Prospects for Rapid Bioluminescent Detection Methods in the Food Industry – a Review

Pavel DOSTÁLEK and Tomáš BRÁNYIK

Department of Fermentation Chemistry and Bioengineering, Faculty of Food and Biochemical Technology, Institute of Chemical Technology Prague, Prague, Czech Republic

Abstract

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This review surveys rapid bioluminescent detection techniques applied in food industry and discusses the historical development of the rapid methods. These techniques are divided into two groups: methods based on bioluminescent adenosine triphosphate (ATP) assay, and on bacterial bioluminescence. The advantages and disadvantages of these methods are described. The article provides the bibliography of fluorescent method applications in food samples.

Keywords: bioluminescence; bacteria; ATP; food samples; food industry; food safety; microbiological techniques

Current trends in nutrition and food technology impose increasing demands on food microbiologists to ensure a safe food supply (Griffiths 1993). The last 25 years have been dedicated to the development of new methods for Quality Assurance, Total Quality Management and the Hazard Analysis and Critical Control Point (HACCP) systems (Ugarova *et al*. 1993). The HACCP system was devised as a procedure to ensure the safety and quality of food products (Jackson & Shinn 1979). It was developed by the U.S. Food and Drug Administration (FDA) for regulating and inspecting food plants (Bauman 1974). The basic premise is to identify possible hazards in advance and set up a system of procedures, inspections, and records to minimise the possibilities of the hazards causing unsafe or off-quality end products. The points at which hazards might be encountered and controlled in the course of preparation and packing of the product are designated as Critical Control Points (CCP). The general categories of hazards include: *Microorganisms* and their toxic products; *Chemicals* such as toxins, heavy metals and pesticide residues, and *Foreign Matter* (Curtis & Huskey 1974).

Although the most effective methods of monitoring CCP are often physical or chemical, many of the hazards associated with the food production are of microbiological nature. Microorganisms of concern to food manufacturers can be divided into two groups: (i) the pathogens and (ii) those that can be used as indicator organisms. It is clear that any direct test for microorganisms must be rapid enough to be compatible with HACCP and it is imperative, therefore, to quit the traditional use of media and plates (Kodikara *et al*. 1991). Of the

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emerging technologies for rapid microbiological analysis, bioluminescence is purported to be the technique giving results in the shortest time. The distinct areas of bioluminescence that are of use in the food industry are: ATP bioluminescence and bacterial bioluminescence (Fung 2002).

BIOLUMINESCENT ADENOSINE TRIPHOSPHATE (ATP) ASSAY

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. Storage at suboptimal conditions may reduce the intracellular ATP levels. In dead cells, ATP is broken down by autolysis within a few minutes. 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.

A very rapid and sensitive ATP assay, based on the firefly (*Photuris pyralis*) ATP luminescent reaction, was developed as an alternative to the traditional plate count techniques in routine microbiological analysis of food and beverages (Leach & Webster 1986).

Firefly luciferase catalyses the ATP-dependent oxidative decarboxylation of luciferin (LH_2) resulting in the production of light as shown in the reaction (where P denotes the product oxyluciferin and hν denotes the light produced):

$$(LH_2) + ATP + O_2 \xrightarrow{Mg^{2+}} P + AMP + PPi + CO_2 + h\nu$$

Three factors led to a greater utilisation of bioluminescent methods for analysis in the 1980s (Leach & Webster 1986), i.e. (1) the availability of commercially prepared reagents, (2) the commercial manufacture of suitable measuring instruments, and (3) the holding of conferences and the publishing of reports and monographs that highlight the advantages of bioluminescent techniques (Leach & Webster 1986).

Commercially available manual or automated luminometers can detect less than 0.1 pg (or 10^{-13} g) of ATP per cuvette, corresponding to approximately 100 bacterial cells. Quantitation of the steady-state ATP levels in a variety of microorganisms revealed 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 (Leach & Webster 1986; Girotti *et al*. 1997; Cross 1992).

