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Use of Fluorescence Spectroscopy to Differentiate Yeast and Bacterial Cells

Hemant Bhatta*
Department of Physics, Division of Information and Communication Sciences
Macquarie University,
North Ryde, NSW 2109
Australia
Ph: 61 (02) 9850 8959
Fax: 61 (02) 9850 8115
Email: hbhatta@ics.mq.edu.au

Ewa M Goldys
Department of Physics, Division of Information and Communication Sciences
Macquarie University
North Ryde, NSW 2109
Australia

Robert P Learmonth
Centre for Systems Biology and
Department of Biological and Physical Sciences
University of Southern Queensland
Toowoomba, QLD 4350
Australia

Abstract

This study focuses on characterization of bacterial and yeast species through their autofluorescence spectra. Lactic acid bacteria (*Lactobacillus* sp.), and yeast (*Saccharomyces sp.*) were cultured under controlled conditions and studied for variations in their autofluorescence, particularly in the area representative of tryptophan residues of proteins. The emission and excitation spectra clearly reveal that bacterial and yeast species can be differentiated by their intrinsic fluorescence with UV excitation. The possibility of differentiation between different strains of *Saccharomyces* yeast was also studied, with clear differences observed for selected strains. The study shows that fluorescence can be successfully used to differentiate between yeast and bacteria and between different yeast species, through identification of spectroscopic fingerprints, without the need for fluorescent staining.

Keywords: Autofluorescence; microorganisms; identification; yeast; bacteria

1. Introduction

Lactic acid bacteria and yeast play very important roles in the dairy (Liu et al. 2004) and wine/brewing industries (Lonvaud-Funel 1999). Usually, specific strains are selected for particular processes and their contamination with foreign species can be detrimental to production. In the fuel alcohol industry, lactic acid bacteria are the most commonly found contaminants, which can inflict serious consequences (Bayrock and Ingledew 2001). Faster-growing bacteria or wild yeast can rapidly outnumber inoculated culture yeast and produce undesirable end products. Dairy products, especially yogurt face serious spoilage problems due to contaminant yeast species (Davis and McLachlan 1974) such as *Saccharomyces cerevisiae, Candida parapsilosis, Candida glabrata and Picihia anomala*. The wine industry also faces similar problems of contamination with bacteria such as *Lactobacillus kunkeei* (Edwards et al. 1999) and undesirable yeast species.

Optical technologies offer a repertoire of fast, simple and reliable techniques that can be applied for the identification and characterization of microorganisms. Alternative traditional methods of microbial identification based on biochemical, physiological and morphological criteria are often time consuming, laborious, involve numerous reagents and the distinction of closely related organisms may be difficult (Barnett et al. 1990). Molecular DNA analysis methods developed recently provide a more reliable differentiation of microorganisms at the species level (Berthier and Ehrlich 1998; Van Reenen and Dicks 1996), however these methods are also reagent- and time-intensive so their applications in industrial settings are not yet widespread. An optics-based method of Fourier transform infrared spectroscopy (FTIR) has been proposed in clinical (Kirschner et al. 2001) and food industry (Amiel et al. 2000; Lefier et al. 2000) applications, with reasonable success. Other techniques in use include infrared spectroscopy, flow cytometry, and chemiluminescence (Gunasekera et al. 2000; Ivnitski et al. 1999). Various earlier studies have proposed the use of fluorescence spectra for rapid microbial identification. Fluorescence labeling is commonly applied (Kosse et al. 1998) along with chromatography, dot-blot and fluorescence *in situ* hybridization (FISH). However, the literature contains very few reports on the use of autofluorescence for microbial

characterization. Some studies included bacterial characterization based on their intrinsic fluorescence (Giana et al. 2003; Leblanc and Dufour 2002) but to the best of our knowledge there are no similar reports for yeast.

Our approach focuses on fluorescence spectroscopy measurements of selected lactic acid bacteria and yeast species, with the aim of verifying that the fluorescence technique can be successfully used to differentiate between yeast and bacteria and between different yeast species, through identification of spectroscopic fingerprints. We aim to establish a differentiation method that would be rapid, simple and would not require fluorescence labeling.

