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Application of a new method to control microbial quality of foods based on the detection of oxygen consumption

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Abstract: New analytical techniques, the GreenLight™ system for rapid enumeration of total viable counts (TVC) were used to estimate the numbers of bacteria inoculated in different levels in broth nutrient media. The new detection methodology was compared with agar plating EN ISO 4833:2003 method showing excellent correlation. The following coefficients of determination R^2 = 0.985 and 0.999 were calculated for aerobic *Pseudomonas aeruginosa* and facultative anaerobic *E. coli*, respectively. After calibration, the system based on the principle of quenching of luminescence intensity and lifetime of an oxygen-sensitive dye by sample O_2 consumed during microbial growth enables to determine the number of microorganisms within less than 24 hours. The higher microbial load the shorter time for determination of viable count is needed. In case of simple food matrix for example, the results can be reached even within one shift of production.

Key words: total viable count, GreenLight™ system

Introduction

The primary responsibility for the setting out the necessary hygiene conditions for production foods, safe and suitable for consumption, has been always directed by the legislation and accepted by manufactures. The compromise approaches or deficiencies in the application of the principles of good manufacturing/hygiene practices have a direct impact on the incidence of microorganisms in the eatables. Therefore, there is a need to obtain the information about microbiological quality in time, as quickly as possible.

Numbers of viable microorganisms are crucial for the evaluation and development of microbiological food quality. An important parameter that determines the quality of food is a total viable count (TVC) (Grexa et al., 2014). According to EN ISO 4833-1:2003, the TVC can be obtained by the incubation of Petri dishes with inoculum at a temperature of 30 °C under aerobic conditions for a period of 72 hours. It is necessary to obtain the results of total viable counts in food matrices for the shortest time, especially, in controlling microbiological quality of raw materials, evaluating the microbial load for processes, monitoring the efficiency of decontamination of equipment and surfaces, which are in contact with foods, and in microbiological analyses of intermediate and completed products before expedition etc. (Grexa et al., 2014; Petruláková et al., 2015). The impetus for the development of many rapid, particularly indirect methods for the enumeration of microorganisms were time demands of classical cultivation methods, and the need to speed up the detection time. In this work, we have the opportunity to introduce the results and describe the experience with the GreenLight™ equipment (Mocon Inc., Minneapolis, MN, USA) based on the detection of metabolic activity of reproduction-competent cells present in a test sample.

The GreenLight™ device continuously detects oxygen consumption during incubation of inoculum of the test sample in liquid culture medium (suitable for determining of TVC). The output is a curve representing the progress of the oxygen consumption as a function of time (similar to growth curve). The exact time to reach a threshold (TT) is related to the initial population size in the sample (Petruláková et al., 2015). Principle of the determination is based on the assumption that the more microorganisms in food, the faster oxygen is consumed from the medium. Based on the mentioned fundamentals, it is obvious that the method can be applied for mesophilic aerobic and facultative anaerobic bacteria, which account for the major part of the microflora in many foods (www.rapidmicrobiology.com, 2016).

Materials and methods

Bacterial strains and food matrices

As a representative of aerobic bacteria, the culture of *Pseudomonas aeruginosa* CCM 3955 (ATCC 27853) was used. Facultative anaerobes were represented by

Escherichia coli BR, isolated from ewes' lump cheese (Faculty of Chemical and Food Technology, Slovak University of Technology in Bratislava, Slovakia). UHT milk (1.5 %) and raw milk were provided by Rajo, a.s. (Bratislava, Slovakia).

The GreenLight™ equipment

Method for the determination of the microbial density is based on the fluorescence detection of oxygen consumption by the population of microorganisms present in a food sample. The GreenLight™ system uses a unique oxygen sensor to determine microbial load. Oxygen is depleted as aerobic/facultative anaerobic microbes grow and respire (www.microbialdetection.com, 2016). This is a mechanisms called the fluorescence quenching, which is accompanied by shortening lifetime of excited state fluorophore molecules (optical sensor on the bottom of the vial) after contact with the quencher (oxygen present in the medium, in vial). Subsequently, the lifetime of the excited state is calculated by the Stern-Volmer equation and determining the amount of oxygen in the medium (Banerjee et al., 2016).