Disturbing factors

Almost all food products contain nonmicrobial sources of "intrinsic" or "somatic" ATP which must be eliminated by sample pretreatment procedures. Somatic cells have to be lysed and incubated with the enzyme apyrase or somase (ATPase) to destroy the released ATP. After this treatment, the microorganisms are chemically disrupted and the released microbial ATP is determined using purified luciferin-luciferase reagent. Another possibility for avoiding somatic ATP interference is to physically separate microorganisms from somatic cells by differential filtration, combinations of exchange resins, centrifugation and filtration, or enzymatic destruction of somatic ATP followed by differential filtration procedures (Cross 1992).

Quenching of emitted light is another factor that can adversely affect microbial ATP determination. Certain compounds from the food samples can strongly reduce the amount of light measured photometrically. Internal standards must then be incorporated in the dilutions to be tested, as has been shown with skimmed milk. Some food samples may also contain inhibitory substances influencing the luciferase activity (Leach & Webster 1986).

APPLICATION OF THE ATP ASSAY IN THE FOOD INDUSTRY

Hygiene monitoring

Probably the most widely used current application of ATP bioluminesce in the food industry is that for the estimation of surface cleanliness. The total amount of ATP present on a surface can be extracted through swabbing and assayed extremly rapidly (i.e. within 5 min) with no less accuracy than that obtained using traditional techniques. The result indicates the overall contamination of the surface because ATP from all microbial sources will be detected. The amount of contamination determined by ATP and plate count methods correlated well in about 80% of samples (Poulis *et al*. 1993). A number of bioluminescent-based hygiene monitoring kits are commercially available (Lumac Hygiene Monitoring Kit, Lumac Water Microbial Kit) and are used routinely to monitor critical

points in many food processing operations worldwide (Anonym 1996).

Milk and milk products

ATP bioluminescent methods can be used for monitoring raw milk quality from the point of view of somatic cell count and microbial count. An indication of the somatic cell concentration in milk can be obtained from the concentration of ATP in milk following the treatment with non-ionic detergent (Webster *et al.* 1988). This can be used as an index for mastitis infection. Recent ATP bioluminescent procedures can be used to detect as few as 10^4 CFU/ml of bacteria in milk in 5 to 10 min. This makes the technique useful as a rejection test for the incoming milk tankers at milk processing plants (Griffiths & Phillips 1989; Moore *et al.* 2001). These tests are available commercially (Anonym 1996) and marketed by Biotrace (Bridgen, Wales) and Lumac (Laandgraaf, the Netherlands – Raw Milk Microbial Kit). ATP bioluminescent methods can also be used for pasteurised milk quality and sterility testing of UHT and other dairy products. Because ATP is an integral part of the metabolism of bacterial cells, the concentration of ATP may provide a better indication of the activity of lactic acid bacteria for monitoring starter culture activity then the measurement of pH changes. A strong correlation between the acid production and ATP concentration exists for a number of lactic acid bacteria, including *Lactococcus lactis* and *Lactobacillus acidophilus*, during their growth in milk. The monitoring of the change in ATP during the growth in milk rapidly indicated the presence of antibiotic residues or phage (Griffiths 1993). Concentrations as low as 0.005 U/ml of penicillin can be detected in about 90 min. An interesting application of luminescence is emerging that will have implications for the dairy industry in the future, namely the development of bioluminescent assays for detecting a variety of enzymes and other substances of importance in this industry (Griffiths 1993).

Meat and meat products

The estimation of the microbial load on meat by an ATP bioluminescence assay is also hampered by interfering somatic ATP. The ATP background levels in meat samples, for example, may be equivalent to 10^5 to 10^8 CFU/g (based on an ATP content of 1 fg/CFU). A successful application of the ATP assay, therefore, depends on a proper pretreatment procedure (Basol & Gogus 1996). In this case, the lower detection limit was 5×10^4 CFU/g, and good correlations between ATP content and colony counts were found. Good correlations also exist between ATP content and colony counts in vacuum-packed cooked cured meat products. Many cooked meat products appear to contain relatively low contents of somatic ATP. The removal of this somatic ATP is therefore not necessary, and as a consequence, the assay time can be reduced to less than 5 min. The detection limit is 10^5–10^6 CFU/g, which suffices for screening these vaccum-packed cooked cured meat products (Bautista *et al.* 1994, 1995; Siragusa & Cutter 1995; Russel 1995). A commercial kit also exists for checking the microbial quality of raw meat in 30 min – the Lumac Meat Microbial Kit (Anonym 1996).

