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RESEARCH ARTICLE

Cell-Free Identification of *S. cerevisiae* Strains by Analysis of Supernatant Using LC-MS

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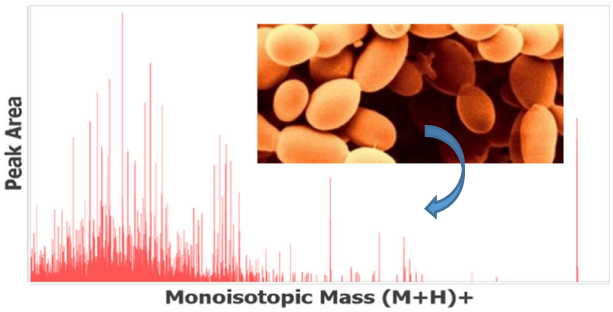

Abstract. Current literature shows a gap for methods which can identify yeast sub-species (strains or serovars) in samples where there are no viable cells remaining. Presented here is a technique for the analysis of yeast supernatant, including solid phase extraction, data-dependent acquisition liquid chromatography/mass spectrometry (LC-MS), and two chemometric methods to identify and classify yeast strains. Five strains of *Saccharomyces cerevisiae* were successfully identified in various stages of growth. In addition, peptide/protein identification was performed, without the need for additional data acquisition.

Keywords: LC-MS, *S. cerevisiae*, Strain identification, Microorganism identification, Secreted peptides, Yeast, Biomarker

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Introduction

Microbial sub-species identification (strains or serovars) has recently gained attention across many fields, thanks to the development of new analytical and biochemical techniques. In the field of medicine, knowledge of the species of a clinical infectious agent is typically enough to determine the course of treatment. However, in some cases, certain infectious strains may require specific treatments. Also, tracking the spread of infectious strains is important for epidemiological studies [1]. In the field of food science, knowledge of the yeast strain used in the production of fermented beverages may be useful for quality control and raw material sourcing [2–11].

Yeast has been historically identified using morphological and biochemical methods. Characterization of the cell and colony traits, utilization of specific nutrients, and survival in the presence of specific antibiotics are used to classify species [3]. These experiments typically require 24 h or more, and the microbiological techniques may not distinguish between closely related species, and rarely between strains. Genomic sequencing techniques are specific enough for strain identification, but are also time consuming and generally not amenable to routine testing [12]. Additionally, these techniques require viable cells or DNA.

In some cases, viable cells may be no longer present in samples, but identification of the previously present microbiological strain is desired. Applications include the beer/wine industry, where yeast cells are often not present in the final product. These include authenticity/fraud detection, reverse-engineering, and yeast contamination during fermentation [4, 8–10]. Other applications include environmental forensics, such as the investigation of drinking water contamination. In such a case, the contaminating microorganism may be non-viable, but determination of the strain could aid in the identification of the source of contamination. For such samples, strain identification using biomarkers in the extracellular environment would be required. Peptides could potentially serve as biomarkers, as there is a wide variety of potential peptide structures, especially when considering sequence variants or differences in relative abundances.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) is routinely used for identification of microorganism species via fingerprint matching with spectral libraries of highly conserved and highly

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abundant proteins [13]. Currently available MALDI-TOFMS libraries may not be applicable for strain identification because the highly abundant biomarkers (e.g., ribosomal proteins) are often conserved across species. Even libraries assembled for the purpose of differentiating strains have been largely unsuccessful [14–16]. MALDI-TOFMS has been shown to differentiate strains of bacteria and yeast in several studies [17–21]; however, these studies required the presence of viable cells.

Initial work on this project utilized MALDI-TOFMS analysis of samples prepared from the yeast strain samples [22]. However, limitations of MALDI-TOFMS analysis include interference and ion suppression. MALDI matrix clusters can interfere with analytes below 1000 m/z. Most importantly, ion suppression can be problematic for analytes at low levels, or with low ionization efficiency, since all compounds are ionized simultaneously. These limitations can be mitigated by using LC-MS. The addition of the chromatographic separation greatly reduces the number of analytes entering the mass spectrometer at any given retention time, resulting in decreased ion suppression and interference. LC-based approaches are often also more quantitative than MALDI, due to the inherent homogeneity of samples in solution. Two recent studies showed bacterial strains could be identified using intact protein LC-MS fingerprinting, but both required viable cells [23, 24].

