Raman Spectroscopy and Chemometrics for Identification and Strain Discrimination of the Wine Spoilage Yeasts Saccharomyces cerevisiae, Zygosaccharomyces bailii, and Brettanomyces bruxellensis

Susan B. Rodriguez, Mark A. Thornton and Roy J. Thornton
Appl. Environ. Microbiol. **2013, 79(20):6264. DOI: 10.1128/AEM.01886-13.**
Published Ahead of Print 2 August 2013.

AEM
Journals.ASM.org

Raman Spectroscopy and Chemometrics for Identification and Strain Discrimination of the Wine Spoilage Yeasts *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, and *Brettanomyces bruxellensis*

Susan B. Rodriguez,[a] Mark A. Thornton,[b] Roy J. Thornton[a]

Department of Enology and Viticulture, California State University, Fresno, Fresno, California, USA[a]; Department of Psychology, Harvard University, Cambridge, Massachusetts, USA[b]

The yeasts *Zygosaccharomyces bailii*, *Dekkera bruxellensis* (anamorph, *Brettanomyces bruxellensis*), and *Saccharomyces cerevisiae* are the major spoilage agents of finished wine. A novel method using Raman spectroscopy in combination with a chemometric classification tool has been developed for the identification of these yeast species and for strain discrimination of these yeasts. Raman spectra were collected for six strains of each of the yeasts *Z. bailii*, *B. bruxellensis*, and *S. cerevisiae*. The yeasts were classified with high sensitivity at the species level: 93.8% for *Z. bailii*, 92.3% for *B. bruxellensis*, and 98.6% for *S. cerevisiae*. Furthermore, we have demonstrated that it is possible to discriminate between strains of these species. These yeasts were classified at the strain level with an overall accuracy of 81.8%.

Wines that develop defects result in reduced revenues for wine companies due to expensive ameliorative treatments, blending, or downgrading. The yeasts *Zygosaccharomyces bailii*, *Dekkera bruxellensis* (anamorph, *Brettanomyces bruxellensis*), and *Saccharomyces cerevisiae* are responsible for the vast majority of spoilage of aging and packaged wine (1). For forensic reasons, i.e., to identify the source(s) of these yeasts, it is important to differentiate *B. bruxellensis* and *Z. bailii* from the yeast that is used to conduct the alcoholic fermentation, *S. cerevisiae*.

B. bruxellensis is commonly associated with red wines that have contact with oak barrels during aging (2). *B. bruxellensis* can alter wine aroma through production of volatile phenol compounds. Some winemakers consider the alteration to be a desirable increase in wine complexity, while others view it as spoilage, using descriptors such as "barnyard" and "Band-Aid" (3). The physiological diversity of this species complicates this situation further (2). Classification at the strain level for *B. bruxellensis* is important, as there is much debate in the wine industry regarding "good" and "bad" strains of *B. bruxellensis*, possibly the result of the diversity of ploidy and chromosome number in *B. bruxellensis* strains (4, 5). Many wineries, as part of their quality assurance/quality control (QA/QC) programs, implement a barrel-sampling schedule to detect *B. bruxellensis*. This QA/QC measure involves plating wine samples on yeast-mold (YM) or Wallerstein Laboratories nutrient (WLN) agar medium. Any colonies that grow on these plates must be identified to determine the spoilage potential. Differential media containing cycloheximide can be used to select for *Brettanomyces*; however, strains of other wine yeasts are also cycloheximide resistant, e.g., *Hanseniaspora* spp., and this yeast is morphologically similar to *B. bruxellensis*.

Winemakers may produce wines with increased levels of residual sugar for those consumers who dislike dry wines, increasing the probability of refermentation of the packaged wine. Arresting fermentations to leave sufficient residual sugar is very costly, involving the use of centrifugation and refrigeration. Thus, wineries often purchase grape juice concentrate to add to dry wines prior to bottling to increase residual sugar levels. *Z. bailii* is a common contaminant of fruit juice concentrates since it is an extremely osmotolerant yeast that is able to grow in up to 72% (wt/vol) glucose (6). *Z. bailii* is very ethanol tolerant (up to 20%, vol/vol) and very resistant to weak acid preservatives used in the food and beverage industries, such as sorbic acid (up to 575 mg liter^{-1}), the sterilant dimethyl dicarbonate (up to 300 mg liter^{-1}), and high levels of SO_2 (3 mg liter^{-1}, molecular) (6). *Z. bailii* is probably the most serious spoilage yeast in wineries and in juice and concentrate plants, causing substantial loss of profits in these industries (1). Substantial quality control efforts in wineries are directed at monitoring concentrate tanks and bottling lines for the presence of *Z. bailii*, since as little as one *Z. bailii* cell can result in refermentation of a bottle of sweet wine, causing turbidity, gassiness, and high levels of acetic acid (7).

S. cerevisiae also poses a spoilage threat for sweet wines. Many commercial *S. cerevisiae* strains have been selected for their ethanol tolerance so that high-alcohol wines can be produced with a reduced probability of stuck or sluggish fermentations. These strains are capable of spoiling sweet wine by initiating a secondary fermentation.