Carbonated beverages and fruit juices

Many carbonated beverages are readily filterable and contain low or undetectable levels of somatic ATP. Simple filtration procedures are sufficient for detecting low levels of yeasts (5 CFU/ml) by ATP assay. Fruit juices, however, contain high levels of somatic ATP and many methods exist for the pretreatment. It has been concluded that the bioluminescent ATP assay offers opportunities for quality control of fruit juice: detection limits are 10^3 CFU/ml for yeasts and 10^5 CFU/ml for bacteria (Ugarova *et al.* 1993). A commercial kit is available from Lumac which checks fruit juice sterility. After a short preincubation period, the presence of microorganisms is tested using a bioluminescent technique. The Lumac Beer Microbial Kit is specifically designed for the rapid detection of microbial contamination or its absence in finished beers (Odebrecht *et al.* 2000). The results are obtained within 20 hours which is faster in comparison to conventional techniques (Anonym 1996).

BACTERIAL BIOLUMINESCENCE

Bioluminescent bacteria (those capable of emitting light) are classified into four major genera: *Vibrio, Photobacterium, Alteromonas*, and *Xenorhabdus*. The bioluminescent reaction, catalysed by the enzyme luciferase, involves the oxidation of a long-chain aldehyde and reduced riboflavin phosphate

($FMNH_2$) and results in the emission of blue green light (Baker *et al.* 1992).

$$FMNH_2 + O_2 + RCOH \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + \text{light (490 nm)}$$

The primary source of energy for the light is supplied by the conversion of the respective aldehyde to the corresponding fatty acid. Long-chain aldehydes are essential for the luminescence reaction and the aldehyde tetradecanal appears to be the natural substrate for the luminescence reaction. The reaction is highly specific for $FMNH_2$ which is formed by the reaction:

$$NAD(P)H + FMN \xrightarrow{\text{FMN oxidoreductase}} FMNH_2$$

Thus, the luciferase reaction may be driven by coupling it to any system that produces $FMNH_2$ (Girotti *et al.* 1990, 1993a; Coulet & Blum 1992; Girotti 1993b; Saul *et al.* 1996; Micková *et al.* 2004; Nivens *et al.* 2004).

Applications of *lux* gene technology in food microbiology

In a review of the potentials of *in vivo* bioluminescence in microbiology, Stewart (1990) states that there are three components necessary for novel developments in gene technology. The first one is the potential of a high level of the light output from individual cells. The second one is the rapidly expanding ability to transfer the biochemistry of the light production by cloning *lux* gene vectors into normally dark microorganisms. The third component involves the use of instruments, originally designed for *in vitro* ATP assays, which permit the detection of light from only a few hundred bacteria per ml. All of these are important in implementing bacterial bioluminescent assays into the realm of microbiological testing. Whereas a number of ATP luminescent techniques are presently employed in various aspects of food microbiology, the application of bacterial bioluminescence is still at the research or prototype levels. Many experiments have been and are currently made, to determine the feasibility and usefulness of the assays proposed. Some of the applications for the use in the food industry include the detection of specific bacterial pathogens and indicator microorganisms, on-line monitoring of hygiene quality, determining the effectiveness of spore destruction, monitoring starter culture integrity, biocide and virucide challenges, and studying the recovery of sublethally injured cells (Baker *et al.* 1992; Hill *et al.* 1993; Meighen 1993).

Detection of specific bacterial pathogens and indicator organisms

At present, in food laboratories the determination of ATP by firefly luciferase is used to detect and enumerate cells, and to assess the shelf life and microbial quality of many kinds of food. The ATP assay is a rapid technique but, for the time being it lacks specificity for the identification of bacteria. This specificity can be achieved with *lux* genes from luminescent bacteria. *Lux* genes can be introduced into bacteriophages, which will then be absorbed by specific bacteria thus transfering the light-emitting genes to those bacteria (Kodikara *et al.* 1991). By knowing what type of bacteria one wishes to detect, it is only the matter of obtaining the host specific phages for that particular organism and performing genetic manipulations. The organisms are mixed with the phages and the light emissions can be quantitatively measured. The research has shown that the bioluminescent assay is rapid (usually less than 1 h), sensitive (luminometers can detect as few as 500 bacteria), simple, specific, and demonstrates a good correlation between cell numbers and bioluminescence (Stewart 1990; Stewart & Williams 1992; Rees *et al.* 1995).

