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ATP bioluminescence

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8.1 Introduction

The food industry is increasingly adopting food safety and quality management systems that are more proactive and preventative than those used in the past which have tended to rely on endproduct testing and visual inspection. The regulatory agencies in many countries are promoting one such management tool, Hazard Analysis Critical Control Point (HACCP), as a way to achieve a safer food supply and as a basis for harmonization of trading standards. For HACCP to be effective, rapid methods for monitoring and verifying performance are desirable. Monitoring usually relies on surveillance of physical or chemical parameters of a process such as time and temperature of heating or pH, whereas validation of HACCP performance requires testing for the absence of specific pathogens such as *Salmonella*, *Escherichia coli* O157:H7, *Campylobacter jejuni, Listeria monocytogenes* and others. There is some controversy as to whether microbiological tests can be used to monitor critical control points (CCPs) because of the length of time needed to generate results and the sampling strategy required to obtain meaningful results. However, considerable advantages may accrue if verification can be achieved quickly. Of all the rapid microbiological tests currently available, the only one that comes close to offering real-time results is ATP bioluminescence.

8.2 Principles of ATP bioluminescent assay

The ATP bioluminescence technique for cell detection was first described in the 1960s by NASA scientists who were interested in clinical applications as well as

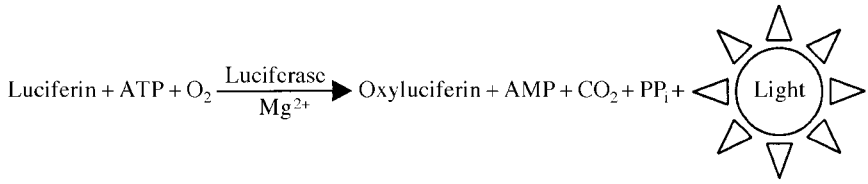

Fig. 8.1 Scheme of ATP-based bioluminescent reaction.

determining if life existed on other planets (Chappelle and Levin, 1968). It makes use of the fact that all living cells contain adenosine triphosphate (ATP), which is the universal energy donor for metabolic reactions. However, after cell death, the ATP content decreases sharply, allowing the intracellular ATP concentration to serve as a measure of biomass and cell viability. An enzyme-substrate complex, luciferase-luciferin, present in firefly tails converts the chemical energy associated with ATP into light by a stoichiometric reaction (Fig. 8.1). The amount of light emitted is proportional to the concentration of ATP present and can be quantified using light detecting devices. Because the quantum yield of this bioluminescent reaction is 0.88 (Seliger and McElroy, 1960), almost 1 photon is emitted per reaction cycle. As instruments are available that can detect even single photons, the theoretical sensitivity of the bioluminescent ATP assay is extremely high (1 amol). The level of ATP in a cell remains relatively constant; thus, the light produced during the luciferase-luciferin catalyzed reaction is directly related to the number of metabolically active cells present in the assay. Assuming that the ATP content of an average bacterial cell is about 1–10 amol, even single cells should be detectable using this method. Sharpe *et al*. (1970) were the first to apply the method to the detection of microorganisms in food but the high level of ATP from non-microbial sources reduced the sensitivity substantially. Although there continued to be an interest in the application of ATP bioluminescence for assessing microbial contamination of food, it was not until the early 1990s that the technique came of age in the food industry (Griffiths, 1993; 1995; Kyriakides and Patel, 1994).