Winery QA/QC programs track wine from the storage tank to the bottle. Samples from a wide range of critical sites, including bottles off the bottling line, are plated on agar media (8). Identification of yeast colonies on these plates is laborious and time-consuming or expensive (if samples are sent to a laboratory for DNA-based identification) and thus is rarely done. Action is taken on the basis of the number of yeast colonies. Packaged wine in which an unacceptable level of yeast colonies have been found is put on extended hold in the warehouse until it has cleared further microbiological testing. Alternatively, the wine may be dumped, refiltered, and rebottled. A delay on the supply chain is a source of

Received 10 June 2013 **Accepted** 30 July 2013
Published ahead of print 2 August 2013
Address correspondence to Susan B. Rodriguez, susanr@csufresno.edu.

doi:10.1128/AEM.01886-13

TABLE 1 Yeast strains used in this study

Species	Strains
Zygosaccharomyces bailii	*Z. bailii* CBS 749,[a] *Z. bailii* CBS 1170,[a] *Z. bailii* CBS 4688,[a] *Z. bailii* IGC 4269,[a] *Z. bailii* IGC 4806,[a] *Z. bailii* Phaff 68-113[b]
Brettanomyces bruxellensis	*B. bruxellensis* CBS 2499,[a] *B. bruxellensis* CBS 4459,[a] *B. bruxellensis* Vin1(1),[a] *B. bruxellensis* Vin8(A),[a] *B. bruxellensis* ETS 11,[c] *B. bruxellensis* ETS 159[c]
Saccharomyces cerevisiae	*S. cerevisiae* UCD 522,[a] *S. cerevisiae* UCD 595,[a] *S. cerevisiae* Maurivin PDM,[d] *S. cerevisiae* Enoferm CSM,[e] *S. cerevisiae* Nobleferm,[f] *S. cerevisiae* VL3c[g]

[a] Viticulture Enology Research Center (VERC) Culture Collection, California State University, Fresno, CA.
[b] Herman J. Phaff Culture Collection, University of California, Davis, CA.
[c] ETS Laboratories, Napa, CA.
[d] Mauri Yeast Australia, Toowoomba, QLD, Australia.
[e] Lallemand, Montreal, QC, Canada.
[f] Chr Hansen, Horsholm, Denmark.
[g] Laffort, Bordeaux Cedex, France.

economic loss. A method which could provide accurate, rapid identification of *S. cerevisiae*, *Z. bailii*, and *B. bruxellensis* would allow the swift implementation of remedial measures, averting economic loss due to spoilage.

In the present study, we used Raman spectroscopy and high-dimensional chemometric analysis to develop a method for identifying the three major wine spoilage yeasts. Raman spectroscopy detects slight differences in the frequency of rare photons of incident light (approximately 1 in 10^7) inelastically scattered by molecules, as opposed to the elastically scattered photons that make up the majority of incident photons. These photons have gained or lost energy in the midinfrared range by interacting with bonds in a compound. Functional groups of biomolecules, such as proteins, lipids, carbohydrates, and nucleic acids, e.g., C—C, C—H, and C═O stretching bonds and H—C—H bending bonds, can be detected as bands in Raman spectra. Whole cells represent a complex mixture of compounds, and the fingerprints (FPs) of all the molecules are superimposed in a Raman spectrum (9). In this case, a statistical approach is needed to interpret the data. One major area of new application of Raman spectroscopy is the identification of microorganisms. Most of the microbial work has been done with medically important isolates, e.g., urinary tract infection isolates, *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus* spp., which have been classified with over 94% accuracy (10), and *Candida* spp. from peritonitis patients, which have been classified with 90% accuracy (11). Raman spectroscopy has been used to a lesser extent to differentiate species of food microorganisms, specifically, lactic acid bacteria found in yogurt, *Lactobacillus acidophilus*, *L. delbrueckii*, and *Streptococcus thermophilus* (12), and in kefir, *L. kefir*, *L. parakefir*, and *L. brevis* (13).

A major advantage of the use of Raman spectroscopy for yeast identification is the low cost. The cost of a Raman spectrometer similar to the one used in this study is approximately the same as that of a PCR thermal cycler. Raman sample preparation involves suspending colonies in phosphate-buffered saline (PBS) and centrifuging. As opposed to DNA-based molecular techniques, no chemical reagents, water baths, freezers, etc., are required. Glass cuvettes, microcentrifuge tubes, pipette tips, and PBS are the only consumables required. Turbidity adjustments of yeast suspensions or exact sample volumes are not required. The presence of microorganisms or DNA in the laboratory environment is not a concern in Raman spectroscopy, as it can be in PCR-based assays.

The Raman spectroscopy assay described in this paper is also very rapid: sample preparation, instrument calibration, reading of the sample, and analysis of imported spectra can be done in 10 to 15 min, enabling rapid identification of yeast from colonies on agar plates. DNA-based molecular techniques take 4 to 5 h; however, some of these techniques, such as those involving Scorpions, can be done directly from wine, obviating the need to culture wine samples.