Dark terrestrial organisms that need to be monitored in food microbiology (pathogens, starter cultures, hygiene indicators) lack the ability to produce luciferase or fatty acid reductase, but they can supply $FMNH_2$. Therefore, all that is needed is the transfer of luciferase genes and, in some cases, fatty acid reductase. Phage P22 is a narrow host range phage infecting only *Salmonella typhimurium*. After infection with this phage, as few as 100 cells of *S. typhimurium* could be detected in 50 min by monitoring light emission. *Lux* containing bacteriophages should be able to target other pathogens, such as *Campylobacter* spp. and *Listeria monocytogenes.* There may still be a need for the recovery and enrichment procedures prior to phage detection, because of zero tolerance (regulatory requirements) for *L. monocytogenes.* However, due to the sensitivity (100 per ml) and specificity of the host and the phage, the enrichment time can be short providing simple same day testing for pathogenic bacteria.

Enteric bacteria are used in the food industry as indicators of poor sanitary conditions. *Lux* genes have been inserted into phages that infect a broad range of enteric bacteria providing a reagent for an on-line hygiene test with a detection limit of 1000 enterics per g or per cm^2. After a 4-h enrichment of the sample, viable enteric counts of 10 per g or per cm^2 can be distinguished from the background (Kodikara *et al.* 1991; Baker *et al.* 1992; Waddell & Poppe 2000).

Sporeforming organisms and bioluminescence

Bacterial luciferase can function in gram-positive organisms, although it originates from gram-negative ones. *Bacillus* spp. are capable of producing heat stable dormant endospores (Cook *et al.* 1993). The spores obtained from phenotypically bioluminescent vegetative cells are dark. When the spores germinate, the onset of electron transport and the initiation of metabolism are the early events. With the *lux* containing spores, germination is accompanied by the emergence of bioluminescence. This provides a sensitive, real-time monitoring of the germination and growth processes. The spores that have been killed or injured, and are thus unable to germinate, produce no light (Baker *et al.* 1992; Hill *et al.* 1993).

Lactic acid bacteria and starter cultures

Many assays based on luminescent bacteria are used in the dairy industry. The presence of bacteriophages or antibiotics can cause starter culture failure in cheese or yoghurt manufacture. Bioluminescent lactic-acid streptococci are suitable for use as an indicator of the presence of lytic phages or antibiotics. This light-emitting component of starter cultures can be monitored for a loss of luminescence that indicates the presence of an inhibitory substance. Using a luminescent derivative of *Lactobacillus casei,* the technique allowed the detection of penicillin G to levels as low as 0.03 μg/ml (0.05 units/ml) in 30 min, and bacteriophages at concentrations as low as 10^5/ml in 100 min (Ulitzur 1986a; Baker *et al.* 1992; Griffiths 1993).

Effectiveness of biocides and virucides

Biocides are used for the microbiological control in food manufacturing or as preservatives or disinfectants. Materials such as food waste, organic soil, dust and organisms can neutralise biocide activity without reducing the apparent chemical activity. For this reason, the monitoring of biocide levels is essential to provide an adequate protection. Current testing techniques (dipslides, dye reduction tests, microbial challenge tests) require more than 18 h and are thus not suitable for on-site evaluation. Bioluminescent microorganisms offer the potential for rapid biocide testing. The test takes only 10–15 min and provides a good correlation with the viable count evaluation. Since dead cells produce no light, the biocidal effect is measured as a decrease in the light emission of the luminescent culture. This method has been used to test the effectiveness of certain biocides on *L. monocytogenes* with good results after 15 min exposure. Assessing antiviral activity usually requires cell culture work or electron microscopy facilities. A rapid bioluminescent test has been developed by inserting *lux* A and *lux* B genes into λ phage. After the exposure to hypochlorite (virucidal agent), the lux^+ phages were unable to infect a nonluminescent *E. coli* (Ulitzur 1986b; Stewart *et al.* 1991; Thomulka *et al.* 1992; Fabricant *et al.* 1995).