In an attempt to improve the sensitivity of ATP bioluminescence, Squirrell and Murphy (1995) proposed the use of the enzyme adenylate kinase (AK) as a cell marker. AK is a key intracellular enzyme found in both prokaryotic and eukaryotic cells that equilibrates AMP, ADP and ATP by the reaction:

$$\mathrm{MgATP} + \mathrm{AMP} \Leftrightarrow \mathrm{MgADP} + \mathrm{ADP}$$

When excess ADP is used as the substrate, ATP will be produced in an amount proportional to the concentration of AK present. This ATP can be assayed using the luciferin-luciferase system. From the turnover number and the cellular ratio of AK to ATP it was predicted that, with 1 minute incubation, 40–50 times more ATP would be available for bioluminescent detection than in assays for ATP alone. This would correspond to a limit of detection between 20 and 25 bacterial cells. The bioluminescent AK assay of bacteria retained the simplicity of ATP

assay and had better sensitivity. However, in practice the instability of ADP and the presence of relatively high admixtures of ATP, even in highly purified ADP preparations, does not allow this theoretical detection limit to be reached.

8.3 Assay for testing the total bacterial count of food products

ATP bioluminescence assays have been developed to estimate the total microbial load in a number of products. As ATP in food can be present in somatic cells, as free ATP, or in microorganisms, microbial ATP must be separated from other sources before assay. The original ATP techniques relied on selective lysis of somatic cells with a non-ionic detergent, such as Triton X-100, followed by enzymatic or chemical destruction of the released ATP. Microbial ATP was subsequently extracted with a cationic detergent and assayed with luciferase-luciferin. The number of cells present was calculated from standard curves relating ATP concentration (or emitted light in Relative Light Units (RLU) to cell count (cfu/ml)). While convenient and rapid, this format suffered from inherent insensitivity (Griffiths, 1991). More recently researchers have utilized ways of concentrating microorganisms from foods before performing the ATP assay. These have consisted of filtration (ATP-F Test, Lumac BV, Landgraaf, The Netherlands; Milk Microbial ATP Kit, Biotrace Ltd., Bridgend, UK) or centrifugation (Enliten reagent, Promega, Madison, Wis.) followed by release of microbial ATP and quantification. The necessity of a two-step procedure for bioluminescent microbial detection was determined by certain instability of luciferase in lysis reagent. Recently a novel single-step bioluminescent reagent was proposed by Promega Corporation (Creswell *et al*., 2002). This has been made possible by using a mutant form of firefly luciferase that was developed through directed evolution to be robust to lytic agents. The stable luciferase was used in the reagent that combined the lytic agent with luciferin-luciferase making possible new simplified assay formats.

For filterable solutions, another approach was introduced by Millipore Corporation (MicroStar^{TM} system) that provides quantitative results in minutes for yeast, and a few hours for bacteria. The assay protocol involves filtering a sample through a specialized membrane that retains and distributes microorganisms evenly across the membrane. The system enables detection of single yeast cells immediately after filtration. Bacterial detection requires a brief incubation of the membrane on the surface of an appropriate nutrient medium. The incubation time required is typically a quarter to one third the time needed for regular plate counts. An ATP releasing agent is sprayed onto the filter and, after a short drying period, bioluminescent reagent is added that produces 'light spots' (photons) corresponding to each yeast cell or bacterial microcolony. Emitted photons are detected by a fast-scan charge-coupled device (CCD camera), and a membrane image with spatial distribution of the light spots is produced. Total photon detection time is two minutes. The system can detect as

few as 1 to 200 cfu per sample. The obtained results are directly comparable with standard plate counts.