The major advantage of the Raman-based identification technique is that highly accurate strain identification is possible. Traditional morphological and physiological tests cannot differentiate among strains. Strain differentiation by molecular techniques, if possible, takes substantially longer than the species-level identification.

MATERIALS AND METHODS

Yeast strains and culture conditions. The yeast strains employed in this study are listed in Table 1. Yeasts were stored at −20°C in Pro-Lab Diagnostics Microbank vials containing cryoprotectant (Austin, TX). Strains were grown from a Microbank bead on Difco YM agar (Becton, Dickinson, Sparks, MD) plates at 30°C. Twenty-four subcultures were streaked from each bead plate. Subculture plates were incubated at 30°C for 48 h for *S. cerevisiae* and *Z. bailii*. Plates of *B. bruxellensis* required 72 h of incubation to reach the same level of growth.

Raman spectroscopy. A loopful of cell mass from a subculture was mixed in 1.5 ml filtered PBS (pH 7.4; Santa Cruz Biotechnology, Santa Cruz, CA) in 1.7-ml microcentrifuge tubes. The suspension was centrifuged at 6,708 × *g* for 3 min. Cell pellets were resuspended in 1.5 ml PBS in 1.7-ml microcentrifuge tubes. The turbidity of the suspensions was not adjusted. One milliliter of suspension was pipetted into a VWR shell vial (Radnor, PA), which was placed in the cell holder of a DeltaNu Advantage 532 Raman spectrometer (DeltaNu, Laramie, WY) with a frequency-doubled neodymium-doped yttrium aluminum garnet laser emitting at 532 nm and a spot diameter of 35 μm. Medium power (30 mW) was used. Each day, prior to running of samples, the spectrometer was calibrated using a polystyrene standard. Cyclohexane was run prior to running of the samples to check the baseline and peaks. The sample holder was covered with optical cloth after the cuvette was inserted into the cell holder to exclude extraneous light. Spectra were acquired for each sample over a Stokes Raman shift range of 3,400 to 200 cm^{-1} with a 15-cm^{-1} resolution. The low-resolution setting was used to optimize the signal to noise in the spectra. Ten spectra, each with a 5-s integration time, were collected and averaged for each of the 24 subcultures of each strain. The averaged spectra were exported from the DeltaNu control software as GRAMS files. A total of 432 spectra were collected.

Preprocessing. Preprocessing and classification were completed using the statistical computing language R (14). Several stages of preprocessing were undertaken in order to maximize the ability to discriminate the yeast samples. First, an automated polynomial subtraction method (15) was used to remove the background fluorescence present due to the biological nature of the samples. In this method, a fifth-order polynomial was re-

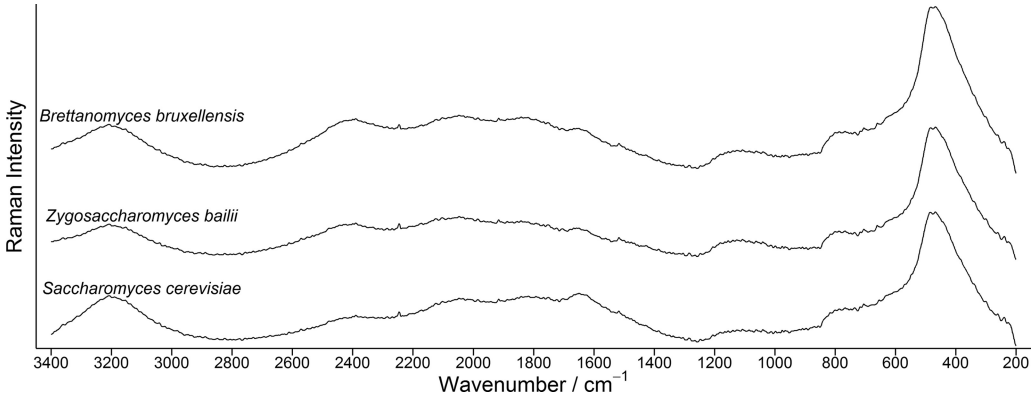

FIG 1 Raman spectra of the three yeast species after fluorescence subtraction. Each spectrum resulted from the averaging of the spectra for 24 samples of six strains (spectra for 4 outlier samples were removed). The spectra have been offset vertically on the intensity axis for clarity.

peatedly fit with least squares to the Raman spectrum of each sample. On each iteration of the algorithm, a new data curve was formed by taking a wavelength-wise minimum of the previous data curve and the polynomial. Convergence was reached, and the algorithm was terminated whenever the number of data points below the polynomial did not change from one iteration to the next. At this point, the final polynomial curve was subtracted from the original sample spectrum to produce the fluorescence-corrected sample spectrum.

The second stage of preprocessing consisted of normalizing wavelengths using a standard normal variate (SNV) transform of each variable. This process scaled all variables so that they had a mean of 0 and a standard deviation of 1 and would thus be equally weighted in subsequent analyses. The final stage of preprocessing consisted of multivariate outlier removal through principal component analysis (PCA). Scores were calculated for each sample on all principal components with eigenvalues greater than 1 (16). The Mahalanobis distance of each sample from the mean spectra was calculated on the basis of these scores, and samples with a distance 3 standard deviations greater than the mean distance were eliminated as outliers. This process resulted in the elimination of four samples.