Recovery of sublethally injured cells

Sublethal injury refers to the state where a microorganism is neither actively growing nor biochemically dead. Microorganisms in this state may arise in food after heating, chilling, freezing, moisture reduction, irradiation, or exposure to preservatives. Sublethally injured cells can retain their pathogenic traits, which makes enumeration of these cells during microbial analysis very important. They usually need a period for the intracellular repair. It is possible to monitor with sensitive luminometers the recovery of sublethally injured cells back to optimum bioluminescence levels without having to observe a single cell division. For example, using a bioluminescent recombinant strain of *S. typhimurium*, researchers have shown that 20% of the population can survive a freeze/thaw cycle with the biochemical system sufficiently intact to permit immediate and sustained bioluminescence. By classical plate techniques, only 2.5% of the original inoculum could be detected. This has important implications for the food industry as these results suggest that appreciable numbers of toxigenic cells, that are not considered viable by conventional enumeration techniques, may continue to produce

toxigenic compounds after processing (Ellison *et al*. 1994; Duffy *et al*. 1995).

ADVANTAGES AND DISADVANTAGES OF THE LUMINESCENT ATP ASSAY AND BACTERIAL LUMINESCENT SYSTEM IN FOOD MICROBIOLOGY

The ATP bioluminescent assay is very rapid but not very specific. The bacterial luminescent assays are rapid – fast enough for near on-line assays that require an immediate action, such as the detection of antimicrobials in milk. The speed of detection may also, in some cases, reduce the costs. The method is simple enough to use so that plant workers could perform the test. It is sensitive – very low numbers of cells can be detected with luminometers and the continued research may lower those numbers even further. In most cases it is advantageous that the *in vivo* bioluminescence assay is noninvasive. No cell disruption is required to measure the light emission. It has been shown to be an accurate method exhibiting good correlation with the cell counts – for both increasing and decreasing numbers.

When using narrow range phages to introduce the *lux* genes, assays can be specific to detect a particular organism. A final advantage is that the technology of bacterial bioluminescence leads to a great deal of innovations in many areas. As with any new technique, it also has disadvantages. There could be a problem with phage or plasmid host ranges being either too specific or too wide, resulting in false negatives or false positives, respectively. Another disadvantage to the food microbiology industry is that the bacterial bioluminescent assays are still at the experimental and prototype stages, in contrast to the ATP bioluminescent assay.

There are also general disadvantages with the luminescent methods. The principal disadvantage is the quenching of emitted light that can adversely affect the measurements. Namely certain compounds from the biological samples can strongly reduce the amount of the light measured photometrically. On the other hand, there are some luminescent non-microbial substances in the biological samples that can increase the intensity of the measured light.

CONCLUSION

A great deal of research in the last 30 years has been directed towards the development of the bioluminescent assays. The resurgence of interest in ATP (firefly) bioluminescence and the dramatic improvements in the reagent quality and instrument sensitivity has led to the availability of applications of direct relevance to many branches of the food industry, and many kits are now commercially available.

The many advantages of assays using bacterial luciferase should increase their use, especially in food microbiology. These assays will not eliminate the classical microbiological methods, but they will enhance the importance of microbial assays in areas such as the HACCP system where rapid results are necessary. The advancements in genetic engineering of microorganisms aimed at making them bioluminescent, coupled with a need for rapid microbiological assays in the food industry, will make *in vivo* bioluminescence a common practice in the future. Bioluminescence, together with low level light detection systems such as photon counting and CCD imaging, will provide the food microbiologist with a very powerful tool for studying food as an ecosystem, and will offer a unique opportunity to study microbial behaviour and interactions in food.

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Corresponding author

Ing. Pavel Dostálek, CSc., Vysoká škola chemicko-technologická, Fakulta potravinářské a biochemické technologie, Ústav kvasné chemie a bioinženýrství, Technická 5, 166 28 Praha 6, Česká republika
tel.: + 420 220 444 037, fax: + 420 220 445 051, e-mail: pavel.dostalek@vscht.cz