LWT - Food Science and Technology 55 (2014) 472–476

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Use of multivariate analysis for the improvement in prediction accuracy of bacterial aerobic plate count by flow cytometry

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ARTICLE INFO

Article history:
Received 16 May 2013
Received in revised form
14 September 2013
Accepted 28 September 2013

Keywords:
Chemometrics
Partial least squares regression
Escherichia coli
Green tea
Culture method

ABSTRACT

Flow cytometry (FCM) and aerobic plate count (APC) by the culture method were performed on green tea samples spiked with *Escherichia coli* type strain NCTC9001 (ATCC11775) solutions of different concentrations. In FCM, fluorescence signals from multiple stained bacteria and other fluorophores are detected using detector channels, and recorded as events with a voltage at each channel. FCM data were analyzed in two ways: conventional and multivariate analysis. In the former, the number of events with voltages larger than the defined threshold values was regarded as the predicted APC. In the latter, voltage histograms of all channels were obtained and merged horizontally to serve as explanatory variables. Then a partial least squares regression (PLSR) model was built to predict APC from the histogram data. The coefficient of determination (R^2) and the root mean square error (RMSE) between APC by the culture method and that predicted by conventional FCM were 0.916 and 1.08 cfu/ml^2. The APC values predicted by the PLSR model and those measured were in good agreement with R^2 of 0.982 and RMSE of 0.417 cfu/ml, which verified the potential of the proposed method for improving APC prediction accuracy by FCM.

1. Introduction

Flow cytometry (FCM) is a method of enumerating particles. In this method, particles are stained with fluorescent dyes and the fluorescence signals are counted one by one in a micro-flow-cell (Haynes, 1988). When applied to bacterial detection, FCM has the advantage of quick inspection over aerobic plate count (APC) by the culture method. Although the culture method needs 24–48 h to culture bacteria so that they could be observed by the naked eye, FCM requires no bacterial cultivation, and inspection can be performed within 1 h (Gunasekera, Attfield, & Veal, 2000; Takenaka et al., 2010). Because of its speed, many studies of FCM application to bacterial inspection of food, such as milk (Gunasekera et al., 2000), yogurt (Bunthof & Abee, 2002), and vegetables (Laplace-Builhé, Hahne, Hunger, Tirilly, & Drocourt, 1993), have been reported.

FCM has a common concept with APC because it depends on the direct count of individual bacterial cells. It is, however, subject to false-positive (FP) and false-negative (FN) results, since bacterial count is dependent on the intensity of fluorescence signals. That is, if the intensity of such signals from materials other than bacterial cells exceeds the detection threshold, the materials are counted as bacterial cells and the number of FPs increases. At the same time, an increase in the threshold would lead to an increase in the number of FNs. Therefore, in the presence of the source of FP, there is always a trade-off between the accuracy of bacterial count by FCM and FP.

Several techniques to eliminate FP and FN have been proposed. One is to remove the source of FP either physically or chemically. For example, in the case of milk, lipid globules can be removed by centrifugation and protein globules can be degraded by enzymatic treatment with proteinase (Gunasekera et al., 2000). These pretreatments decrease the level of interference of materials other than bacteria, thereby enhancing the bacterial signal. However, special equipment in addition to a flow cytometer and reagents are required, which increases system and inspection costs. Also, specific treatments should be carried out according to the nature of the material, which is not realistic when processed food composed of many materials is the inspection target.

Another technique is to stain bacterial cells with multiple fluorescent dyes and detect their fluorescence signals using multiple

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0023-6438/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.lwt.2013.09.030

detector channels. Even if there is an overlap between bacterial and nonbacterial signals in one channel, a clear separation of them is expected in other channels. This approach can increase the selectivity for bacteria without any additional equipment or chemical reagent other than the flow cytometer itself. However, since nonbacterial materials are not removed, there remains a risk of FP or FN, especially in the presence of fluorophores that emit a strong fluorescence, such as catechins (Nagaoka, Toyoshima, & Takeda, 2002), anthocyanins (Drabent, Pliszka, & Olszewska, 1999) and chlorophylls (Schoefs, 2002).

To overcome the above-mentioned FP/FN problem, the potential of multivariate analysis (MVA) for the analysis of FCM data has been investigated in this study. MVA has been extensively used in the field of spectroscopy such as mid-infrared (Al-Jowder, Defernez, Kemsley, & Wilson, 1999), near-infrared (Roggo et al., 2007), Raman (López-Díez, Bianchi, & Goodacre, 2003), and fluorescence spectroscopy (Sadecka & Tothova, 2007). MVA is a method of modeling the relationship between the sample spectrum and its attributes such as protein content (Delwiche, 1998), moisture (Wold & Isaksson, 1997), Brix (Tsuta, Sugiyama, & Sagara, 2002), and mycotoxin content (Fujita, Tsuta, Kokawa, & Sugiyama, 2010). In this method, the whole pattern of spectra, not a single intensity at a specific wavelength, is taken into account to predict sample attributes, which makes it an accurate and robust method. Among the many techniques for MVA, partial least squares regression (PLSR) has been the most used because of its high prediction power and interpretable model (Adams & Allen, 1998; Egesel & Kahriman, 2012; Wentzell & Vega Montoto, 2003; Zhang et al., 2012). The objective of this study was to reduce the number of FPs and FNs to improve the accuracy of bacterial count using FCM by applying PLSR to FCM data analysis.