8.4 The use of assays for particular foods

8.4.1 Raw milk

ATP bioluminescence assays for the determination of microbial load in raw milk within 10 min have been described (Bautista *et al.*, 1992; Griffiths, 1991; Reybroek and Schram, 1995; Sutherland *et al.*, 1994; Frudzhyan *et al.*, 1999; Brovko *et al.* 1999; Samkutty *et al.*, 2001). The milk is incubated in the presence of a somatic cell-lysing agent and then filtered through a bacteria-retaining membrane. The microorganisms retained on the filter are then lysed and the lysate assayed for ATP activity. Microbial populations as low as 10^4 cfu/ml can be detected and the methods have a greater precision than the plate count. The technique is useful for screening incoming tanker milk at processing sites and monitoring quality during prolonged storage. Although the detection limit achieved by these modifications may be sufficient for practical use, the accuracy of analysis was not significantly improved. The reported values for accuracy estimates for cfu/ml in raw milk (S_{xy}) by the bioluminescent methods were in the range of 0.27–0.87 log units. The main reason for this inaccuracy was the error in estimation of cfu/ml introduced due to bacterial clumping which was dependent on both the level of bacterial contamination and composition of the milk itself. To improve correlation between ATP and cfu, it may be more appropriate to obtain separate correlation curves for milk samples from different sources with relatively stable bacterial populations (Brovko *et al.*, 1999). The accuracy of the ATP bioluminescence assay of bacterial quality of raw milk can also be improved by the introduction of a preliminary incubation of the raw milk sample at 15.6ºC for 18 h (Samkutty *et al.*, 2001). This approach assumes that low-temperature incubation of milk allows the growth of only the psychrotrophic microflora and the normal flora of the udder will not grow. Coefficient of linear regression of ATP bioluminescent assay versus standard plate counts improved significantly after incubation from 0.58 to 0.80.

A 'concentrating' reagent, Enliten, has been described that clarifies milk and allows the removal of microorganisms by centrifugation. Combination of this treatment with an ATP assay enabled detection of microbial levels down to 2×10^4 cfu/ml to be detected within 6–7 min (Pahuski *et al.*, 1991). However, this method has not found wide application in comparison with the filtration techniques mentioned above.

Incorporating a pre-incubation step before the ATP assay provides a useful 25 h test for the keeping quality of pasteurized milk (Griffiths, 1993). This principle was used in the Rapid Pasteurized Milk Screen and Shelf Life Determination test from Celsis Inc. (Evanston IL, USA). ATP bioluminescence can also be used for sterility testing of UHT products, saving two to five days over conventional methods (Griffiths, 1993). A sensitive and rapid (1.5 h) test

for antibiotics in milk has also been described that relies on the detection of the growth of *Streptococcus thermophilus* in milk by ATP bioluminescence (Hawronskyj *et al*., 1993).

8.4.2 Drinking water, brewing, beverage, fruit and fruit juice

ATP bioluminescence provides a very fast alternative (< 5 min) for detection of bacteria in drinking water in comparison with the conventional plate count technique that takes up to seven days (Deininger and Lee, 2001). Spoilage of beer is mainly brought about by yeasts and *Lactobacillus* spp. Yeasts contain about 100 times more ATP than bacterial cells and, thus, can be detected at lower numbers. However, beer contains high levels of non-microbial ATP as well as components that quench the light reaction, so a filtration step is necessary before the luciferase-luciferin reaction can be applied (Kyriakides and Patel, 1994). Simpson *et al*. (1989) described such a method for the sensitive detection of microorganisms in filtered, unpasteurized beer that could be performed in 60 min. They suggested that the method could be carried out on continuous in-line filters providing a rapid indication of process efficiency. Takashi *et al*. (2000) applied MicroStar^{TM}-RMDS (Millipore Corp.) for detecting beer-spoilage bacteria (*Lactobacillus brevis*). Judging from the detection time and detection limit, 16–24 h of cultivation for the RMD System corresponded to 40–96 h of cultivation for the standard plate count method. Unlike beer, carbonated beverages generally contain low levels of non-microbial ATP. Since they can be easily filtered and spoilage is mainly due to yeasts, ATP bioluminescence assays are a useful indication of product quality (Kyriakides and Patel, 1994).

A filtration system using vortex mixing filtration technology, the Haemocell Rapid Microbial Quality Assurance (RMQA) system (GEM Biomedical, Inc., Hamden, Conn.) achieves rapid and gentle filtration by creating a turbulent flow across a dimpled membrane. The final sample can be retrieved into a disposable syringe by back washing the filter with ATP-free water. When combined with an ATP bioluminescence assay, the filtration system allowed low-level microbial contamination of brewing products to be detected in 60 min (Davies *et al*., 1995). The RMQA system in conjunction with ATP bioluminescence has also been applied successfully to carbonated beverages (Heying, 1995).

