

Determination of bacterial contamination of milk using fluorometry

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Abstract:

The aim of this paper is to determine the feasibility of using fluorometric methods as an indicator for quality and contamination of milk with *E.coli* bacteria, and selection the suitable wavelength to be used with laser induced auto fluorescence. Three groups of milk samples were used in this study: Fresh pasteurized milk samples, milk samples containing different concentration of *E.coli* bacteria which were added artificially, and milk samples that were kept in refrigerator for 3-5 days. Thirteen excitation wavelengths were used to get the emission spectra for all milk samples using spectrofluorometer .The results showed that the emission spectra at 275nm excitation wavelength gave a good differentiation between these three groups. The data analysis demonstrated that the proposed method can bring progress in identification of milk quality and contamination with rapidness, high sensitivity and low cost diagnostic tool. Laser light at this wavelength that transmitted by optical fiber can be used for milk samples examination in situ and for more precise results.

Key words: Milk fluorescence, Bacterial contamination

Introduction:

Good quality measurements are essential to control and maintain products and processes quality, both in manufacturing, trade and research. Milk products are a very important human nutrient since their consumption has increased in recent years[1]. Raw milk in process and end-product quality control are crucial areas for quality managements in the dairy industry .Psychotropic microorganisms are of particular importance to spoilage of milk and dairy products [2] .Fluorescence spectroscopy has been extensively exploited for studies of molecular structure and function in chemistry and biochemistry. However, its effectiveness in microbial identification and characterization has only been recently recognized in the last two decades[3].The potential of using fluorescence in food research

had increased during the last few years with the propagated application of chemo metric and with the chemical and optical development of spectrofluorometers[4].Some studies have proposed the use of fluorescence spectra for rapid bacterial identification[5]. Bacterial intrinsic fluorescence has been also shown to allow tracing for sources of bacterial contamination in milk , water and another sources[6]. Fluorescence spectroscopy applied on milk and dairy products has previously been investigated in a few studies to monitor changes in milk due to heating , oxidation ,storage and contamination; Wold and Coworkers[7] showed that front face fluorescence spectroscopy could be used to measure light induced oxidation in various dairy products . Milk coagulation induced by acids was investigated using fluorescence

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spectroscopy[8]. A rapid fluorometric method to estimate the heat treatment of liquid milk was introduced by Birlouez *etal.*[9] Fluorometry were also used for determination of skim milk powder adulteration in fresh milk[10]. X.Liu and L. Ematzgue[11] have introduced a fluorescence spectroscopy method for characterization of nonfat dry milk caused by manufacturing and storage conditions. A rapid analysis of bacterial contamination of milk during its production process in performed with the use of the bioluminescence based ATP metric[12]. The aim of this study is to investigate the feasibility of using auto fluorescence as an indication for contamination of milk by bacteria and finding the suitable laser with certain wavelength to be used in laser induced auto fluorescence.

Materials and Methods:

1) Bacterial isolates: *E.coli* Bacteria were collected in different dates and from human biology feces with dairy poisoning, the bacterial species were identified by conventional phenotypic procedures based on biochemical, physiological and morphological criteria according to microbiology standardized techniques[13]. Once accomplished the identification, appropriate biochemical culture media were used to identify with accuracy the bacteria species, using commercial strips (API 20E, BioMerieux Inc, France)[14].

2) Milk samples preparation

The experimental set up where designed to include three groups of milk samples to be investigated for fluorescence spectroscopy

A-Three fresh pasteurized milk samples were obtained with valid expiry date. These samples are considered as control groups.

B-In this group, the bacteria were added artificially to the milk samples thus this group were considered as the

contaminated samples, in this regard; fresh cultures *E.coli* were prepared. Using a septic techniques in which three to five colonies of bacteria were harvested by centrifugation and suspended in a small volume of saline solution. Then saline solution was removed by centrifugation at 500rpm for 10 min, washed with 5ml phosphate buffer saline and suspended in the same buffer saline following McFarland scale using an automatic apparatus SP3000 UV-Visible spectrophotometer (Optima) to standardize its concentration, representing 300,000 bacteria/ml. This suspensions was used for fluorescence experiments. Then five milk samples were inoculated with 100 μ l, 200 μ l, 300 μ l, 400 μ l, and 500 μ l) of the bacterial suspension.

