillus* is the ω-alicyclic fatty acids serving as major membrane lipid components, which contributes to their survival at low pH and high temperature (Chang, 2003; Sinigaglia et al., 2003). The absorption peaks at 2960, 2929, and 1740 cm^{-1} are believed to be at least in part from ω-alicyclic fatty acids in the *Alicyclobacillus* cell membrane (Fig. 1).

The spectral region between 1500 and 800 cm^{-1}, or the "fingerprint region" conveys bacterial strain specific information (Choo-Smith et al., 2001; Schmitt and Flemming, 1998). This range contains important deformation-, bending- and ring-vibrations from various biochemical functional groups (Schmitt and Flemming, 1998). The vibrational combination bands of this "fingerprint" region are important for identification of biochemical compounds because they can be linked to single molecular bond or to a particular functional group.

FT-IR spectral features often look similar because bacterial cell constituents have very subtle compositional differences. Therefore, data pre-processing algorithms, such as smoothing, second derivative transformation, and normalization were used to

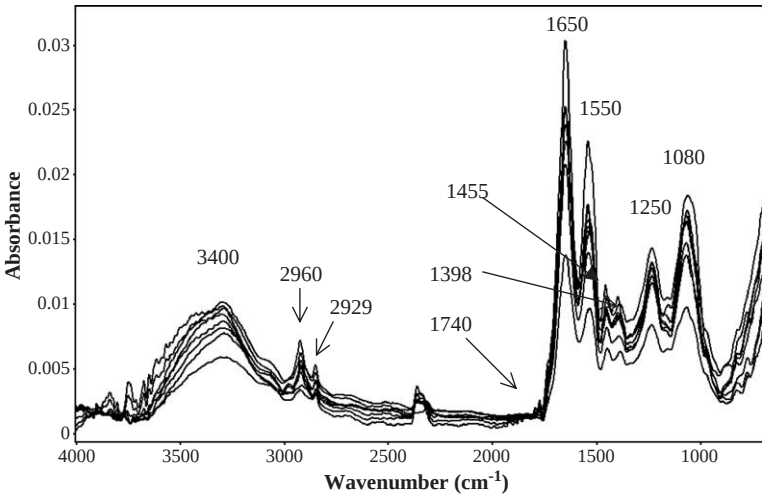

Fig. 1. Typical FT-IR ATR absorbance spectra (before data pre-processing) for eight *Alicyclobacillus* strains.

enhance these spectral differences (Huang et al., 2002). Smoothing eliminates high frequency instrumental noise by averaging neighboring data points. Second derivative transformation separates overlapping absorption bands, removes baseline offsets and provides an estimate of the number of overlapped bands within the region (Lin et al., 2003). In FT-IR, a layer containing higher amounts of bacterial cells absorbs more infrared energy than a layer containing lesser amounts of cells resulting in greater peak heights. Therefore, normalizing sample spectra can compensate for this pathlength effect and allow for a comparison of the spectra from samples containing different concentrations of bacteria.

Fig. 2 presents clear strain-dependent differences in the spectral features between the *Alicyclobacillus* strains. A peak at 1455 cm^{-1} corresponds to the asymmetric CH_3 bending mode and one at 1398 cm^{-1} to the symmetric vibrations of protein methyl groups (Zeroual et al., 1994; Maquelin et al., 2002). Among the strains, WAC had a very prominent peak at 1398 cm^{-1} which may be used for differentiating this strain from the others. The peak around 1303 cm^{-1} can be linked to the C–N functional group of proteins; those around 1240 cm^{-1} to the asymmetric stretching of the P=O in phosphate and the peak around 1080 cm^{-1} to the symmetric stretch of P=O of nucleic acids. The peaks between 1200 and 900 cm^{-1} are believed to correspond to stretching vibrations of the phosphate and the vibrations of polysac-