Biotrace Ltd. produces a reagent kit (Bev-Trace^{TM}) designed to detect rapidly the presence or absence of spoilage contaminants in all filterable beverages that are pasteurized or sterile filtered. According to the developed protocol, the required volume of beverage is first filtered through a 47 mm sterile filter with 0.2–1.2 μm pore size followed by a rinsing step. To concentrate and increase microbial contamination, an initial filtration step is followed by an enrichment phase on a suitable growth medium. After enrichment, the membrane is treated with ATPase for 60 min to remove non-microbial ATP. Extraction and detection of microbial ATP with luciferin-luciferase reagent takes another two minutes. If the light output registered from the test sample is three times higher than for the control (sterile) sample, it indicates the presence of microorganisms.

An ATP bioluminescence assay was used for estimating total plate counts of surface microflora of whole cantaloupe and determining efficacy of washing (Ukuku *et al*., 2001). A high correlation ($r^2 = 0.995$) was observed between bioluminescence and aerobic plate count of unwashed and washed (with water) cantaloupe. A reliable minimum detection level obtained with the bioluminescent method was 10^4 cfu/cm^2, but that was not sufficient for evaluation of cantaloupe washed with hypochlorite or hydrogen peroxide.

Attempts have been made to apply ATP bioluminescence to fruit juices, with varying results. Problems are encountered due to high non-microbial ATP levels associated with pulp cells, and the low pH of the products. When reconstituted orange juice concentrate was analyzed immediately, the results were poor, but they improved after storage of the reconstituted product at 25ºC for 24 h (Kyriakides and Patel, 1994). A rapid method for the microbiological assessment of fruit juices and fruit concentrates marketed by Celsis-Lumac involves 48 h pre-incubation of product at 28–30ºC, followed by treatment with a nucleotide-releasing solution (F-NRS) to lyse somatic cells and with a hydrolytic enzyme (Somase) that destroys ATP released from these somatic cells prior to assay of microbial ATP with luciferase-luciferin.

8.4.3 Poultry

Bautista *et al*. (1995b) have described a method for the enumeration of surface contamination of poultry carcasses by ATP bioluminescence. Chicken carcass rinse water was treated with a somatic cell-lysing agent containing lipase. The treated solution was then filtered, first through a coarse filter and then through a bacteria-retaining filter. The cells trapped on the filter were lysed and assayed for ATP. Good correlations were obtained with plate count and levels of 5×10^4 cfu/ml of rinse water (corresponding to about 2×10^3 cfu/g carcass weight) could be detected in less than 15 min.

A simplified version of the poultry carcass ATP test was used to monitor CCPs identified at a poultry processing plant. The test, which involved swabbing and then extracting and assaying the ATP on the swab, took about two minutes to complete. ATP levels were elevated on carcasses directly after evisceration but dropped to low levels after the pre-chill and chill tanks. This work suggests that this simple test may provide a way for microbiological monitoring of CCPs in poultry processing operations (Bautista *et al*., 1996).

A rapid ATP bioluminescence test to monitor the microbial load in poultry processing waters within 15 minutes has also been described (Bautista *et al*., 1994). This provides a near real-time indication of water quality and allows water use in the plant to be minimized.