2. Materials and Methods

Cell culture

Lactobacillus casei were cultured in Lactobacillus/Elliker broth from Fluka® at room temperature (25°C). Yeast strains (Table 1) from a variety of industrial backgrounds were chosen to investigate the range of applicability of the technique. The yeasts studied included *Saccharomyces cerevisiae* strains A9 (baker's yeast), A13 (distillery yeast), K7 (sake yeast), and *Saccharomyces pastorianus* Y275 (brewer's yeast). The yeasts were obtained from A/Prof. Robert Learmonth at the University of Southern Queensland. An overnight culture of the cells was set up at room temperature (25°C) in Yeast Extract Peptone in Dextrose (YEPD) medium which contains in g/L: Yeast extract - 5, bacteriological peptone - 5, $(NH_4)_2SO_4$ - 3, KH_2PO_4 - 3, Dextrose – 10. The cells were oscillated in an orbital shaker at 120 o.p.m. The culture optical density at 600 nm was measured after 24 hours culture before cells were transferred to Yeast Nitrogen Base (YNB) (Difco^{TM}) medium to final optical density at 600 nm (OD_{600nm}) of 0.1 to ensure standardized inoculations. The YNB medium contains in g/L: Yeast Nitrogen Base– 6.7, Dextrose – 10. The YNB medium was chosen initially because of lower autofluorescence compared to YEPD. In preliminary studies, cells were analyzed in the low fluorescence medium YNB. However given the relatively low intensity of the autofluorescence we found it necessary to wash cells free of media and suspend them in distilled water for fluorescence measurements. With incorporation of the washing step, any laboratory or industrial growth medium could be used for cell growth prior to analyses. Each strain was then inoculated into YNB medium in three different flasks to prepare culture in triplicates for comparison and consistency.

Washing the cells:

Cells were extracted from the growth medium, and suspended in non-fluorescent distilled water for auto-fluorescence measurements. One mL of the cell culture was aseptically taken in a microfuge tube and centrifuged at 2,400 r.p.m. (700×g) for three minutes. The supernatant was removed and cells resuspended in 1mL of distilled water and centrifuged again for three minutes. This washing procedure was repeated three times to ensure complete removal of the medium, which could potentially interfere with the desired cell spectrum. Bacterial cells were treated similarly; the centrifugation time was however eight minutes as bacterial cells are smaller in size and take longer to sediment.

Experimental sample preparation and data collection:

Prior to measurement, cell density was adjusted to OD_{600nm} of 1.0 to standardize comparative cell densities. At such high cell densities inner filter effects distort spectra, especially at lower wavelengths. We emphasize that all samples were carefully adjusted to the standardized OD_{600nm}, so the distortion was identical for all samples. Notwithstanding the distortion, clear differences were observed in the spectra.

We applied fluorescence excitation-emission spectral techniques to: (i) differentiate yeast and bacterial species; and (ii) differentiate between various yeast *Saccharomyces* strains. The washed cells were suspended in 3mL distilled water in 10mm x 10mm quartz cuvettes and their autofluorescence examined. Differentiation of yeast and bacterial samples was studied with a Fluorolog-Tau3 system from JY Horiba. Differentiation of yeast species from each other was studied with a PC1 Spectrofluorometer from ISS Inc. with confirmation on the Fluorolog system. The data were collected with a photomultiplier in photon-counting mode. The slits were adjusted in order to maximize readings from the naturally low intensity samples without exceeding the photomultiplier upper limit of 2×10^6 photon counts/sec, with slit widths 3 nm (Fluorolog system) and 8 nm (PC1 system). The cells were constantly stirred with a magnetic stirrer to ensure uniform cell distribution during the experiment. Spectra were corrected using correction files suitable for unpolarized light to account for the instrument transfer function. During this experiment we analyzed the fluorescence emission spectra ranging from 310nm to 420nm for the excitation wavelength of 290nm and fluorescence excitation spectra ranging from 200nm to 320nm for the 340nm emission wavelength.

In preliminary experiments for comparison of yeast strains (data not shown), 1 mL of cell solution each was taken out of the culture flasks at intervals of 26 h, 48 h and 72 h for analyses. The fluorescence intensity variation between strains was studied at the different culture ages and maximum variation was identified in the 48 h culture in all yeast strains tested. The spectra from yeast cells at 26 h, 48h and 72 h were peocessed by the statistical technique called Principal Component Analysis (PCA) in order to categorize the closely related spectral curves into distinct groups.