Current literature shows a gap for identification of yeast strains in samples where there are no viable cells remaining. The study presented here describes a potentially cell-free technique to identify five *Saccharomyces cerevisiae* strains using LC-MS by analysis of the yeast supernatant. Solid phase extraction (SPE) was used to concentrate analytes, while removing sugars, salts, and polar small molecule metabolites, thus improving peptide detection and reducing potential ion suppression. Non-targeted analysis was performed using data-dependent acquisition (DDA), and the data was simplified into "pseudo-spectra" for classification. Two classification methods were evaluated: spectral pattern matching (k-nearest neighbors), and biomarker matching. Yeast in various stages of growth were tested to examine the robustness of the technique.

Experimental

Yeast Growth and Supernatant Preparation

Five strains of dried *S. cerevisiae* beer brewer's yeast were obtained from Fermentis (Lille, France): K97, S33, T58, US05, WB06. Each strain was prepared separately six times (3 biological replicates for the 4-day incubation samples, and additional preparations for 1-day, 2-day, and 7-day incubation samples). For each preparation, 150 mg of yeast was rehydrated in 20 mL of 25 mg/mL glucose solution, in sealed vented vials, to maintain anaerobic conditions. After the incubation time, the suspension was centrifuged at 4696×*g* for 5 min, and the supernatant was purified and concentrated using solid phase extraction. Waters (Milford, MA) C18 Sep-Pak Light SPE cartridges were conditioned using 2 mL strong eluent (60:40 acetonitrile/0.1% formic acid) followed by 2 mL weak eluent (0.1% formic acid). Ten milliliters of yeast supernatant was loaded onto the cartridge, followed by a 2 mL wash of weak eluent. The analytes of interest were eluted from the cartridge using 1 mL of strong eluent. The solution was dried off by vacuum in a Speed-Vac (Thermo Scientific, Waltham MA) and reconstituted with 200 µL 0.1% formic acid. All chemicals were purchased from Fisher Scientific (Waltham, MA). Blanks were concurrently analyzed, which consisted of glucose solution subject to the same incubation times and solid phase extraction procedure. A set of yeast samples was also prepared using water instead of glucose solution, with an incubation time of 1 h, to evaluate whether compounds in the supernatant were components of the residual growth media in the dehydrated yeast samples rather than products of yeast metabolism.

Solid Phase Extraction Optimization

A single sample of 4-day K97 supernatant was prepared as above using different solid phase extraction (SPE) loading volumes (1–20 mL) to evaluate the impact on the number of identified peptides/proteins. In addition, the SPE effluent during sample loading was collected in 1-mL fractions to evaluate SPE cartridge saturation. The approximate concentration of peptides in the effluent was determined in duplicate by UV at 280 nm, using an Unchained Labs (Pleasanton, CA) Lunatic DropSense spectrophotometer.

LC-MS Parameters

LC-MS was performed on a Thermo Scientific Vanquish UHPLC and Q-Exactive Plus MS, using a Waters Acquity CSH C18 column (150 mm × 2.1 µm, 1.7 µm particle size) at 30 °C with a 5 µL injection volume. A gradient of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was used from 0 to 40% B over 60 min, at 0.2 mL/min, followed by a wash of 95% B. Data-dependent acquisition (DDA) parameters include a 1-microscan full MS (resolving power 70,000, 400–2000 m/z) followed by MS/MS for the top three most abundant ions using a normalized collision energy of 35 (resolving power 17,500, 200–2000 m/z), with exclusion of isotopes, unassigned/+ 1 charges, and a dynamic exclusion of 4 s. The exclusion of precursors with low m/z or + 1 charge state was included to limit chemical noise and to preferentially select larger peptides, which are more likely to be unique. Mass calibration was performed prior to analysis, as per manufacturer's recommendations, using Pierce LTQ Velos ESI positive ion calibration solution (no lock-mass).