GreenLight 930 Measurement

To verify and detect the measurement range, the pure cultures of aforesaid bacterial strains with the initial concentration of 10^1—10^9 CFU · mL^{-1} were used. As a medium for the measurement of GreenLight™, the GTYE broth (Merck, Darmstadt, Germany) was used. In a volume of 10 mL, the appropriate ten-fold dilution of a sample was placed into special GreenLight APCheck™ vials with fluorescent agent and they were incubated at 30 °C in GreenLight™ equipment until a set threshold of oxygen consumption was reached. The required time to reach the threshold is inversely proportional to the initial number of microorganisms in a sample. The obtained times for each microbial strains were evaluated in relation to their numbers provided by a reference standard plate count cultivation method (EN ISO 4833-1).

Statistical evaluation

Statistical evaluations were carried out using Microsoft Excel 2007 linear regression package (Microsoft, Redmond, WA, USA).

Results and discussion

Responses of individual microbial populations were recorded by GreenLight™ as time to reach the threshold (*TT*) that was dependent on initial concentration (*log N*) of microorganisms in specific vial. The actual concentration of microorganisms in each vial was also determined by classical cultivation method according to EN ISO 4833-1. The time period necessary for reaching the *TT* is positively determined by the density *E. coli* and *P. aeruginosa* as it is shown in Fig. 1 and expressed also by equation 1—2. Naturally, higher microbial counts achieved faster times required to cross the pre-set limit — threshold.

$$TT_{PA} = -2.3153 \log N_{PA} + 17.558, (R^2 = 0.9849) \quad (1)$$

$$TT_{EC} = -1.3027 \log N_{EC} + 10.117, (R^2 = 0.9985) \quad (2)$$

For the comparison of microbial responses, the slope values of linear regression and coefficients of determination R^2 were taken into consideration. In view of differentiation of bacteria with various demands on the presence of oxygen, we were interested in the values of directives for the linear dependence between *TT* measured by GreenLight system and bacterial load as determined by agar plate counting. Assumption that different oxygen depending bacteria will have different slope of the calibration curve was confirmed (see Fig. 1). As was the microorganisms more aerobic, the more negative value of curve directive was obtained. In another word, the absolute value of curve directive was higher and the slope of the trend line was steeper. It can be also expected, that the dissimilarity between the values of curve directives in the case of anaerobic bacteria can be a result of lag phase duration of proliferating population. The high correlation between variables, the microbial load and the time to threshold, was also confirmed by the coefficient of determination R^2 that ranged from 0.98 to 0.99 in all cases. This means, that 98—99 % of the variability of *TT* was explained by a linear relationship with TVC and a high degree of tightness between dependent and independent variables (log CFU/mL) was achieved finally.

In further experiments, the suitability and accuracy of the GreenLight™ 930 system was verified for food commodity that contains wide spectrum of microbial groups. As an example, we inoculated UHT milk with raw milk to various initial levels. In this way, we expected the answer if the system could be applied in TVC determination in raw milk or in detection of UHT milk unsterility. Generally, UHT milk process control is based on detection TVC in the samples after certain incubation period. This approach is well established, but time demanding (a few days) and the necessary control measures have to be delayed. The equipment GreenLight™ has an internal incubator and thus its application could be achieved by shortening the incubation period before the actual detection of microbial contaminants in UHT products. In our case, the *TT* was positively