C-Three samples of pasteurized milk were opened and were left in refrigerator for 3 to 5 days. The milk in this group was considered as old milk samples

3) Fluorescence spectroscopy: Fluorescence spectra of eleven milk samples were collected at excitation wavelengths (200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450 and 500nm) using a spectrofluorometer mode SL174 (fluorescence spectrophotometer, India) connected to a PC (personal computer), fitted with 1ml cells and xenon lamp source. The choice of the excitation and emission wavelength range was based on automatic laser spectrofluorimeter described by Zangaro *et al* [15]. The milk samples were placed in a quartz cuvette, detector sensitivity was set at 900 volt, the excitation and emission slits were adjusted to provide a 20nm spectral band pass, and the scan speed was 600nm/min. Data plots were made using software Excel 2003.

4) Data analysis:

The data of emission spectra were analyzed and normalized using origin

lab 7.5 program. The plotting of data was achieved using Excel 2003. Both intensity and shifts in peaks were considered in results

Results:

E. coli bacteria were gram negative, circular smooth pink in color on Mackonky agar, small pink in blood agar. They are Indol and catalase positive, oxidase and coagulase negative, and positive for lactose fermentation. The results of APi 20 E system came to ensure the identification. The emission spectra of milk samples using 225 nm and 275 nm excitation wavelength are illustrated in figure (1) and figure (2) in their respective order.

The data were normalized in order to explore the differences in peaks regardless the intensity. The average of emission spectra for each of control milk samples and old milk samples were determined, while the contaminated milk samples were taken into account according to concentration of bacterial inoculation in each sample. As it is clear from figure (1), all samples share the same peak of emission at 335 nm wavelength and slight differences in peaks between samples can be noticed.

Figure (2) shows the emission spectra of samples using 275 nm wavelength. Two peaks of emission can be noticed for all samples. The first peak is around 330 nm for control sample. Slight shift to the left can be seen for the old milk samples to give peak at 325 nm. On the other hand, most of bacterial contaminated milk samples tend to give peaks at 340 nm. Second peak can give a clear differentiation between samples, especially for old milk samples that gave a noticeable different peak at 430 nm. Most of other peaks of bacterial contaminated milk samples tend to give peaks at 425 nm.

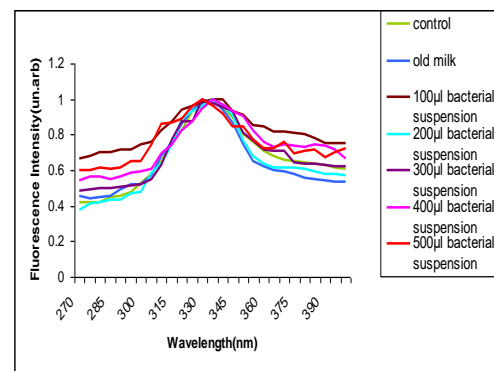


Fig.1: Normalized fluorescence spectra of milk samples at 225 nm Excitation wavelength

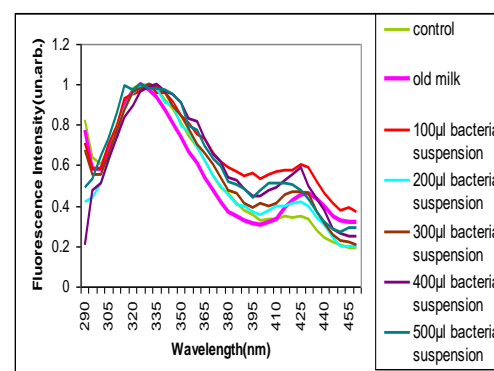


Fig. 2: Normalized fluorescence spectra of milk samples at 275 nm excitation wavelength.

The data in figure (3) and figure (4) represent the emission spectra at 225 nm and 275 nm in their respective order, in these two figures. The data were not normalized and the real intensities were taken into account. The average of control, old and bacterial contaminated milk samples were plotted. As it's clear from figure (3), clear variation in peak intensities can be noticed at 335 nm; control milk gives higher intensity, 170 nm, and less intensity was for bacterial contamination milk samples at 180 nm, and lower intensity was for old milk 140 nm. Figure (4) shows that there are clear differences between groups in terms of peaks and intensities at two emission areas.

It's clear that bacterial contamination milk gives a distinctive peak at the first

area of emission at 340nm while control group give peak at 330nm , and old milk group give peak at 325nm .Control groups give higher intensity at 500nm in comparison with 400nm in each of old and bacterial contamination group.

Very clear differences appeared in the second emission in term of peaks especially for old milk at 430nm wavelength in comparison with around 425nm in both of control and bacterial contaminated milk.

The intensity of bacterial samples tends to be higher than old and control group at this emission area.