PCA is used to reduce the dimensionality of a data set consisting of a large number of interrelated variables, while retaining as much as possible of the variation present in the data set. In order to achieve this, the data is transformed to a new set of variables called the principal components (PCs) which are uncorrelated, and which are ordered so that the first few retain most of the variation present in all of the original variables. The first few variables can be more conveniently used to study the problem of interest in a subspace of lower dimension (Jolliffe 2002). The first two principal components that represent over 90% of the variance of the spectra were used for spectral differentiation among the three yeast strains. A total of nine curves, three for each strain, were analysed by the PCA test for ages 26 h, 48 h and 72 h. The XLSTAT (Addinsoft) software was used for the PCA analysis and the plots were made using Origin (OriginLab Corporation).

3. Results

Differentiation of yeast and lactic acid bacteria:

Lactic acid bacteria (*Lactobacillus casei*), and yeast (*Saccharomyces cerevisiae*) strain A9 were studied for variations in their autofluorescence, particularly in the area representative of tryptophan residues of proteins, around the excitation/emission wavelengths of 290nm/340nm. Clear differences were observed for cells harvested after 24-hour culture. Figure 1 reveals the differences between yeast and bacterial tryptophan fluorescence for cells excited at 290nm and emitting in the 310 – 405nm range. We noted that the bacterial emission peaks at 325nm and yeast emission peaks at 328nm. The yeast emission curve is characterized by ~3nm red shift compared to the bacterial emission. The bottom plot shows variation in intensity between the two curves. The intensity variation in percentage was calculated according to:

$$Variation(\%) = \frac{IntensityDifference}{IntensityMean} \times 100\% = \frac{Intensity(Bact - Yeast)}{Intensity(Bact + Yeast)/2} \times 100\%$$

For the intensity in the emission range 305 to 405nm, the minimum variation was at 327nm. The percentage variation reached 18% at 310 nm and -27% at 405nm.

Figure 2 shows a normalized excitation scan for yeast and bacteria at 340nm emission. The excitation plot reveals a notable spectral shift between yeast and bacteria ranging from about one to four nanometers. The yeast emission curve is again characterized by a red shift compared to the bacterial emission. The area around 287nm provides the best indication of spectral variation between the species. The variation curve, (Figure 2, bottom curve) gives a clear indication of the wavelength range optimized for microbial differentiation. A variation of 12% is observed at 260nm excitation wavelength and 36% at 300nm. Higher variation values were observed beyond 305nm but the data at the edges are less reliable due to higher degree of noise at lower intensity levels.

The above observations establish clear differentiation between yeast and bacteria by emission curves (~ 3nm shift, ~ 15% average difference). The best differentiation is seen in the region of 340 – 460nm emission. While the excitation curve showed relatively less difference, the region of 285 -290nm may also be useful for species differentiation.

Different yeast strains:

Yeast strains A9, A13, K7 and Y275 grown in culture flasks were studied for differences in their autofluorescence spectra. The emission spectra for yeast strains A13 and K7 cultured for 48hrs in YNB, excited at 290nm, are shown in Figure 3. Unlike in Figure 1, we did not observe a consistent spectral shift throughout the wavelength range but differences in intensity at a range of wavelengths. This difference, characterized by the variation curve is more pronounced at the blue end of the spectrum, where it reaches a maximum value of 13% at 310nm emission wavelength. By analyzing the spectral pattern of the curves we note that the difference is greater at lower wavelength, the limit of which is defined by the

instrumental range and scatter from the exciting illumination. The emission spectra of yeast strains A9 and K7 were almost identical, but significantly different from the spectra of Y275 and A13, which were also nearly identical to each other (data not shown). Thus the emission spectra may be useful to classify yeasts into groups, but not to distinguish individual strains.