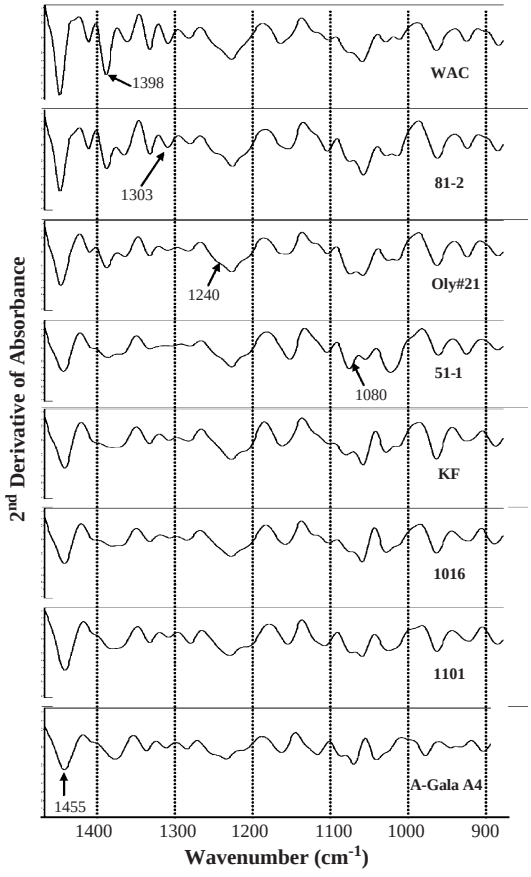

Fig. 2. Second derivative transformation (12-point gap) of FT-IR ATR absorbance spectra of eight *Alicyclobacillus* strains in the "fingerprint" region.

charide moieties (Zeroual et al., 1994; Schmitt and Flemming, 1998; Kansiz et al., 1999). Strains 1016 and 1101 (*A. acidoterrestris* ATCC 49025) showed a high degree of similarity in spectral features. These strains of *A. acidoterrestris* are phenotypically and biochemically closely related (Chang, 2003).

PCA could differentiate the eight *Alicyclobacillus* isolates based upon differences in FT-IR spectral features. PCA is a common multivariate statistical analysis technique widely employed in the interpretation of infrared spectral data variance. It reduces a multidimensional data set to its most dominant features, removes the random variation (noise), and retains the principal components (PC) that capture the related variation (Nilsen et al., 2002). PCA shows whether there are natural clusters in the data and similarities or differences from multivariate data sets (Goodacre et al., 1998). A mean centered PCA was conducted on the second derivative spectra over the entire wavenumber range. Fig. 3 shows the PCA clustering results for the *Alicyclobacillus* strains. Clear segregations with distinct sample clusters were observed between most strains. The clustering of 1016 and 1101 were partially overlapped, which confirms the high degree of similarity between these strains.

PCA clustering results for the strain WAC were noticeably different from the apple juice control and the other seven isolates (Fig. 3). These results agree with Chang (2003) who reported that WAC demonstrated a very different behavior from other strains during studies conducted to optimize incubation temperature, pH, acid, and use of various nutrient supplements to improve the recovery of *Alicyclobacillus* spp. from juice products. This strain may have a somewhat different cell wall composition than the other strains, as indicated by a much stronger absorbance by WAC at 1398 cm^{-1}, corresponding to the symmetric deformation CH_3 and CH_2 of proteins or peptidoglycan components in the bacterial wall (Zeroual et al., 1994) (Fig. 2).

Fig. 4 shows the first and second loading plot from PCA analysis for the *Alicyclobacillus* strains. Loading weights indicate the contribution of each variable (wavenumber) to a principal component and provide an indication of which spectral region makes the most significant contributions to data variation (Kansiz et al., 1999). Variables between 1700 and 900 cm^{-1} provided the greatest contribution to the total variance in the FT-IR spectral data for these *Alicyclobacillus* strains. In this spectral region, loading 1 accounted for 47% of the total variability; while loading 2 accounted for 11% of the total variability.

Fig. 5 shows SIMCA classification results for guaiacol-producing (triangles) and non-guaiacol pro-