8.4.4 Meat

Siragusa and co-workers at the US Department of Agriculture's Meat Animal Research Laboratories in Nebraska have developed ATP bioluminescence

assays for beef and pork carcasses (Siragusa and Cutter, 1995; Siragusa *et al*., 1995). A 500 cm^2 area of beef carcass or a 50 cm^2 area of pork carcass were swabbed using an ATP-free, sterile, microbiological sampling sponge moistened with saline and Tween 80. After swabbing, the liquid was removed from the sponge and the somatic cell ATP was extracted and removed using a special filtration device, the Filtravette (New Horizons Diagnostics, Columbia, Md.). The bacteria were retained in the device, which could then be placed in the luminometer (New Horizons Diagnostics), where the microbial ATP was extracted and assayed. The assay could be performed in about 5 min and the lower limits for detection were 1×10^2 cfu/cm^2 and 1.6×10^3 cfu/cm^2 for beef and pork carcasses, respectively. An ATP bioluminescence method for assessing the microbiological quality of beef carcasses sampled by excising a 5 cm × 5 cm area of the surface has been described. The excised sample was rinsed and processed in somatic cell extractant containing lipase. The bacteria were removed by filtration and their ATP content determined. Microbial numbers down to 4×10^4 cfu/cm^2 on the beef carcass were detectable in 15 min (Bautista *et al*., 1995a). The sensitivity could be improved dramatically by sampling a larger area. If the same area sampled in the USDA study had been used in the latter study, the sensitivity would be about 2×10^3 cfu/cm^2. A similar approach was used by Celsis-Lumac (Netherlands) for screening the total microbial contamination level of beef and pork carcasses. The developed method allows testing of total bacterial contamination of beef and pork carcasses within 10 min with a sensitivity of 100 cfu/cm^2 and 500 cfu/cm^2, respectively (Zwartkruis *et al*., 1999). The reported correlation of the method with the plate count technique was 0.95 and 0.93 for beef and pork, respectively.

A method for assessing the bacterial contamination of minced meat was developed by Frudzhyan *et al*. (2002). Due to the high level of non-bacterial ATP in raw minced meat that corresponds to 10^7–10^8 bacterial cells per g of meat, a novel method for its elimination was proposed. Treatment with pancreatin – Neonol mixture at 45ºC for 60 min followed by filtration through a Filtravette™ (New Horizons Diagnostics, Columbia, Md.) was used to destroy somatic ATP from the sample and allowed detection of 10^3–10^5 cfu/g of minced meat. The correlation with standard plate count was 0.99 and the analysis time was 2 h in comparison to 48–72 h required for the traditional method.

8.5 The use of assays for hygiene monitoring

ATP bioluminescent techniques are increasingly used for measuring the efficiency with which surfaces and utensils are cleaned. A survey of 500 food manufacturing businesses in the UK revealed that 48 per cent of respondents used swabbing followed by bacterial culture, while 27 per cent used ATP bioluminescence (Davidson *et al*., 1999). To adequately sanitize a food contact surface, it must first be washed to remove any food residues that could act as a nutrient source for subsequent growth of microorganisms. Following washing,

the surface is sanitized to kill the residual microflora. If either process is not done properly, food particles and/or microorganisms could remain on the surface and may constitute a risk to the quality and safety of foods to be processed afterward. Normal swabbing and plate counting procedures would detect only microbial contamination of the surface and may not tell you whether the surface has been cleaned properly. However, ATP bioluminescence detects contamination from both sources within two minutes and is a more reliable indicator of the overall hygienic condition of the area tested (Hawronskyj and Holah, 1997). Several studies have compared the results obtained by standard microbiological techniques and ATP bioluminescence for assessing surface cleanliness. Some reported a good correlation between these methods (Seeger and Griffiths, 1994; Kyriakides *et al*., 1991; Bautista *et al*., 1992). Others have obtained a poor correlation (Griffith *et al*., 1997; Poulis *et al*., 1993; Carrick *et al*., 2001). Such discrepancy in findings could be explained either by the different nature of the surface and surface contamination (presence of spores, for example) or inability/inconsistency of swabs to pick up microorganisms effectively. Loss in bacterial viability during drying could also have an impact on both ATP bioluminescence and plate count results. In addition, the presence of detergents, sanitizers or other chemicals may interfere with bioluminescent reaction (Velazquez and Feirtag, 1997) leading to false positive or false negative results. Despite these difficulties ATP bioluminescence has been used successfully as an initial step in hygiene monitoring, especially within HACCP plans. Most of the ATP-based reagent kits produced for rapid surface cleanliness (Enliten® Total ATP rapid bio-contamination detection kit, Promega, WI; Clean-Trace™ Rapid Cleanliness kit, Biotrace, UK) tests rely on the use of a baseline cut-off value that should be determined for every tested environment. Protocols have been proposed for evaluation of ATP-based hygiene monitoring systems and they were tested for three different single-shot systems manufactured by Biotrace Ltd (UNILITE Xcel), Charm Sciences (Luminometer K, PocketSwab) and IDEXX (Light-NING) (Colquhoun *et al*., 1998). It was anticipated that a described test protocol could be the first tier of a more extensive assessment programme which could include, in addition to sensitivity and accuracy, also direct surface swabbing comparison and the effect of sanitizers on system responses.

