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Review

Determination of microbial load for different beverages and foodstuff by assessment of intracellular ATP

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Traditional detection methods available for microbial analysis of food are time consuming and not sufficiently sensitive to meet food industries requirements as rapidity, on-site applicability, and cost-effectiveness. Among the more recent rapid methods for detection of microorganisms in food, adenosine triphosphate (ATP) bioluminescence is very suitable for on-line monitoring of bacterial contamination in food and beverages due to no need for a culturing step or large equipment to fulfill the measurement, rapidity and sensitivity. The availability of sensitive luminometers as well as many commercial ATP-bioluminescent kits allowed the development of various applications in industrial microbiology for rapid *in situ* determinations. This review summarizes the scientific literature available to date on the use of microbial ATP to determine the microbial load for different beverages and/or foodstuff.

Introduction

Food quality and food safety, from primary producer to final consumer, are ensured by the food industries through the hazard analysis and critical control point (HACCP) system, which focuses on prevention and control of every stage in the food chain (Buchanan, 1990; Notermans, Zwietering, & Mead, 1994; Ropkins & Beck, 2000). The HACCP system approach to the management of the microbial hazard suffers from slow and cumbersome conventional methods in microbiology which allow neither a rapid evaluation of raw materials nor real-time monitoring of the measures taken during processing or on the final product (Dostalek & Branyik, 2005). Faster microbiological methods are required to contribute to a better control of raw materials as well as finished products and to provide a better reactivity throughout the manufacturing process. Over recent years, there has been a concerted effort toward the development of rapid methods for the estimation of microbial counts in food, including, microscopy cell counting, automated immunoassays, quantitative real time PCR and biochemical based enumeration methodologies such as impedometric detection or bioluminescence (Jasson, Jacxsens, Luning, Rajkovic, & Uyttendaele, 2010; Johnson *et al.*, 2014). Of the emerging technologies for rapid microbiological analysis, bioluminescence is proposed to be the technique giving results in the shortest time (Hawronskyj & Holah, 1997). The distinct areas of bioluminescence that are of use in the food industry are: adenosine5'- triphosphate (ATP) bioluminescence and bacterial bioluminescence (Fung, 2002). ATP assay is a rapid and sensitive alternative to the traditional plate count techniques in routine microbiological analysis of food and beverages (Leach & Webster, 1986). Based on the firefly (*Photinus pyralis*) ATP luminescent reaction, it uses the chemical energy contained in the ATP molecule to drive the oxidative decarboxylation of luciferin (LH_2), with the resultant production of light (McElroy, 1947) as shown in the reaction (where P denotes the product oxyluciferin and *hv* denotes the light produced):

$$(LH_2) + ATP + O_2 \xrightarrow{\text{Luciferase},Mg^{2+}} P + AMP + PP_i + CO_2 + hv$$

The light emitted as a result of the above reaction is measured using a luminometer and expressed as relative light units (RLU). All living cells contain intracellular ATP needed for the regulation of the stored metabolic energy, for the maintenance of the enzyme systems, and for biosynthesis of cellular constituents during all phases of growth. Damaged or dead cells are not able to synthesize ATP which is broken down within a few minutes after

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death, thus intracellular ATP measurement is an optimal indicator of the number of viable cells (Venkateswaran, Hattori, La Duc, & Kern, 2003).

Bioluminescence reaction activates at very low levels of ATP. This made it possible to apply this reaction, with appropriate preparation protocols, to the evaluation of the presence of microorganisms in different sorts of samples and with very low or lacking degrees of contamination. ATP can thus be used as a measure of microbial biomass. Indeed, linear relationships have been found between intracellular ATP levels and total number of colony-forming units (CFU) with bacteria as well as with yeasts (Dostalek & Branyik, 2005). However no discrimination of species can be done. The concentration of ATP per cell varies between bacterial species, from cell to cell within the same species, and at different points in the bacterial life cycle (Vogel, Tank, & Goodyear, 2014). Quantitation of the steady-state ATP levels in a variety of microorganisms revealed that ATP ranges from 0.1 to 4.0 fg/CFU (average ca. 1 fg or 10^{-15} g/CFU) in bacteria and from 10 to 100 fg/CFU in yeasts (Cross, 1992; Girotti *et al*., 1997; Leach & Webster, 1986). Commercially available manual or automated luminometers can detect less than 0.1 pg (or 1×10^{-13} g) of ATP per cuvette, corresponding to approximately 100 bacterial cells.