Classification. Classification of samples was performed with a one-against-one multiclass linear support vector machine (SVM) classifier from the LIBSVM extension in R (17, 18). The SVM classifier is a type of classifier that functions by maximizing the margin between the hyperplane dividing two classes and the nearest examples of each class. The relative insensitivity of this type of classifier to nonmarginal cases allows it to deal with high-dimensionality data with minimal overfitting and no need for dimensionality reduction as in linear discriminant analysis. SVM classifiers have previously been used to great effect in classifying lactic acid bacteria on the basis of Raman spectra (12). In the present case, each strain of yeast was treated as a separate class, for a total of 18 classes. Full leave-one-sample-out cross-validation was employed to assess the performance of the model in terms of generalization accuracy.

RESULTS AND DISCUSSION

The aim of this study was to determine if the Raman spectra of wine spoilage yeasts grown on YM agar could be used to obtain an accurate prediction of their identity. The spectra, an average of 144 per species with outliers and fluorescence contribution removed, were very similar for the three yeast species (Fig. 1). Significant differences among the three species were observed when the SNV transforms of the spectra were compared (Fig. 2). Similarly significant differences among the strains of each of the three species were observed (Fig. 3).

Overall, the SVM classifier achieved very high performance across all classes using the entire spectrum, 3,400 to 200 cm^{-1}. Accuracy was 94.9% at the species level and 81.8% overall at the strain level (Table 2). Chance classifier performance was approximately 33.3% at the species level and 5.6% at the strain level, and binomial tests confirmed the statistical significance of the observed accuracies ($P < 0.001$). These results also supported our choice of preprocessing techniques. An alternative approach using dimensionality reduction through PCA combined with feature selection through analysis of variance produced a substantially lower overall classification accuracy at the strain level with both linear discrimination analysis (72.7%) and linear SVM classification (68.2%).

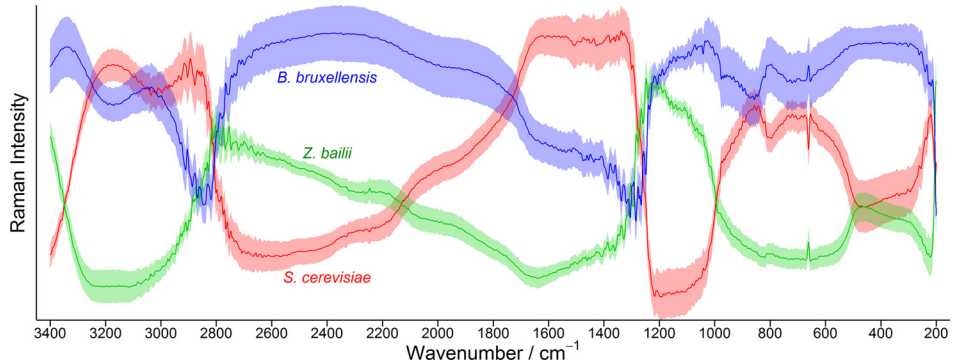

FIG 2 Raman spectra of the three yeasts after fluorescence subtraction and standard normal variate transformation of each wave number. The width of the shaded area around each spectrum indicates the 95% confidence interval for the mean spectrum of that species.