Therefore we explored the fluorescence excitation spectra of the strains to investigate the potential to detect greater differences. The variation in fluorescence excitation spectra is more pronounced and three different classifications were possible. Figure 4 shows fluorescence excitation spectra of yeast cells at 48 h growth for the emission wavelength of 340nm. Here, we notice that the variation is highest at the blue and red ends with values of ~45% and 19% at 200nm and 320nm respectively, for strains A13 and K7 and also for A13 and A9. It was difficult to classify strain Y275 as the intensity values fluctuated between that of K7 and A13 (data not shown). The excitation and emission curves for different yeast strains show that fluorescence excitation provides a better tool for differentiation between the strains as compared to emission.

The excitation curves for cells at 26 h and 72 h evidenced some distinctions between strains, however they were not as clearly pronounced as those for 48 h. Hence, we applied principal component analysis (PCA) to the spectra. The PCA clearly assorted the cells into three distinct groups. Figure 5 shows the PCA analysis of the yeast strains A9, K7 and A13 for the ages 26 h, 48 h and 72 h. It can be seen that, three regions are formed, with cells K7 falling in the left region, A9 falling in the middle and A13 falling in the right. This is the case for all ages. Yeast strains A9 and K7 at 26 h tend to form groups at close proximity; however the distinction is still possible. For older age cells the grouping is quite definite.

4. Discussion

The observations reported above indicate that fluorescence spectroscopy can be successfully applied to differentiate between bacteria and yeast and between different yeast strains, the latter being difficult even using advanced biochemical methods. We chose to collect the emission spectra at 290nm excitation wavelength because it coincides with a typical excitation range for aromatic amino acids in microorganisms (Lehninger *et al.* 1993), particularly tryptophan, with some contribution from tyrosine and phenylalanine. It is well documented that the indole groups of tryptophan residues are the dominant source of UV absorbance and emission in proteins. For example pure tryptophan in water at neutral pH emits at 353nm with 60nm bandwidth when excited at 295nm (Lakowicz 1999). The emission of tryptophan is known to be highly sensitive to its local environment, and spectral shifts in its emission have been observed as a result of a range of phenomena, such as binding of ligands and protein-protein association (Lakowicz 1999).

The differences in emission and excitation spectra noted in Figure 1 and 2 between bacterial and yeast cells could be due to the presence of different environments experienced by tryptophan residues in specific proteins. The greatest differences were seen in emission scans in the region of 340 – 460nm; although the excitation scans showed relatively less difference, the region of 285 -290nm may be provide a useful ancillary measurement. In

contrast, the differentiation between yeast strains (Figures 3, 4) was better achieved by analyzing the excitation spectra, with emission spectra of limited value. This may be expected, as relative differences in tryptophan distribution in proteins would likely be higher in different species as compared to different members of the same species.

A difference due to instrumentation precision was also noted. The experiments for yeast and bacterial differentiation were performed with a high precision Fluorolog Tau3 system that uses double grating monochromators for improved reduction of stray light. However, yeast - yeast differentiation was more prominent with the PC1 spectrofluorometer, which incorporates single grating monochromators and is thus more susceptible to collection of scattered light. Hence, we see that less sophisticated instruments are actually more effective to differentiate between the various yeast strains. Thus we have identified how the relative limitations of lower precision equipment may actually be turned to an advantage. We have also demonstrated that the closely related spectra, yeast strains at 24 h and 72 h in our case, can also be categorized using Principal Component Analysis. We believe that the findings presented here will be useful in designing standardized, simple and inexpensive equipment which would be applicable as a standard platform for investigations in industrial settings.

5. Conclusion:

We demonstrate that yeast and bacterial cells show differences in fluorescence spectra in the ultraviolet to visible region. The difference between yeast and bacteria is easily noticed in normalized fluorescence excitation/emission spectra in the region characteristic of tryptophan emission. In this region, the bacterial and yeast emissions peak at 325nm and 328nm, respectively, displaying ~3nm red shift for yeast cells compared to bacteria. The differentiation in selected yeast strains was observed as a result of combined fluorescence excitation/emission and scattering light, with better differentiation in excitation spectra. Thus it is shown that yeast and bacterial species can be successfully characterized using fluorescence spectroscopy and statistical analysis (PCA) without the need for fluorescence labeling.