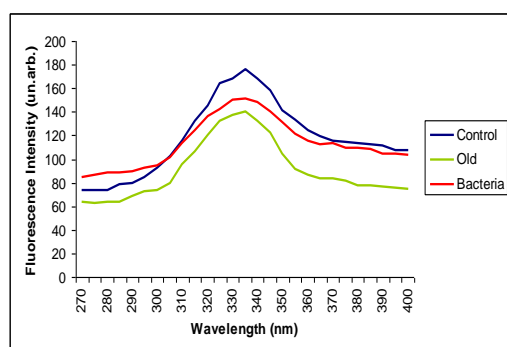


Fig. 3: Average fluorescence spectra of milk samples at 225nm excitation wavelength

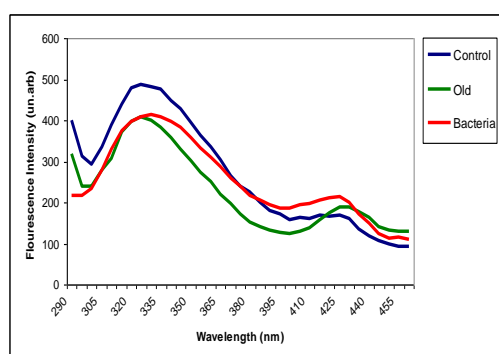


Fig. 4: Average fluorescence spectra of milk samples at 275nm excitation wavelength

Discussion:

The fluorescence emission spectra of milk samples may belong to presence of a variety of amino acids in milk like tryptophan residue that has

excitation around 200nm and emission at 340nm[14]. That may give the contribution of the peaks that appeared at around 340nm when 225nm excitation wavelength was used .Also tryptophan has emission between 300-400 nm when 270nm excitation wavelength is used[15]. The reasons behind variations in fluorescence spectra profile at both of 225nm and 275nm excitation wavelength may be explained in terms that the fluorescence of tryptophan has been shown to be highly sensitive to its local environment, and spectral shift in its emission have been observed as a result of range of phenomena, such as binding of legends and protein –protein association [16]. These facts fit with our result since that the local environment of old milk is under changing due to degradation of milk components and proteins. On the other hand, protein-protein association may be occur due to presence of other tryptophan in bacterial cells. It was found that live bacteria own numerous intracellular biological molecules associated with energy yielding reactions .The fluorescent characters of these endogenous molecules at specific excitation and emission wavelengths make them very attractive props for biological detection and characterization. These endogenous fluorophores include amino acids like, tryptophan, tyrosine, phenylalanine, enzymes and coenzymes like FAD, flavin, NADH AND NADPH[3]. AT 275nm excitation wavelength second peak appears between 425-440nm wavelengths. This peak is not distinguished in control groups, but it is clear in both of old milk and milk with bacterial contamination. This peak is almost due to presence of NADH in bacteria not in milk. This coenzyme has emission wavelength at around 440nm[17,18,19].Probably using of lasers as an excitation source

at these used excitation wavelength which give more distinguished emission spectra , especially for tryptophan residues and NADPH due to the selective absorption of laser light by these fluorophores and the coherence of laser light .Also, easily transmitting of laser light through optical fiber ,make it possible and more practical to use laser induced fluorescence spectroscopy to examine the milk samples in situ.

Conclusion:

-This study demonstrated that using of fluorometry method at 275nm excitation wavelength has potential as a rapid analytical technique for detection the bacterial contamination of milk.

-Laser light at this wavelength that transmitted by optical fiber can be used for milk samples examination in situ and for more precise results.

-Excimer laser, dye laser and fourth harmonic generation Nd: YAG laser can be used for this purpose.

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تحديد التلوث البكتيري للحليب باستخدام الفلورة

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الخلاصة:

الهدف من هذا البحث هو لتحديد امكانية استخدام طرق التفلور كمؤشر لنوعية الحليب وتلوثه ببكتيريا الاشريشيا القولونية وامكانية تحديد الطول الموجي المناسب لاستخدامه في التفلور المحتث بالليزر. استخدمت في هذه الدراسة ثلاث مجاميع من الحليب ، حليب ميستر وحليب تمت اضافة البكتريا اليه بتراكيز مختلفة ونماذج حليب تم حفظها بالتلاجة لمدة ثلاثة الى خمسة ايام. تم استخدام ثلاثة عشر طول موجي كأطياف تحفيز للحصول على اطياف الانبعاث. اظهرت النتائج بأن اطياف الانبعاث عند الطول الموجي 275 نانومتر ممكن ان تعطي تمييز واضح بين المجاميع الثلاثة . اظهر تحليل البيانات بأن هذه الطريقة يمكن أن تعطي فعالية في التمييز بين عينات الحليب بدقة وسرعة وكلفة قليلة. كما يمكن استخدام الليزر المنقول عن طريق الليف البصري لفحص نماذج الحليب في الموقع بدقة عالية.