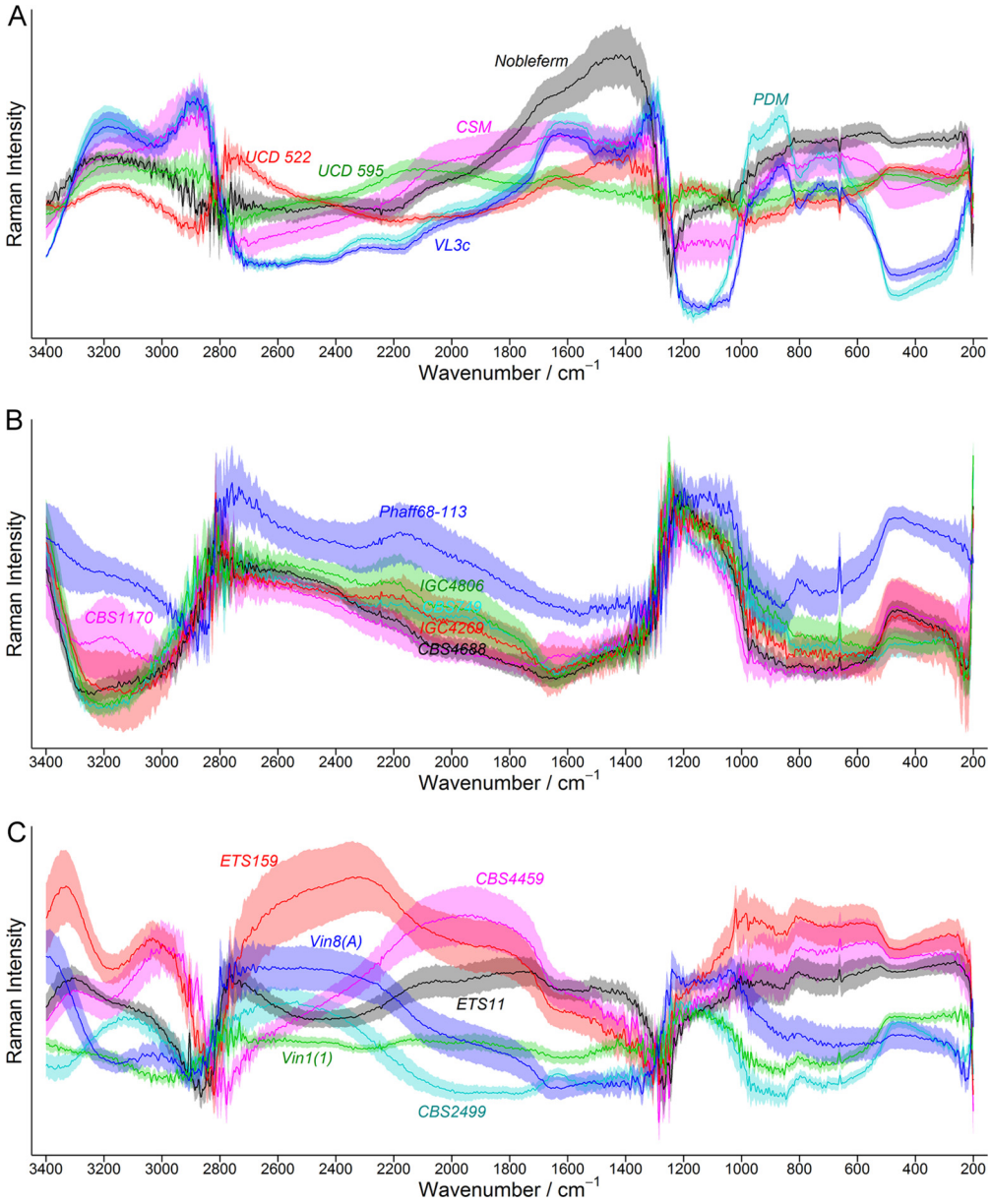

FIG 3 Raman spectra with outliers removed. Spectra were averaged, fluorescence was subtracted, and wave numbers were SNV transformed. Shaded areas indicate 95% confidence intervals. Raman spectra for six *S. cerevisiae* strains (A), six *Z. bailii* strains (B), and six *B. bruxellensis* strains (C) are shown.

Sensitivity and PPV. The sensitivity and positive predictive value (PPV) for each class are reported in Table 2. Sensitivity indicates the proportion of samples in a class (144 samples per species class and 24 samples per strain class) that were accurately classified. PPV indicates the proportion of samples classified as a certain class that actually belong to that class. Other class-specific performance metrics, such as accuracy, specificity, and negative predictive value, are highly dependent on overall performance and thus of little diagnostic value, due to the large number of classes in the analysis.

In the full validation confusion matrix, the strain intersection boxes show the number of samples of a yeast strain correctly classified (Table 3). For example, in the first row, one can observe that 23 of the 24 *S. cerevisiae* PDM samples were correctly classified as PDM. One of the samples was incorrectly classified as *S. cerevisiae* VL3c. Thus, the *S. cerevisiae* PDM sensitivity is 23/24, or 0.958. Although 24 samples were classified as *S. cerevisiae* PDM (column in Table 3), 1 of these was a VL3c sample incorrectly classified as PDM. Thus, *S. cerevisiae* PDM has a PPV of 0.958.

The confusion matrix reflects that there were few between-species errors: 5% for all the strains (Table 3). A disproportionate number (64.3%) of within-species errors was found in *Z. bailii*. Five of the six *Z. bailii* strains had within-species misclassified samples.

Spectral regions. A region of the Raman spectrum (1,800 to 200 cm^{-1}) is called the fingerprint (FP) region due to bonds of many important biomolecules that can be detected there. Sugars and polysaccharides are detected by multiple bands in the 1,200-

TABLE 2 Sensitivities and PPVs for yeast SVM classification

Yeast species or strain	Sensitivity	PPV
Yeast species		
S. cerevisiae	0.986	0.940
Z. bailii	0.938	0.958
B. bruxellensis	0.923	0.949
Strains		
S. cerevisiae PDM	0.958	0.958
S. cerevisiae CSM	0.826	0.731
S. cerevisiae Nobleferm	0.913	0.913
S. cerevisiae UCD 522	0.958	0.920
S. cerevisiae UCD 595	0.958	0.821
S. cerevisiae VL3c	0.875	0.913
Z. bailii CBS 749	0.667	0.640
Z. bailii CBS 1170	0.792	0.792
Z. bailii CBS 4688	0.583	0.609
Z. bailii IGC 4269	0.792	0.826
Z. bailii IGC 4806	0.542	0.565
Z. bailii Phaff 68-113	0.750	0.783
B. bruxellensis CBS 2499	1.000	1.000
B. bruxellensis CBS 4459	0.833	0.800
B. bruxellensis ETS 11	0.870	0.952
B. bruxellensis ETS 159	0.833	0.769
B. bruxellensis Vin1(1)	0.875	0.840
B. bruxellensis Vin8(A)	0.696	0.941