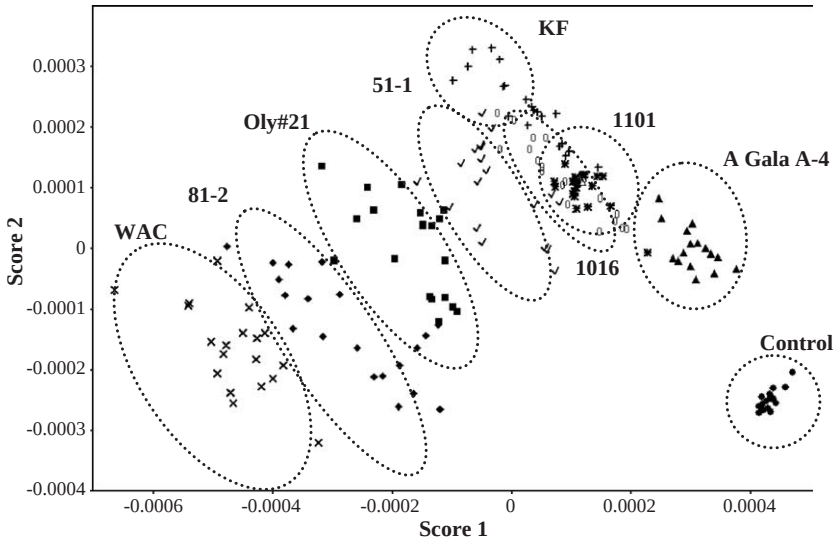

Fig. 3. PCA clustering results for Control (●), WAC (x), 81-2 (♦), Oly#21 (■), 51-1 (✓), KF (+), 1101 (*), 1016 (0), A-Gala A4 (▲).

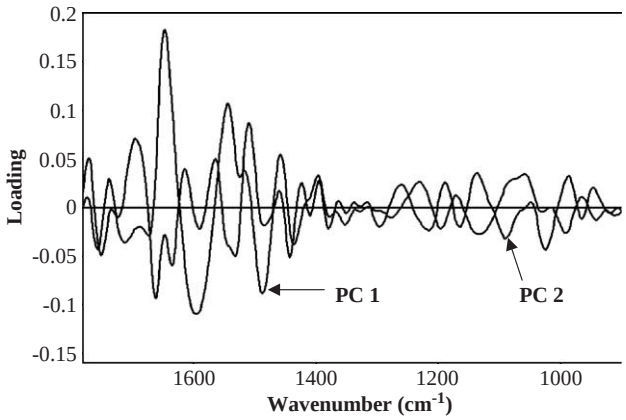

Fig. 4. Loading plot of first and second principal components (PC) obtained from PCA results of FT-IR spectra of eight *Alicyclobacillus* strains.

ducing (circles) *Alicyclobacillus* strains. SIMCA is based upon a PCA model generated for each class in the training set. Then, test samples are compared to the class models, and assigned a class according to their analogy to the training samples (Hampton et al., 2001–2002). When building a SIMCA model based upon the spectral data collected from guaiacol-producing *Alicyclobacillus* strains, approximately 89% of guaiacol-producing *Alicyclobacillus* strains were correctly classified; while approximately 38% of the non-guaiacol producing strains were incorrectly classified. Similar results were obtained when building a SIMCA model based upon the spectral data collected from non-guaiacol producing *Alicyclobacillus* strains. Thus, this technique can be used to classify guaiacol-producing and non-guaiacol producing *Alicyclobacillus* strains with a reasonable degree of accuracy, although additional strains should be tested to confirm this result.

Evidently, FT-IR analysis can differentiate between strains of *Alicyclobacillus* even when they are phenotypically very similar. Subtle differences in the cell wall composition of different strains can be used to classify guaiacol-producing and non-guaiacol producing *Alicyclobacillus* strains based upon differences in spectral features. However, more work will be needed to establish a comprehensive spectral reference database for identification of unclassified *Alicyclobacillus* and to determine if characteristics such as guaiacol production can be predicted from spectral measurements.

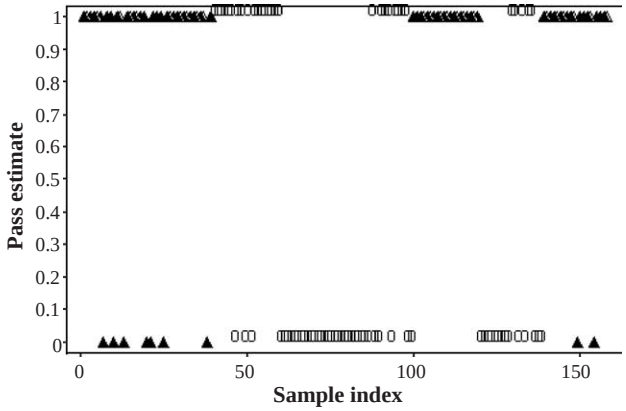

Fig. 5. SIMCA classification of guaiacol-producing (▲) and non-guaiacol producing (○) *Alicyclobacillus* strains.

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