Data Pre-processing

Analyte lists were generated using Thermo Scientific BioPharma Finder software for all peaks above the software-defined signal-to-noise. Average and monoisotopic masses were calculated for each isotopic distribution, and peak area was determined by integrating the extracted ion chromatogram (XIC) of the most abundant isotopic peak. Only one analyte

entry is generated for each isotopic distribution within a single-charge state, but the presence of multiple charge states results in multiple entries. This is not problematic as the multiple entries are recombined in the next data processing step (Mass-Up inter-sample peak matching). Separate entries are generated for analytes with the same mass but different retention times. The following filters were applied prior to data export: +2 or higher charge state, assignable monoisotopic mass, and XIC peak area > 10,000.

The analyte lists were exported as csv files, and reduced to include only monoisotopic mass and peak area for the most abundant 3000 analytes (by XIC area of the selected m/z peak). For PCA, only the top 1000 peaks were included, to account for long computation times. For k-NN classification, peak area normalization was performed by translating peak area into peak rank, from 3000 to 1.

Mass-Up Processing

The csv files were input into Mass-Up [25] (http://sing.ei.uvigo.es/mass-up, SI4 Next-Generation Computer Systems Group, Vigo, Spain) as analyte lists. Inter-sample peak matching was performed using 10 ppm mass error. PCA was performed using 0.95 variance. Biomarkers were identified using the 4-day samples, and matched against each 1-day, 2-day, and 7-day sample. The matched biomarker lists were exported for biomarker percent matching calculations performed in Excel. k-NN classification (weka.classifiers.lazy.IBk) was performed using 10-fold cross validation.

Protein Identification

Peptide sequencing and protein identification were performed using Protein Metrics Inc. (San Carlos, CA, v2.16.11) Byonic software against all *S. cerevisiae* proteins in the UniProt database (www.uniprot.org, 80,097 proteins). Parameters include non-specific cleavage, mass tolerance 4/10 ppm (precursor/fragment) with no modifications, and 1% false discovery rate. Peptide identification required MS/MS score ≥ 200.0. No proteins were identified which had a probability rank less than the top 20 reverse-sequence decoys.

Results and Discussion

Sample Preparation Optimization

The use of SPE for concentration of yeast supernatant analytes was essential for the identification of high numbers of peptides. Preliminary injections of neat supernatant yielded chromatograms with few discernable peaks in the MS total ion chromatograms (TIC). Subsequent optimization of the SPE procedure evaluated various sample loadings of a single K97 4-day yeast supernatant sample from 1 to 20 mL. The number of identified peptides, and number of proteins from which they originated, increased with increasing sample loading as shown in Table 1. Separately, by measuring the peptide content of the SPE effluent during sample loading, it was determined that the

Table 1. Number of identified peptides, and originating proteins, as a function of SPE sample loading volume

SPE sample loading (mL)	Identified peptides	Originating proteins
1	179	49
2	573	132
5	1394	251
10	2168	308
20	3002	377

SPE cartridge nears saturation after approximately 10 mL loading (see Fig. 1). This sample loading volume was selected for the method. An additional effect of employing SPE during sample preparation is the reduction of polar small molecules and salts, which are not retained on the cartridge. This may also aid in increasing peptide identifications through improving ionization efficiency, reduction of salt adducts, and reduction of analytes competing for DDA peak selection.

Data Pre-Processing

The yeast supernatant LC-MS chromatograms are too complex to use for direct strain classification (see Fig. 2). There is extensive peak co-elution, and each analyte results in many m/z peaks due to multiple charge states and isotopic distributions. Therefore, BioPharma Finder was used to simplify each raw data file into an analyte list by deconvoluting the isotopic distributions, and integrating the respective XICs. These deconvoluted analyte lists were entered into Mass-Up software as pseudo-spectra containing monoisotopic M+H mass (x-axis) vs. XIC peak area (y-axis), as shown in Fig. 3. Mass-Up is a software designed for the analysis of MALDI-MS spectral data, so each pseudo-spectrum is analogous to a MALDI-MS spectrum (peak intensity vs. m/z).