2. Materials and methods

2.1. Escherichia coli solution for sample spiking

The experimental procedure in this study is shown in Fig. 1. The *E. coli* NCTC9001 strain (Easy QA Ball *E. coli* designation NCTC9001/ATCC11775, BTF Pty Ltd., Australia) was cultured in a liquid medium for 18 h at 35 °C. The liquid medium was then centrifuged for 360 s at 4.2×10^4 m/s^2 and the supernatant liquid was discarded. The sediment was diluted from 10^3- to 10^8-fold with sterile water to prepare a series of *E. coli* solutions.

2.2. Green tea samples

A bottle of green tea was purchased at a local supermarket in Ibaraki Prefecture, Japan. It was tenfold-diluted with sterile water to reduce the effect of autofluorescence on FCM by catechins in green tea. *E. coli* solutions were added to it to make green tea samples spiked with *E. coli* solutions of different concentrations. Nine replications were prepared for samples spiked with 10^4- and 10^5-fold *E. coli* solutions, and 10 replications were prepared for those spiked with other solutions and those without spiking. Sixty-eight samples were prepared for analysis by the culture method as well as by FCM.

2.3. APC by culture method

One milliliter of each sample was prepared for the pour plate method in a plate count agar (Standard Method Agar "Nissui", Nissui Pharmaceutical Co., Ltd., Japan) with incubation at 35 °C for 48 h to determine APC. APCs were then converted into a logarithm scale and used as reference data or objective variables in the following MVA.

2.4. Flow cytometer

A flow cytometer (Cassette Lab® One, Hitachi Power Solutions Co., Ltd., Ibaraki, Japan) was used to analyze the green tea samples. It was equipped with two laser diodes for excitation and three detector channels (Chs A, B and C), whose voltage changes proportionally to incoming fluorescence intensity. Blue and near-infrared dyes (SYTO®41 and LDS751, Life Technologies Corporation, USA) were used to stain both live and dead bacterial cells, while orange dye (SYTOX®ORANGE, Life Technologies Corporation,

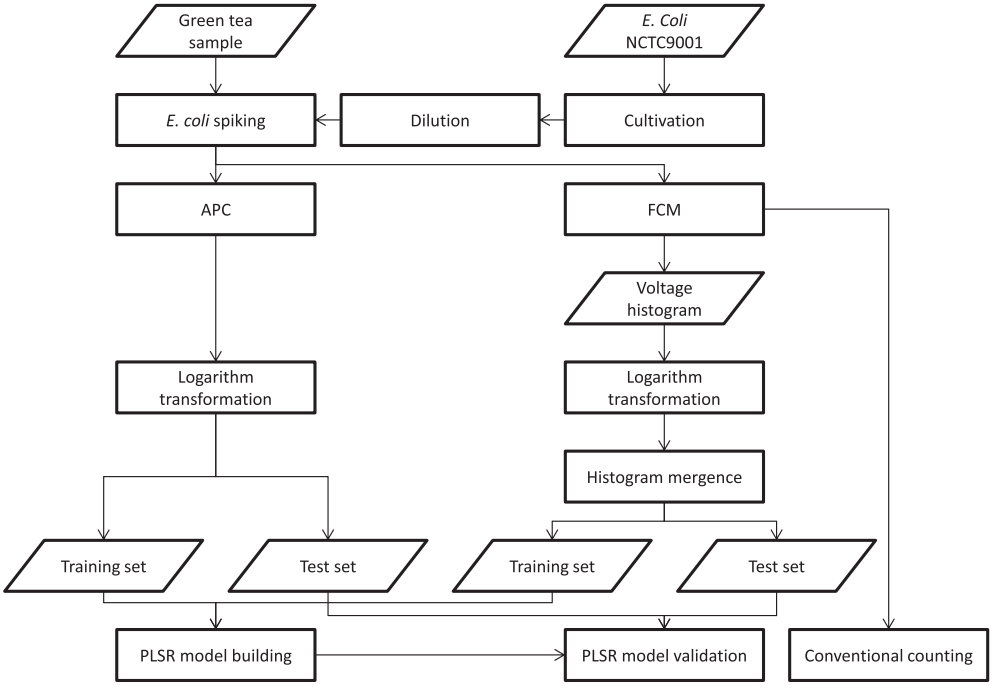

Fig. 1. Experimental procedure.