Bioluminescent assay is broadly used to monitor air and surface cleanliness and product quality mainly in food industries and in less extent in pharmaceutical industries (Aycicek, Oguz, & Karci, 2006; Bautista, Vaillancourt, Clarke, Renwik, & Griffiths, 1995; Davidson, Griffith, Peters, & Fielding, 1999; Dostalek & Branyik, 2005; Girotti *et al*., 1997; Hawronskyj & Holah, 1997; Whitehead, Smith, & Verran, 2008).

Availability of sensitive luminometers as well as many commercial ATP-bioluminescent kits has allowed the development of various protocols and applications in industrial microbiology for rapid *in situ* determinations.

Extractions

To be able to specifically detect living organisms by ATP-bioluminescence, the first step is to extract ATP from cells. This step is critical and impacts directly the reliability of the detection (Selan, Berlutti, Passariello, Thaller, & Renzini, 1992). Various methods for ATP extraction have been developed, including continuous DC voltage (Lee & Cho, 2007; Wang, Bhunia, & Lu, 2006), microwave (Tsai, 1986), ultrasound (Law, Derrick, & Higson, 2003), organic solvent (Romanova, Brovko, & Ugarova, 1997), strong acid (Contin, Nobili, & Brookes, 1995; Ishida, Yoshikawa, Nakazawa, & Kamidate, 2002; Janaszek, Aleksandrowicz, & Sitkiewicz, 1987; Narsaiah *et al*., 2012), and surfactant (Dukhovich, Rodriguez, Gaona, Levashova, & Ugarova, 1995). For chemical extraction, reagents as trichloroacetic acid (TCA), Tris–EDTA buffer, cetyltrimethyl ammonium bromide (CTAB), sodium dodecyl sulfate (SDS), commercial bacterial releasing reagent (BRA) can be used at different concentration. Coupling CTAB as the proper ATP extractant and β -cyclodextrin (β-CD), as the relevant neutralizing reagent, has been shown to be the best combination to efficiently extract microbial ATP and easily consume the residual extractant without inhibit firefly luciferase (Luo *et al*., 2009). The concentration of the ATP extractant is also important as it can strongly affect the response of the used sensors.

As the ATP is also present in a variety of foodstuffs in the form of non-microbial ATP, a procedure to separate the somatic-cell ATP from the bacterial-cell ATP is necessary. A somatic-cell-releasing agent can be used to lyse all non-bacterial cells and release their ATP. After the removal of non bacterial ATP, bacterial-cell-releasing agent has to be added to lyse the bacterial cells and release their ATP. Extracellular ATP can be removed by enzymatic reaction between adenosine phosphate deaminase and apyrase resulting in ATP and relative adenosine derivatives conversion to inosine monophosphate (Hattori *et al*., 2003; Shinozaky *et al*., 2013).

ATP measurements disturbing factors

ATP tests are reported to be able to reveal the presence of both somatic cells (like food residues) and microbial contamination with no distinction (Caputo, Ferri, Girotti, Gozzi, & Saracino, 2011) generating bioluminescence background and hampering a reliable detection. Almost all food products contain non-microbial sources of "intrinsic" or "somatic" ATP which must be eliminated by sample pretreatment procedures, especially when the samples are rich in ATP-containing tissues, such as meat, fruit, etc. This limitation has been overcome with recently developed technology that allows the extrinsic ATP to be eliminated with enzymes and other methods. After this treatment, the microorganisms are chemically disrupted and the released microbial ATP is determined using purified luciferin-luciferase reagent. Fig. 1 shows an example of procedures to improve assay sensitivity in raw milk samples (Griffiths, 1993). Another possibility for avoiding somatic ATP interference is to physically separate microorganisms from somatic cells. This is particularly feasible for liquid samples as, besides removing extracelluar ATP, it increases the concentration of microbial cells during analysis, eliminates compounds that quench light emission and stops bioluminescence inhibitors being passed into tests, thus leading to an improvement of sensitivity (Thompson, 1999). However, problems can occur when filtering drinks with high levels of suspended particles such as high pulp content fruit juices. In these cases, sample pretreatments may be done with differential centrifugation allowing the selective removal of non microbial particles bigger than 50 μm. Samples of clear liquid can be directly filtered through 0.45 μm pore size filters, which retains microbial cells, while samples containing suspended particles such as orange juices, can be double filtered, with coarse filtration followed by fine filtration (Thompson, 1999). In