The optimal number of analytes in the sample analyte lists for subsequent classification experiments was considered. Each of the 4-day biological replicates, which collectively were used as the "library" for classification, contained 5000–12,000

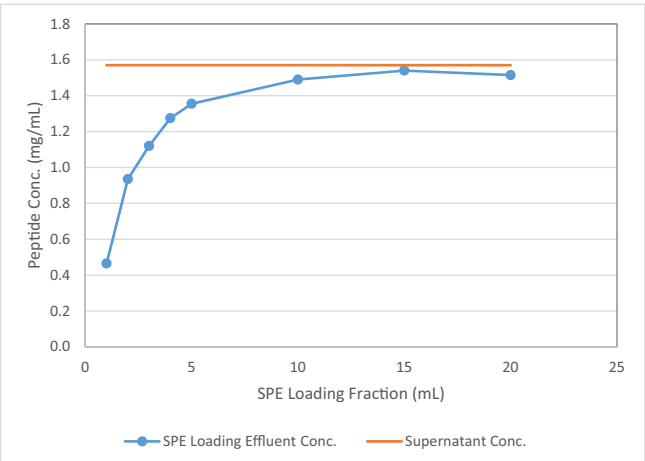

Figure 1. Approximate concentration of peptides in the SPE effluent during sample loading, as measured by UV

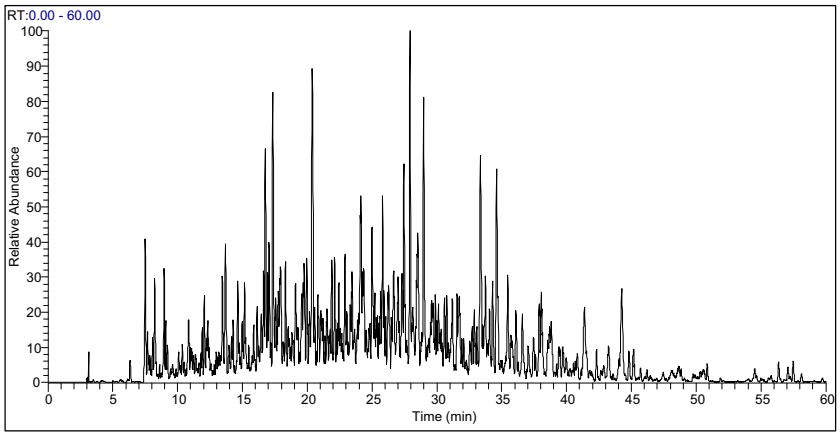

Figure 2. Example LC/MS base peak chromatogram for the K97 4-day sample

analytes. However, inclusion of all analytes was not found to be beneficial to classification. Each yeast strain had a different optimal number of analytes to maximize the number of biomarkers identified (see Fig. 4). Criteria for being a biomarker is that the analyte must be present in all three biological replicates of a single yeast strain, and absent in all preparations of all other yeast strains (using 10 ppm mass tolerance). The number of biomarkers initially increases as more low-intensity analytes are considered, but at some point the number of biomarkers begins to decrease when more analytes with the same/similar mass are observed in other strains. This includes both (1) analytes which are identical to biomarkers, but at much lower levels, and (2) analytes which have the same/similar mass as biomarkers in other strains, but are different as shown by retention time. The strain with the fewest biomarkers (T58) had a maximum number of biomarkers when 3000 analytes were used; therefore, this value was selected for the subsequent classification work.

There are notable visible differences in the pseudo-spectrum profiles between the strains, as shown in Supplemental Fig. S1. There are also minor differences in the profiles of each strain over time, which stabilize by day 2. This may be explained by the slowing of metabolic activity after 2 days, as evidenced by reduced visual carbon dioxide bubble formation.

PCA

Principal component analysis (PCA) was used to visualize the differentiation of the yeast strains in Mass-Up with incubation times from 2 to 7 days. As shown in Fig. 5, the five strains were well separated from each other and tightly grouped. This indicates that there are distinct and reproducible differences in the compositions of the strain supernatants. Inclusion of the 1-day samples resulted in poorer differentiation, suggesting that a minimum amount of time and metabolism is needed for reproducible profiles of analytes to accumulate in the extracellular environment.

