Identification of lactobacillus casei-BDI and Streptococcus thermophilus by fluorescence spectra

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Fluorescence analysis applied in the study of lactic acid bacteria (LAB) provides a new method and theory to study probiotics and realize the detection and identification of the strains. It is also possible to achieve automation and computerization. In this letter, the differences between the fluorescence spectra of lactobacillus casei-BDI (Lc-BDI) and Streptococcus thermophilus (St) are shown, and the second-order derivative spectra are used to further study the diversity of these two strains. According to the results, with the excitation wavelengths of 285 and 340 nm, there are significant differences between them. The experiment is repeated for 6 times, showing good repetitiveness.

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Probiotics is the live microbial feed additives which are beneficial to the hosts via improving the balance of intestinal microflora. They have many kinds of other normal gut flora of incomparable physiological functions to organism. Probiotics used both at home and abroad is various in styles. Japan is one of the earliest nations to develop probiotics and has formed commercial scale products. The species of probiotic products are of varieties in European countries. Though the USA starts relatively late in this filed, it has already developed a series of probiotic products rapidly. The research and products of probiotics in China are relatively backward. In recent years, probiotics is widely used in commercial production, which promotes the research of probiotics[1−3].

At present, a global upsurge of application and research of microbial ecological agents has emerged. From original morphology to cell and molecular level, many research technologies have been used in this filed. Infrared and fluorescence spectra have also been applied[4,5]. Yang has reported the method to detect Alicyclobacillus gen, the common spoilage bacteria in apple juice[4]. Ammor reviewed recently developed methods using bacterial intrinsic fluorescence for identification and characterization[6]. Giana *et al.* reported the identification of bacterial species by fluorescence spectroscopy and classification through principal component analysis, and the results showed that the bacterial identification was very efficient with such methodology[7]. There are so many fluorophores in cell that the spectra of each chromophore overlap with each other, and as a result, the direct use of spectral data causes data lost and difficulty in improving accuracy. The valley of the second-order derivative spectra related to the fluorophore can effectively isolate spectra from superposed bands and accurately determine the location of fluorescence peak of fluorescent material in cells[8−10]. In this letter, the zeroth- and second-order derivative fluorescence spectra are used to study lactobacillus casei-BDI (Lc-BDI) and Streptococcus thermophilus (St). To obtain the derivative spectra, there are two methods: by hardware[11] and software. The method in this letter is related to the latter. The second-order derivative spectra are obtained by the numerical differentiation accomplished by the program model in Origin 7.5, and the calculation expression of fluorescence intensity differentiation to excitation wavelength is

$$\frac{1}{2}\left(\frac{y_{i+1}-y_i}{x_{i+1}-x_i}+\frac{y_i-y_{i-1}}{x_i-x_{i-1}}\right), \tag{1}$$

where y_i is the fluorescence intensity corresponding to emission wavelength x_i.

For the preliminary exploration, the facile bacteria are the best choice for the experiment. The commonly used Lc-BDI and St are selected. The samples came from the State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University. The bacteria grew for 12±1 h on liquid media, according to the growth conditions of de Man, Rogosa, and Sharp (MRS) medium at 37.5 °C. The culture medium was removed by centrifugation at 5000 rpm for 6 min, and the cells were washed twice with 5 ml of saline solution (NaCl, 9 g/L), and centrifuged under the same conditions. Then the cells were kept in saline solution at 4 °C in fridge.

Cells as the basic unit of bacteria contain the same main fluorescent biochemical constituents: the amino-acid residue including tryptophan, tyrosine, and phenylalanine; the reduced form of nicotinamide-adenine dinucleotid (NADH); the reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH); and the flavin compounds. The fluorescence spectra of these biomacromolecules have been well characterized in literatures. Since the fluorescent compounds in cells of different bacteria are various in number and species, the measurement

of the fluorescence emission spectra can realize the discrimination between different bacteria.