to 300-cm^{-1} region. Amide I and amide II bands of peptides and proteins are detected at ca. 1,650 and 1,550 cm^{-1}, respectively. The vibration for the C═O stretching bond of lipids is detected at ca. 1,740 cm^{-1}. DNA and RNA are detected at ca. 1,300 cm^{-1}. Bands for phospholipids are found in the 1,500- to 1,200-cm^{-1} region. The FP region is commonly used for bacterial identification and human tissue characterization. Within the FP region, the three yeast species differed significantly ($P < 0.05$, uncorrected for multiple comparisons) in most of the 1,700- to 1,000-cm^{-1} range (Fig. 2). In two of the carbohydrate absorption regions, two of the yeasts were differentiated from the third: *B. bruxellensis* from *Z. bailii* and *S. cerevisiae* in the 500- to 200-cm^{-1} region and *Z. bailii* from *S. cerevisiae* and *B. bruxellensis* and in the 900- to 500-cm^{-1} region (Fig. 2).

Information from the high-wave-number (HW) region (3,400 to 2,400 cm^{-1}) has not typically been used in characterization of biological matter; however, recently, medical diagnosis research has found valuable information in this region (19). The C—H stretching modes of lipids and proteins are detected in the 3,100- to 2,800-cm^{-1} range. Within the HW region, the three yeast species differed significantly ($P < 0.05$, uncorrected for multiple comparisons) in the 2,800- to 2,200-cm^{-1} range (Fig. 2). The intensity of the band at ca. 3,010 cm^{-1} has been used to correlate with the degree of unsaturation of fatty acids. The ratio of unsaturated to saturated fatty acids of yeasts grown in the presence of ethanol has been implicated in ethanol tolerance (20). The intensities for *S. cerevisiae* and *B. bruxellensis* in this region are very similar and significantly higher than that of *Z. bailii* (Fig. 2). However, all three yeast species are extremely ethanol tolerant. Ethanol tolerance may be further understood by study of this membrane-rich region in spectra of yeasts sampled during fermentation.

When classification performance at the strain level was studied as a function of spectral regions, we found that the performance with the full spectrum, 81.8%, was better than the performance

TABLE 3 Validation confusion matrix from SVM classification

	No. of samples classified as follows:																	
Strain present	*S. cerevisiae* PDM	*S. cerevisiae* CSM	*S. cerevisiae* Nobleferm	*S. cerevisiae* UCD 522	*S. cerevisiae* UCD 595	*S. cerevisiae* VL3c	*Z. bailii* CBS 749	*Z. bailii* CBS 1170	*Z. bailii* CBS 4688	*Z. bailii* IGC 4269	*Z. bailii* IGC 4806	*Z. bailii* Phaff 68-113	*B. bruxellensis* CBS 2499	*B. bruxellensis* CBS 4459	*B. bruxellensis* ETS 11	*B. bruxellensis* ETS 159	*B. bruxellensis* Vin1(1)	*B. bruxellensis* Vin8(A)
S. cerevisiae PDM	23					1												
S. cerevisiae CSM		19			2	1											1	
S. cerevisiae Nobleferm		1	21												1			
S. cerevisiae UCD 522			1	23														
S. cerevisiae UCD 595		1			23													
S. cerevisiae VL3c	1	2				21												
Z. bailii CBS 749							16		3		4						1	
Z. bailii CBS 1170								19	2	1	2							
Z. bailii CBS 4688		1					3	1	14	3	2							
Z. bailii IGC 4269								3		19	1			1				
Z. bailii IGC 4806							6	1	4		13							
Z. bailii Phaff 68-113				2	1							18				1	2	
B. bruxellensis CBS 2499													24					
B. bruxellensis CBS 4459					1									20		2		1
B. bruxellensis ETS 11			1									2			20			
B. bruxellensis ETS 159														4		20		
B. bruxellensis Vin1(1)		1										2					21	
B. bruxellensis Vin8(A)		1			1						1	1				3		16

with the HW region removed, 77.3% ($P = 0.017$), suggesting that the HW region does contribute unique information to the overall classification accuracy of the yeast strains. However, the performance with wave numbers 200 to 2,400 cm^{-1} (77.3%) was significantly better than the performance with just the FP region, 73.1% ($P = 0.001$), suggesting that the 1,800- to 2,400-cm^{-1} region also contributes to strain classification accuracy over and above that achieved with the fingerprint area. The performance with the HW region, 67.1%, was significantly worse than the performance with the FP region, 73.1% ($P = 0.027$).