Biomarker Classification

The first classification approach was based on identification of biomarkers unique to each strain. A list of biomarkers was generated in Mass-Up using the 4-day sample analyte library. Each strain was found to contain between 166 and 457 biomarkers which were present in all three biological replicates, and absent in all other biological replicates of the other strains. Refer to Supplemental Table S2 for the list of biomarkers identified for each yeast strain. The 1, 2, and 7-day sample analyte lists were screened as unknowns against the biomarkers from the 4-day samples. A percent match was calculated for

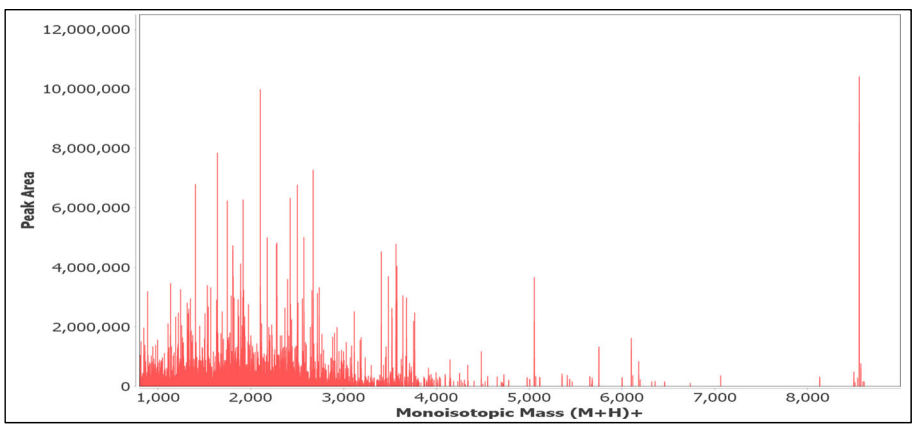

Figure 3. Example pseudo-spectrum for the K97 4-day sample

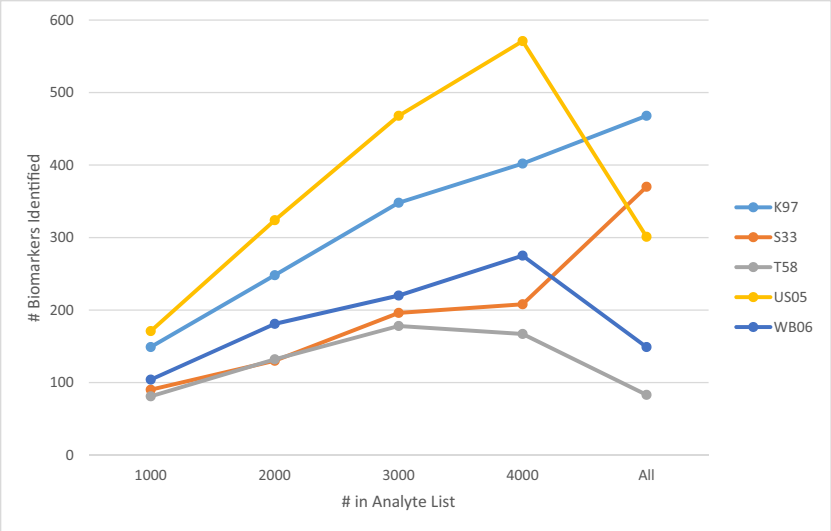

Figure 4. Number of biomarkers identified versus number of top analytes considered

each sample against each strain's biomarker list using the equation below.

$$\%\text{Match} = \frac{\#\text{Peaks in Unknown matching Strain X Biomarkers}}{\#\text{Biomarkers in Strain X}} \times 100\%$$

Samples were then classified into the strain with the highest biomarker percent match. This classification technique correctly identified the yeast strains for all 1-day, 2-day, and 7-day samples, as shown in Fig. 6.

It was important to determine whether the biomarkers were truly products of yeast growth and metabolism, and not simply components of the cell culture media prior to dehydration and packaging. This was established by rehydrating a set of yeast samples using water instead of glucose solution, and a very short incubation time of 1 h. Without glucose as a source of energy, reduced metabolism occurs, and the resulting supernatant chromatograms were visually less complex (similar to SPE blank samples). These samples show low percent matching to their strain's respective biomarkers, and have very low numbers of identified peptides as compared to the 4-day samples rehydrated with glucose solution (refer to Table 2). This