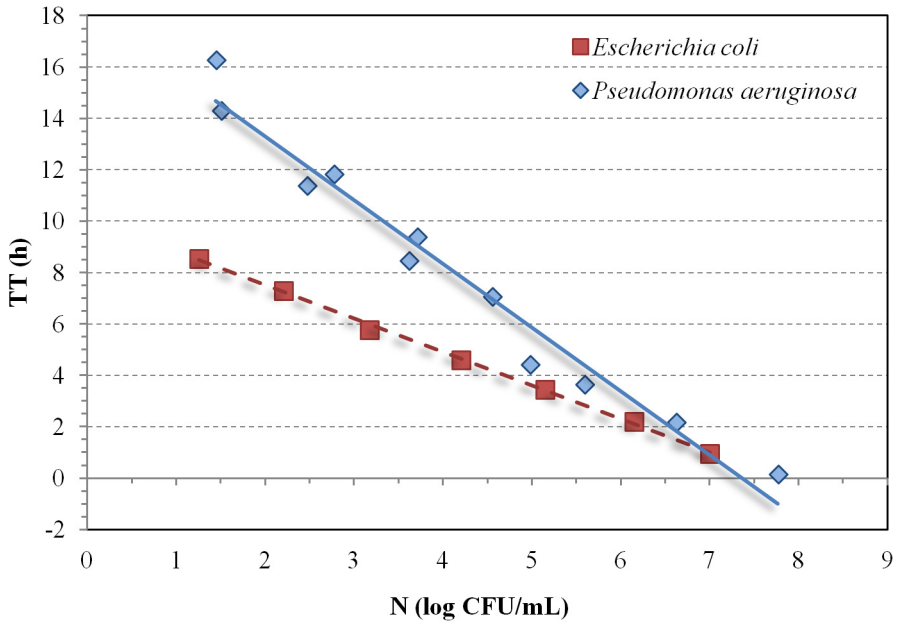

Fig. 1. The correlation between TT (h) measured by GreenLight™ 930 system and TVC in broth nutrient medium as determined by reference method (EN ISO 4833-1).

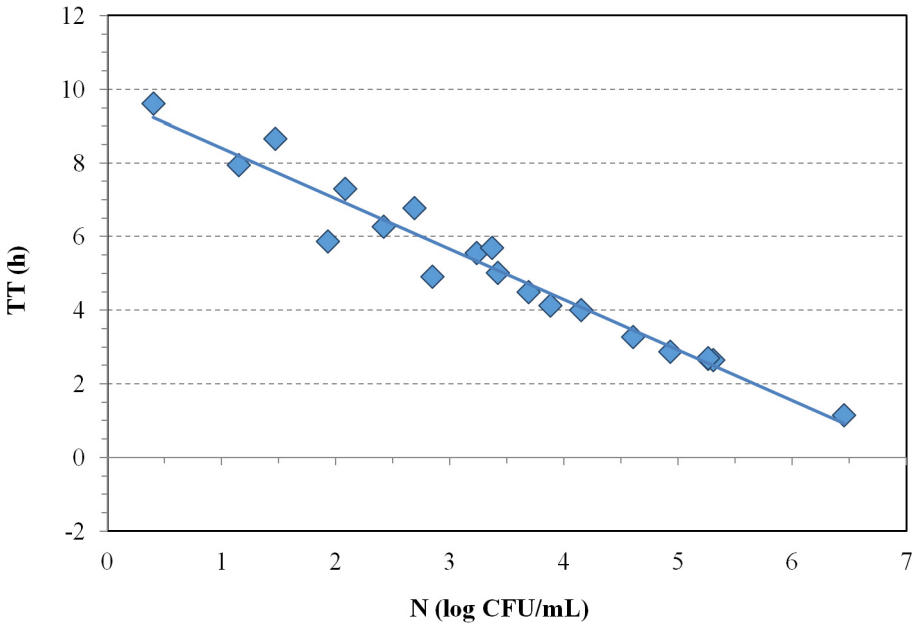

Fig. 2. The correlation between TT (h) measured by GreenLight™ 930 system and TVC in inoculated UHT milk as determined by reference method (EN ISO 4833-1).

determined by the microbial load, as a described by equation 4 (with lower $R^2 = 0.949$). Slightly less significant correlation was probably caused by the presence of microbial groups with various nutrition and oxygen demands during their growth.

$$TT = -1.3725 logN + 9.7768, (R^2 = 0.9493) \quad (3)$$

As it is seen from Fig. 2, the results were obtained within less than 10 h, even the TVCs were lower than hundreds of CFU · mL^{-1}. On the other hand, if TVC was higher than 10^6 CFU · mL^{-1} the *TT* was achieved in a few minutes. During applications of this method in foreign dairy/laboratories (n = 2), we observed also another positive fact that their results fully fitted with our linear relationship (3). With increasing numbers of data the relevant R^2 become higher and the relation statistically more significant.

Conclusions

Based on our results, we can conclude that in terms of time, the GreenLight™ system provided TVCs statistically highly correlated with standardized classical cultivation methods. If the sample contained a higher numbers of microorganisms, results were obtained within 2–6 hours, even after few minutes in the case of real matrices. In the food practice, the reduction of the time of microbiological analyses would prevent from unacceptable decision of managers due to the lack of information regarding microbial contamination of raw materials, intermediate or finished food products. In some cases, the calibration process may require specific approach of experienced microbiologists. Nevertheless, we can confirm that after the calibration, the system GreenLight™ is applicable in wide variety of food matrices.

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