Z. bailii strains did not differ significantly across the FP region, with the exception of strain Phaff 68-113, which differed significantly from the other five strains across most of the spectrum. More heterogeneity was evident among strains of *S. cerevisiae* and *B. bruxellensis* in the FP region (Fig. 3). *B. bruxellensis* strains were divided into two groups in the 1,000- to 200-cm^{-1} region, where carbohydrates and proteins are detected. In the HW region, *S. cerevisiae* strains were differentiated in the 2,700- to 2,400-cm^{-1} range, and the *B. bruxellensis* strains were differentiated in the 3,400- to 2,900-cm^{-1} range.

Z. bailii is a poorly characterized yeast, with the exception of its wide-ranging chemical resistances. Its genome has not been sequenced, and some believe that it is a heterothallic haploid (8), but others believe that it is a diploid that conjugates and undergoes mitotic sporulation (21). The strain confusion in *Z. bailii* observed by the Raman spectra (Table 3) could be the result of the major biochemical changes associated with conjugation and mitotic spore formation. In this study, conjugation was observed in all *Z. bailii* strains within a week's incubation on YM agar. In a study of carbon source assimilation and oxidation (Biolog microplates), seven *Z. bailii* strains were correctly identified only 50% of the time, whereas seven *S. cerevisiae* and seven *B. bruxellensis* strains were correctly identified 100% of the time (22). In another study, four *Z. bailii* strains were spread across three classes of spoilage yeasts on the basis of the variable growth on pantothenate-free and lysine media (23). In our study, although the species identification of *Z. bailii* by SVM classification was very high (sensitivity, 0.938), strain differentiation was the least sensitive for *Z. bailii* strains at 68.8%, as opposed to 91.5% for *S. cerevisiae* and 85.1% for *B. bruxellensis*. Thus, *Z. bailii* strains appear very different from *S. cerevisiae* and *B. bruxellensis* strains by Raman spectroscopy, but strains of this yeast appear to be more similar to each other than do strains of the other two yeast species. Strain identification, however, is the least important for this yeast, since any *Z. bailii* organism isolated from bottled wine would be considered a threat regardless of the strain.

The widespread diversity of *B. bruxellensis* strains in terms of physiology and morphologically is well documented. In this study, strain Vin8(A) was selected for its extensive pseudomycelial growth compared to that of the other strains. Strain ETS 159 was selected as a high 4-ethyl guaiacol producer rather than a 4-ethyl phenol producer. The genetic diversity of this yeast is also being demonstrated. The chromosome number of 30 *B. bruxellensis* strains was found to vary from four to nine (5). Eight genotypes were discovered in a study of 244 *B. bruxellensis* strains collected from 31 Australian wine regions (24). Recently, the genome of a *B. bruxellensis* wine isolate, AWRI 1499, was sequenced (4). This yeast is reportedly a heterozygous triploid. Another wine isolate which has been sequenced, CBS 2499 (25), was the only strain of the 18 in this study to be identified with 100% accuracy (Table 3).

S. cerevisiae wine strains are homothallic diploids, polyploids, or aneuploids with very low levels of heterozygosity. These strains typically require the standard method of growth on rich medium, such as glucose nutrient agar (GNA), followed by starvation on potassium acetate agar (PA) to induce meiotic sporulation. Many strains require repeated rounds to induce significant sporulation (26). In this study, CSM was the only *S. cerevisiae* strain observed to sporulate on YM agar. This characteristic may be associated with the difference in the width of the 95% confidence interval for this yeast's spectrum, especially in the polysaccharide region (Fig. 3).

The difficulty in discriminating between and identifying fermentation yeast strains in wine populations has hampered monitoring of the fermentation kinetics of *S. cerevisiae* inocula in grape must. Six *S. cerevisiae* wine yeasts were identified by this Raman spectroscopy assay at a sensitivity averaging 91.5%, indicating the assay's potential as a tool to track the fate of inoculated strains through fermentation.

In this study, the Raman spectra of *Z. bailii*, *B. bruxellensis*, and *S. cerevisiae* strains differed sufficiently that highly accurate classification at the species and strain levels were obtained using an SVM classifier. This discrimination, combined with the ease, rapidity, and low running costs of this assay, has the potential to provide a significant improvement in the ability of QA/QC personnel in wineries to identify yeast colonies isolated from wine samples.

ACKNOWLEDGMENTS

Project funding for S.B.R. and R.J.T. was provided by the American Vineyard Foundation and the California State University Agricultural Research Institute (ARI). M.A.T. was supported by a graduate research fellowship from the National Science Foundation.