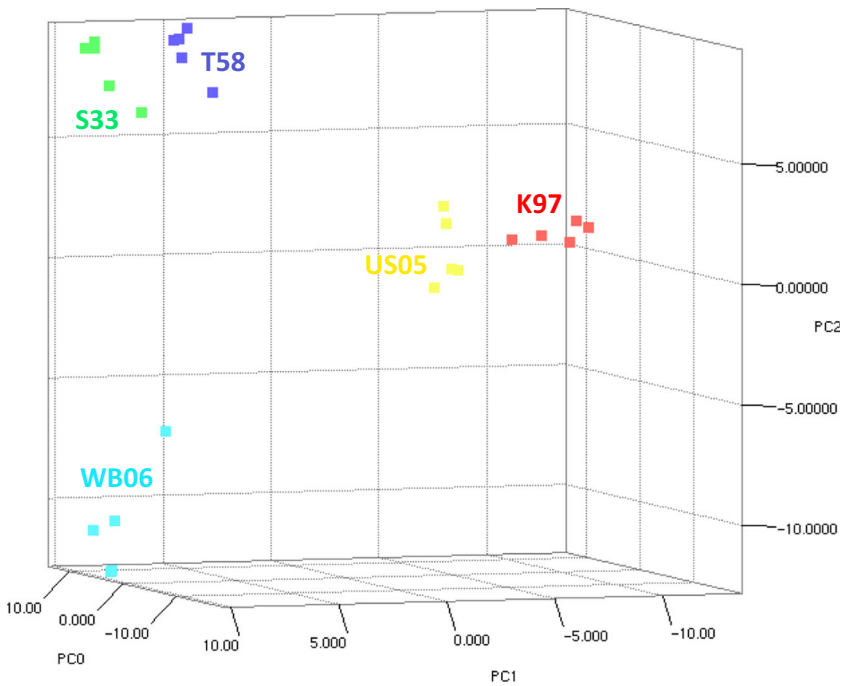

Figure 5. PCA plot (including the first three principle components) demonstrating clear differentiation of the strains with 2- to 7-day incubation times

indicates that the majority of biomarkers are therefore products of the cells.

LC-MS XICs were inspected to evaluate whether several randomly selected biomarkers are truly unique to one strain of yeast. It was observed in some cases that the biomarkers are often present in other strains, but at lower relative abundances, and therefore a lower likelihood of being in the 3000 most abundant peaks. These types of biomarkers can be used for classification as long as they are consistently at relatively high abundance in one strain and not in others.

Table 2. Biomarker percent match to their respective strain for samples reconstituted without glucose, and comparison of identified peptides with and without glucose

Sample	biomarker% match	Identified peptides–no glucose	Identified peptides–with glucose
K97	8	48	3179
S33	4	32	3697
T58	16	56	4140
US05	7	88	4948
WB06	8	25	2527

k-NN Classification

An alternative classification analysis was performed on the pseudo-spectra using a k-nearest neighbors (k-NN) algorithm, which is a non-parametric pattern recognition method. Peak area normalization was performed prior to classification by translating peak area into peak rank, from 3000 to 1. This prevents the algorithm from over-weighting the largest peaks, since it was observed that relative peak areas vary based on incubation time.

Using 10-fold cross-validation, as shown in Table 3, the analysis correctly classified all 4-day yeast samples (3 biological replicates of each strain), and these were used as the "library". The 1-day, 2-day, and 7-day samples (one separate preparation of each strain) were then added as separate unknown classes, and the cross-validation was repeated. There was one misclassification in the 1-day samples. This classification technique may require sufficient time and metabolic activity to achieve sufficient analyte concentrations and stable peak profiles. The use of alternative statistical models (other than k-NN) may also improve the accuracy of the classification of samples in earlier stages of growth. In total, the k-NN algorithm correctly classified 93% of the samples (14 of 15).