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Strain	Details	Source / description
A9	*Saccharomyces cerevisiae* wild-type baking strain	Lewis *et al.* 1997
A13/Y270	*Saccharomyces cerevisiae* Distillery yeast, high optimal growth temperature	ATCC 4132
K7	*Saccharomyces cerevisiae* Saké yeast	ATCC 26422
Y275	*Saccharomyces pastorianus* Brewing yeast	ATCC 2345

Table 1: Description of the yeast strains used.

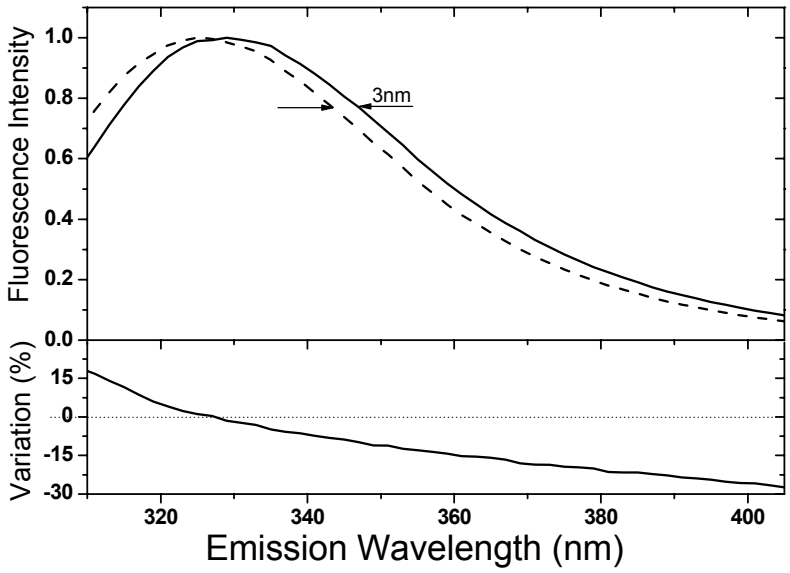

Figure 1: Top: Normalized emission spectra for excitation wavelength of 290nm for 3 replicates of each of the yeast (strain A9 —) and the bacteria (L. *casei* ---) cultures. Bottom: Difference/mean percentage intensity variation between bacteria and yeast, presented as mean with error bars as standard deviation.

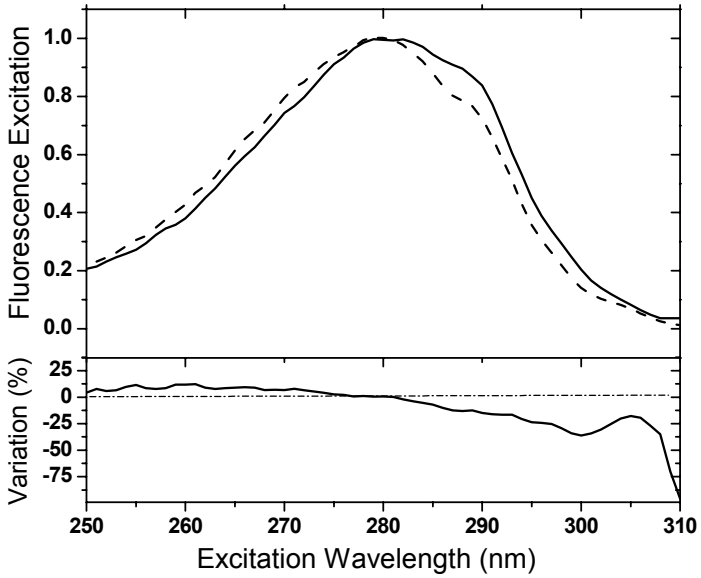

Figure 2: Representative normalized excitation spectra for emission at 340nm for Yeast (Strain A9—) and bacteria (*L. casei*---) cultures. Bottom: Difference/mean percentage intensity variation between bacteria and yeast.