REFERENCES

1. **Loureiro V, Malfeito-Ferreira M.** 2003. Spoilage yeasts in the wine industry. Int. J. Food Microbiol. **86:**23–50.
2. **Oelofse A, Pretorius IS, du Toit M.** 2008. Significance of *Brettanomyces* and *Dekkera* during winemaking: a synoptic review. S. Afr. J. Enol. Vitic. **29:**128–144.
3. **Lattey KA, Bramley BR, Francis IL.** 2010. Consumer acceptability, sensory properties and expert quality judgements of Australian cabernet sauvignon and Shiraz wines. Aust. J. Grape Wine Res. **16:**189–202.
4. **Curtin CD, Borneman AR, Chambers PJ, Pretorius IS.** 2012. De-novo assembly and analysis of the heterozygous triploid genome of the wine spoilage yeast *Dekkera bruxellensis* AWRI 1499. PLoS One **7:**e33840. doi: 10.1371/journal.pone.0033840.
5. **Hellborg L, Piskur J.** 2009. Complex nature of the genome in a wine spoilage yeast, *Dekkera bruxellensis*. Eukaryot. Cell **8:**1739–1749.
6. **Zuehlke JM, Petrova B, Edwards CG.** 2013. Advances in the control of wine spoilage by *Zygosaccharomyces* and *Dekkera/Brettanomyces*. Annu. Rev. Food Sci. Technol. **4:**57–78.
7. **Deak T, Reichart O.** 1986. Unacceptable levels of yeasts in bottled wine, p 215–218. *In* King AD, Pitt JI, Beuchat LR, Corry JE (ed), Methods for the mycological examination of food. Plenum Press, New York, NY.
8. **Fugelsang KC, Edwards CG.** 2007. Wine microbiology, practical applications and procedures, 2nd ed. Springer, New York, NY.
9. **De Gelder J, De Gussem K, Vandenabeele P, Moens L.** 2007. Reference database of Raman spectra of biological molecules. J. Raman Spectrosc. **38:**1133–1147.
10. **Kastanos EK, Kyriakides A, Hadjigeorgiou K, Pitris C.** 2010. A novel method for urinary tract infection diagnosis and antibiogram using Raman spectroscopy. J. Raman Spectrosc. **41:**958–963.
11. **Ibelings MS, Maquelin K, Endtz HP, Bruining HA, Puppels GJ.** 2005. Rapid identification of *Candida* spp. in peritonitis patients by Raman spectroscopy. Clin. Microbiol. Infect. **11:**353–358.
12. **Gaus K, Rösch P, Petry R, Peschke K-D, Ronneberger O, Burkhardt H,**

Baumann K, Popp J. 2006. Classification of lactic acid bacteria with UV-resonance Raman spectroscopy. Biopolymers **82:**286–290.
13. **Mobili P, Araujo-Andrade C, Londero A, Frausto-Reyes C, Tzonchev RI, De Antoni GL, Gómez-Zavaglia A.** 2011. Development of a method based on chemometric analysis of Raman spectra for the discrimination of heterofermentative lactobacilli. J. Dairy Res. **78:**233–241.
14. **R Development Team.** 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
15. **Lieber CA, Mahadevan-Jansen A.** 2003. Automated method for subtraction of fluorescence from biological Raman spectra. Appl. Spectrosc. **57:** 1363–1367.
16. **Kaiser HF.** 1960. The application of electronic computers to factor analysis. Educ. Psychol. Meas. **20:**141–151.
17. **Chang C, Lin C.** 2011. LIBSVM: a library for support vector machines. ACM Trans. Intelligent Syst. Technol. **2:**1–27.
18. **Meyer D, Dimitriadou E, Hornik K, Weingessel A, Leisch F.** 2012.R package version 1.6-1. e1071: miscellaneous functions. Department of Statistics, Technische Universität Wien. Vienna, Austria.
19. **Bergholt MS, Zheng W, Huang Z.** 2013. Development of a multiplexing fingerprint and high wavenumber Raman spectroscopy technique for realtime in vivo tissue Raman measurements at endoscopy. J. Biomed. Opt. **18:**030502. doi:10.1117/1.JBO.18.3.030502.
20. **Huffer S, Clark ME, Ning JC, Blanch HW, Clark DS.** 2011. Role of alcohols in growth, lipid composition, and membrane fluidity of yeasts, bacteria and archaea. Appl. Environ. Microbiol. **77:**6400–6408.
21. **Rodrigues F, Ludovico P, Sousa MJ, Steensma HY, Corte-Real M, Leao C.** 2003. The spoilage yeast *Zygosaccharomyces bailii* forms mitotic spores: a screening method for haploidization. Appl. Environ. Microbiol. **69:**649–653.
22. **Praphailong W, Van Gestel M, Fleet GH, Heard GM.** 1997. Evaluation of the Biolog system for the identification of food and beverage yeasts. Lett. Appl. Microbiol. **24:**455–459.
23. **Rodriguez SB.** 1987. A system for identifying spoilage yeasts in packaged wine. Am. J. Enol. Vitic. **38:**273–276.
24. **Curtin CD, Bellon JR, Henschke PA, Godden PW, de Barros Lopes MA.** 2007. Genetic diversity of *Dekkera bruxellensis* yeasts isolated from Australian wineries. FEMS Yeast Res. **7:**471–481.
25. **Piskur J, Ling Z, Marcet-Houben M, Ishchuk OP, Aerts A, LaButti K, Copeland A, Lindquist E, Barry K, Compagno C, Bisson L, Grigoriev IV, Gabaldon T, Phister T.** 2012. The genome of wine yeast *Dekkera bruxellensis* provides a tool to explore its food-related properties. Int. J. Food Microbiol. **157:**202–209.
26. **Thornton RJ, Eschenbruch R.** 1976. Homothallism in wine yeasts. Antonie Van Leeuwenhoek **42:**503–509.