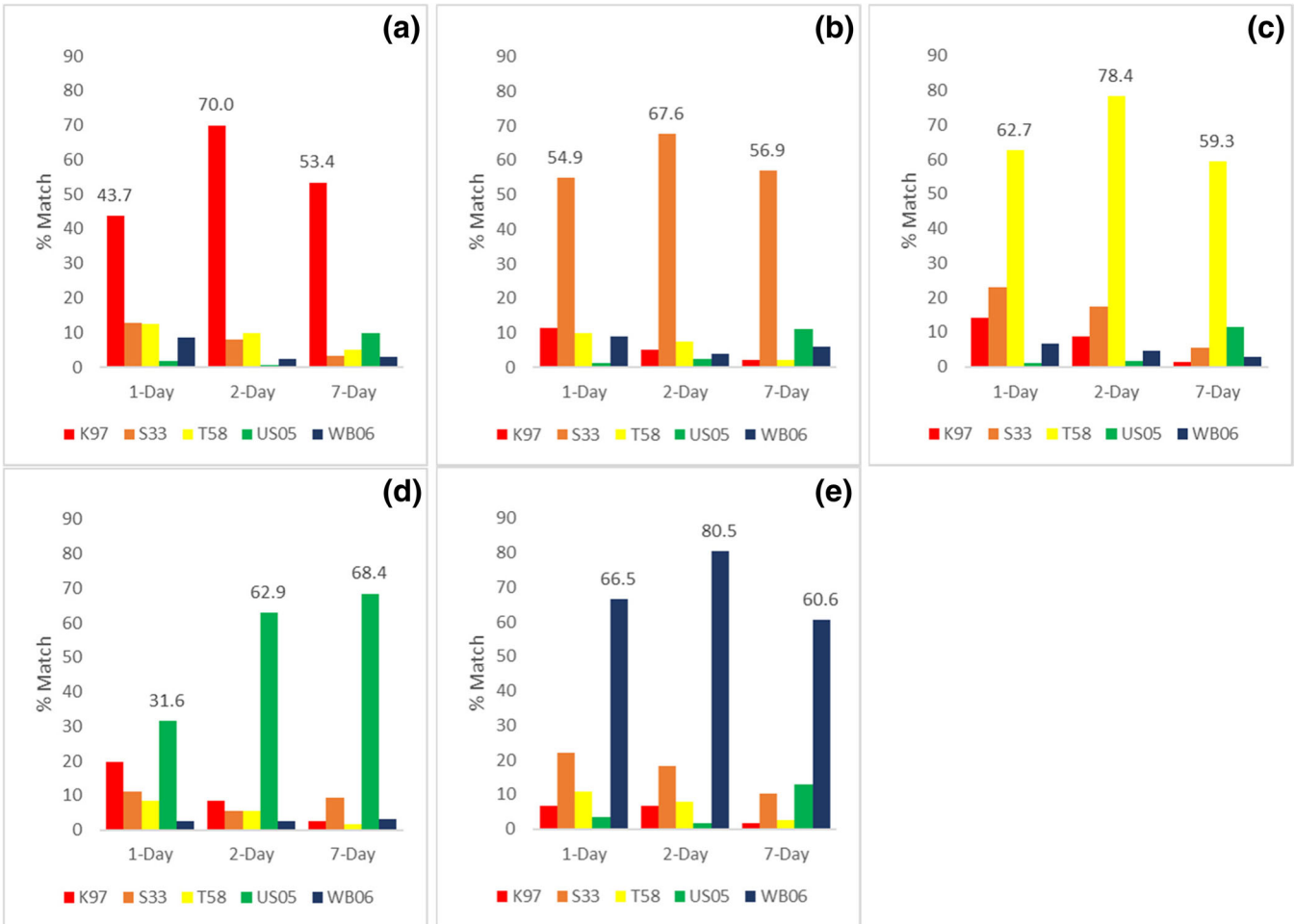

Figure 6. Classification by biomarker percent match was correct for all 1-day, 2-day, and 7-day samples of (**a**) K97, (**b**) S33, (**c**) T58, (**d**) US05, and (**e**) WB06

Table 3. k-NN classification results against 4-day library

Sample	Classification (correct = Y)		
	1-Day	2-Day	7-Day
K97	Y	Y	Y
S33	Y	Y	Y
T58	Y	Y	Y
US05	N (K97)	Y	Y
WB06	Y	Y	Y

Protein Identification

Peptides originating from 288 to 446 proteins were identified in each strain supernatant after 4 days, when screened against *S. cerevisiae* proteins in the UniProt database. No additional data acquisition was needed for protein identification, since the original LC-MS data contain DDA fragmentation, and those MS/MS spectra were used for peptide sequencing and protein mapping. Refer to Fig. 7 for an example spectrum. Complete lists of peptides identified in each strain supernatant, and their respective protein sources, are located in Supplemental Table S2.

Interestingly, one of the proteins which was identified in 4 of the 5 strains is glyceraldehyde-3-phosphate dehydrogenase 2 (GADPH, UniProt P00358), whose peptides have been shown to have antimicrobial properties against other species of yeast and bacteria during wine fermentation [26]. The results from similar future studies may provide further insight on peptides or proteins which may be secreted by the yeast for competitive advantage or quorum-sensing, in addition to normal metabolic processes [27, 28].