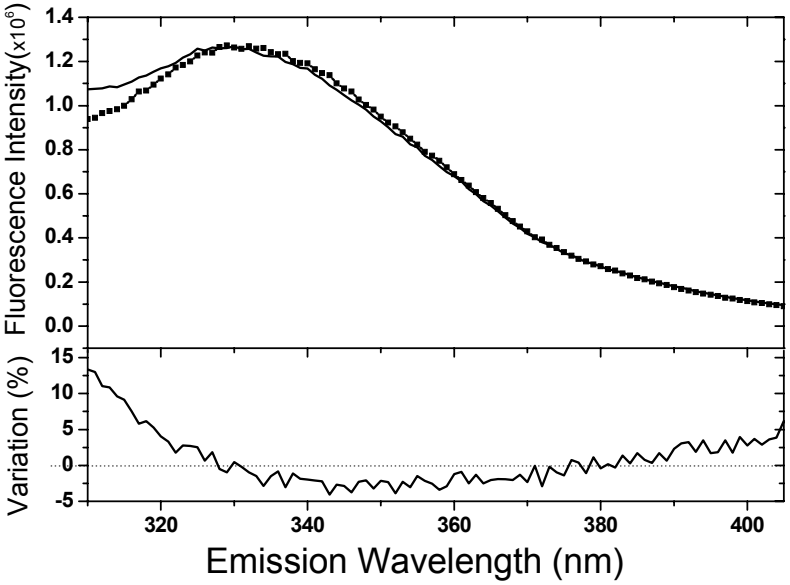

Figure 3: Representative emission spectra for excitation wavelength of 290nm for yeast (strain A13 —, and strain K7 ---) cultures. Bottom: Difference/mean percentage intensity variation between yeast strains A13 and K7.

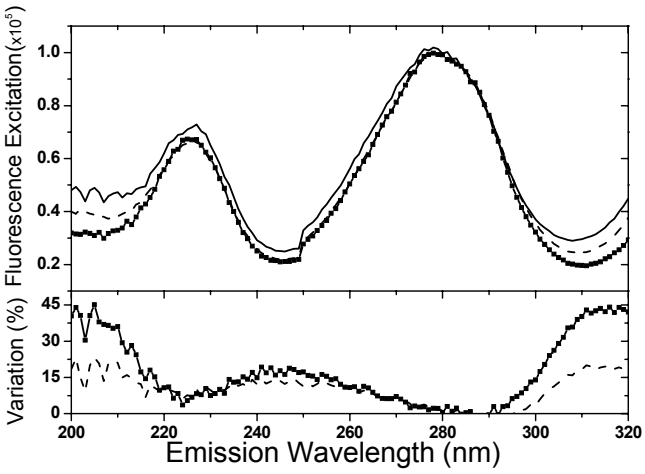

Figure 4: Representative excitation spectra for emission at 340nm for yeast cultures (strain A13—, strainA9--- and strain K7 ■). Bottom: Difference/mean percentage intensity variation between yeast strains (A13 vs K7 ■) and (A13 vs A9 ---).

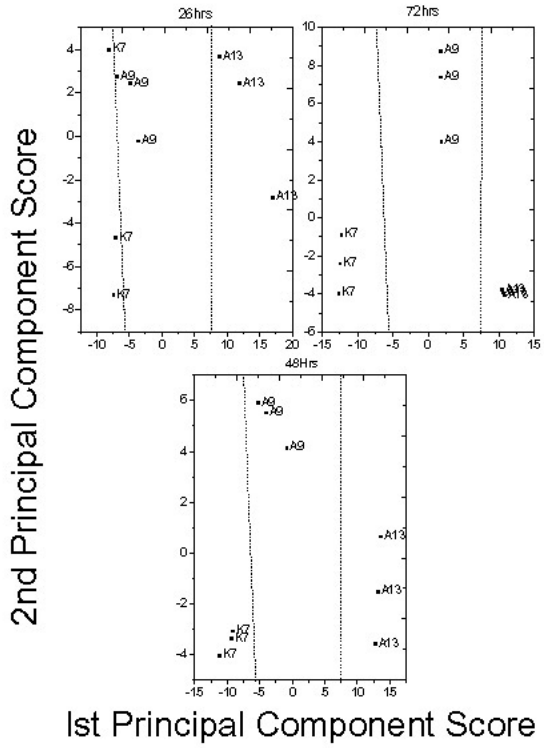

Figure 5: Principal component analysis (PCA) results of the yeast strains at 26 (top left), 48 (bottom), and 72 h (top right) of growth. Strain K7 tends to fall in the left region, strain A9 in the middle region and strain A13 in the right region for all ages.