The fluorescence spectra were measured by an SP-2558 multi-functional spectrometer system made by Roper Scientific Inc., USA. The light source was a xenon lamp. The light beam chosen by a monochromatic system irradiated the sample in a quartz colorimetric cuvette placed in the sample cell. The fluorescence emitted by the sample went through another monochromatic system and was collected by a charge-coupled device (CCD). Then a computer acquired the real signals and outputted the experimental results after data processing. The used grating in the exciting monochromatic system was a 1200-g/mm blazed grating with the blazed wavelength of 300 nm. While a 150-g/mm blazed grating with the blazed wavelength of 500 nm was used in the emitting monochromatic system. A filter with the cut-off wavelength of 320 nm was used to filter out the Rayleigh scattering in our experiment when the sample was irradiated with excitation wavelength below 320 nm.

As reported in the literature, the best excitation wavelengths for amino acids, NADH or NADPH, and flavin compounds are around 280, 340, and 450 nm, respectively[5]. The fluorescence spectra with appropriate excitation wavelengths were measured with an integration time of 2 s. The spectra data were differentiated through the software Origin 7.5, and the second-order derivative spectra were obtained.

Figure 1(a) shows the spectra of Lc-BDI with excitation wavelengths around 285 nm. When the sample is excited by 260-nm light, there are two main peaks occurring at approximately 342 and 470 nm. With the increase of excitation wavelength, another peak around 390 nm begins to emerge. When the excitation wavelength increases to 285 nm, the intensities of peaks at 342 and 390 nm reach the maximum, and then they decrease with the increase of wavelength above 285 nm[12].

Similar results can be seen from the spectra of St shown in Fig. 1(b). Comparing the spectra excited by 285-nm light, there are a few differences: the second peak in Fig. 1(b) is located about 395 nm but it is at 390 nm in Fig. 1(a), and the intensity ratios of these two peaks are different.

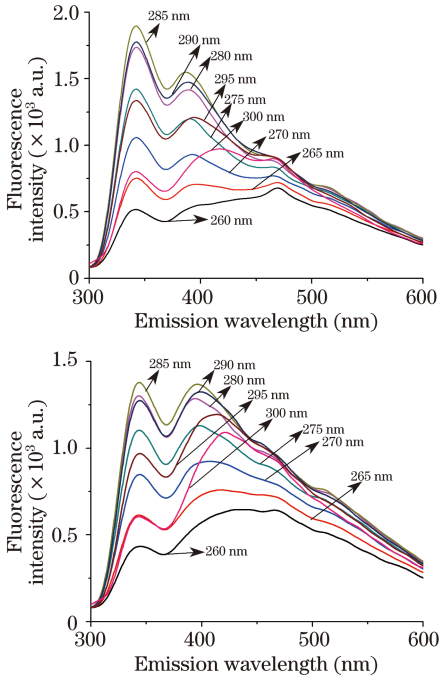

Fig. 1. Zeroth-order derivative spectra of (a) Lc-BDI and (b) St with different excitation wavelengths.

In order to verify the feasibility and effectiveness of the method, the results of duplicate tests are shown in Table 1. It can be seen that the ratios of the first peak intersity to the second one are about 1.0 for St and 1.2 for Lc-BDI, respectively[13].

The excitation wavelength of 340 nm was selected to irradiate the samples. Figure 2 shows the spectra of Lc-BDI and St with 340-nm excitation. In this figure St has only one obvious peak but Lc-BDI has five peaks. The biggest difference is in the range of $400 - 530$ nm, and the fluorescence spectra are the compound spectra of protein, NADH, NADPH, flavin compounds, and so on. The fluorescence in this range is so strong that the peak at 520 nm is not manifestative in the spectrum of St, but there is an obvious peak in the spectrum of Lc-BDI.