Conclusion

A technique for identification of *S. cerevisiae* strains using LC-MS by analysis of the yeast supernatant was developed involving solid phase extraction, non-targeted analysis, and data simplification into pseudo-spectra. Two chemometric classification techniques (k-NN and biomarker matching) were shown here to successfully differentiate yeast strains in various stages of growth. Since this technique can identify yeast strains without disruption of the yeast cell membrane, it has potential for use in applications where there are no remaining cells. This technique may be valuable even if viable cells are present, due to the fast turnaround time (since cell culturing is not required). This technique may be more powerful than MALDI-TOFMS to identify closely related strains due to the increased number of detected analytes due to reduced interference and reduced ion suppression. This technique also allows for peptide and protein identification, without additional testing, as DDA MS/MS is included in the initial data acquisition.

The classification method based on biomarker percent match was shown to be slightly more accurate for the five strains tested here (100% accuracy, versus 93% accuracy for k-NN). However, as more yeast strains are added to databases, it will become less likely that there will be biomarkers associated with only one strain. To deal with this, biomarkers may be identified which are reproducibly detected in multiple strains, rather than in just one strain. An ongoing effort would be required to maintain an up-to-date list of biomarkers. Therefore, a spectral-pattern method of classification, such as k-NN, may prove to be more useful as databases grow.

It may be interesting to identify the peptide sequences of identified biomarkers, to better understand the proteomic differences between strains. This was not performed in this study, as many true biomarkers are likely to contain sequence variants. The PMI software, used for peptide and protein identification against a protein database, has a limited capacity to identify sequence variants in large databases such as the yeast proteome. However, de novo sequencing could be performed using the same dataset.

The technique could potentially be adapted for mixtures of strains. Presumably, if one strain is much more abundant than the other, it will be classified, since only the top 3000 peaks are considered. For equal mixtures, the biomarker classification

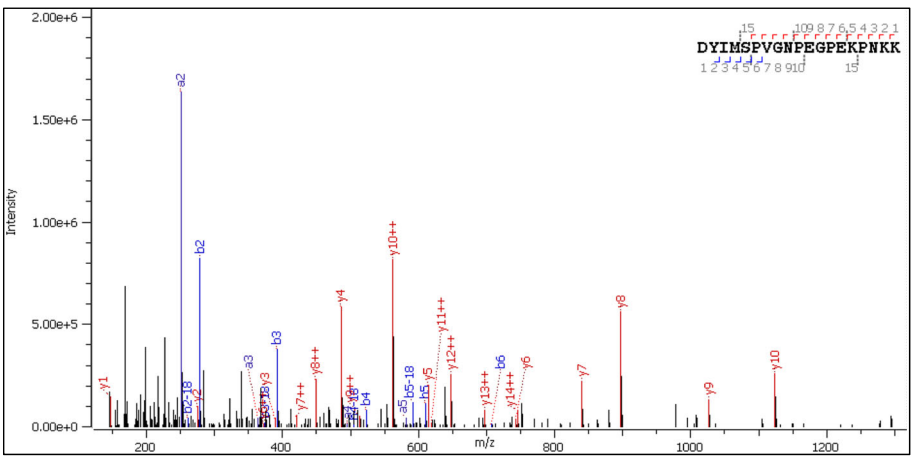

Figure 7. Example MS/MS spectrum used for identification of the 526.02 m/z peak (+ 4 charge state, 20.4 min) observed in the K97 4-day sample

may effectively classify the two strains with high percent match scores for each strain. The k-NN classification would only classify as one strain. This will be evaluated in future studies.

Additional planned studies include evaluation of this technique to classify yeast strains in more complex environments with varied carbon and nitrogen sources, and ultimately beer and wine. It would also be beneficial to utilize or develop a classification tool which can incorporate the use of peak retention times. The current classification techniques described here only utilize two variables to define the analyte profile (monoisotopic mass and XIC peak area). Additional utilization of retention times would allow distinction of different peptides with the same mass, potentially increasing the number of biomarkers identified. It is also feasible that this technique could be transitioned from LC-MS/MS to LC-MS for simplified analysis after libraries are established, as was previously demonstrated for yeast extracts [29].

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