Table 1. Duplicate Test Results: Peak Wavelength, Intensity, and Intensity Ratio for Two Strains

Strain	Peak (nm)	Intensity (a.u.)	Peak (nm)	Intensity (a.u.)	Intensity Ratio
St	343.742	1429.1	397.841	1381.2	1.03468
Lc-BDI	342.679	1936	387.126	1581.6	1.224077
St	344.526	1513	394.182	1506.3	1.004448
Lc-BDI	342.958	1990.3	388.955	1634.6	1.217607
St	346.094	1744	394.705	1746.3	0.998683
Lc-BDI	345.3	2307.8	389.217	1980.2	1.165438
St	344.265	1593.6	394.705	1584.2	1.005934
Lc-BDI	343.742	1741.8	388.433	1426.9	1.220688
St	343.742	1438.8	397.318	1387.9	1.036674
Lc-BDI	342.958	1734.8	390.523	1378.9	1.258104
St	344.003	1374.9	397.804	1347.7	1.020183
Lc-BDI	343.742	1181.3	389.478	964.69	1.224538

Derivative fluorescence spectra excited by 285- and 340-nm wavelengths are obtained through Origin 7.5. Figure 3 shows the second-order spectra of Lc-BDI and St. These two strains show the same peaks and valleys, which means that the fluorophores in different cells are fairly similar, and the main differences are the number and the ratio of different fluorophores. Figure 3(a) shows four relatively obvious valleys located at about 335, 390, 470, and 515 nm when the sample is irradiated by 285-nm light, and Fig. 3(b) shows five relatively obvious valleys located at about 410, 440, 465, 490, and 525 nm when the sample is excited by 340-nm light. Coinciding with the past data of bacterial fluorescence location, the changes at about 340, 440, 470, and 520 nm are respectively the results of intracellular matter's fluorescence such as protein, NADH, NADPH, flavin compounds, and so on. There are also some variances located at around 400 and 490 nm which are not well explained, and in the following research, the problem will be further studied. Although the locations of peaks and valleys are the same, the relative intensities are obviously different.

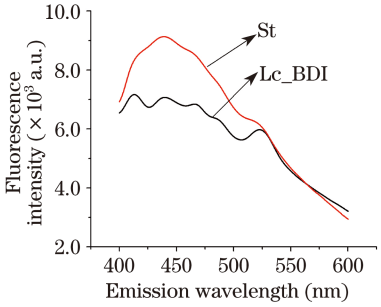

Fig. 2. Spectra of Lc-BDI and St under 340-nm excitation.

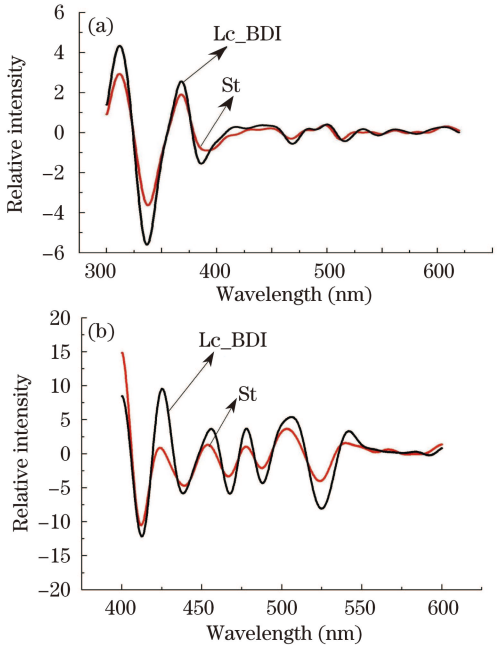

Fig. 3. The second-order fluorescence spectra of Lc-BDI and St under (a) 285- and (b) 340-nm excitation.

In conclusion, we can get the following results. When the bacteria are excited by 285-nm light, they emit fluorescence in the range of 300 − 500 nm. The locations of peaks are different between these two strains. The second peak of Lc-BDI is located at the range of 386−391 nm, but for St it is in the range of 394 − 398 nm. The peak intensity ratio of Lc-BDI is larger than that of St. With different concentrations, the ratios are not constant, but the ratio of Lc-BDI always keeps larger than that of St. Under 340-nm excitation, there is one peak in the spectrum of St, while five peaks appear in Lc-BDI's spectrum. This difference can be used to distinguish Lc-BDL and St. The second-order spectra tell us that the differences of relative intensities and some locations of valleys can be used to identify these two strains. The method proposed in this letter is not only suitable for the identification of Lc-BDI and St, but also applicable to the identification of other strains.

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