Although the existing ATP bioluminescent assays are sufficient for the needs of 90 per cent of the food industry, in certain situations there is a demand to detect low levels of bacteria that may still be present. For these cases, an ATP recycling system that uses a cocktail of enzyme to amplify low ATP levels was developed (Hawronskyj *et al.*, 1994). The amplification reagent consists of a mixture of firefly luciferase, myokinase and pyruvate kinase together with their substrates (luciferin, AMP and phosphoenolpyruvate), which effectively 'amplifies' all AMP to ATP (Fig. 8.2). The time for the reaction to reach half of the maximum light output is directly related to the log of ATP and can be used as an indicator of cleanliness.

A combined index of ATP, AMP and RNA was proposed by Sakakibara *et al.* (1999) for hygiene monitoring. Simultaneous bioluminescent detection of all

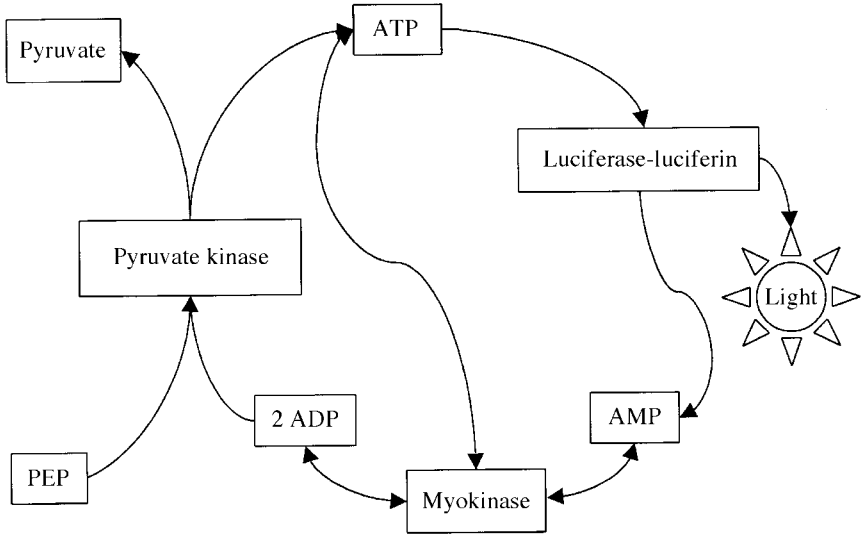

Fig. 8.2 Reaction scheme for ATP amplification.

these metabolites was achieved by coupling the reaction catalyzed by pyruvate orthophosphate dikinase (PPDK) with firefly luciferase (Fig. 8.3). The sensitivity of the detection of food residues on surfaces was several-hundred-fold better than with usual methods using ATP as an index.

Adenylate kinase (AK) amplification of ATP bioluminescence was also used for hygiene monitoring in the food industry (Corbitt *et al.*, 2000). It was shown

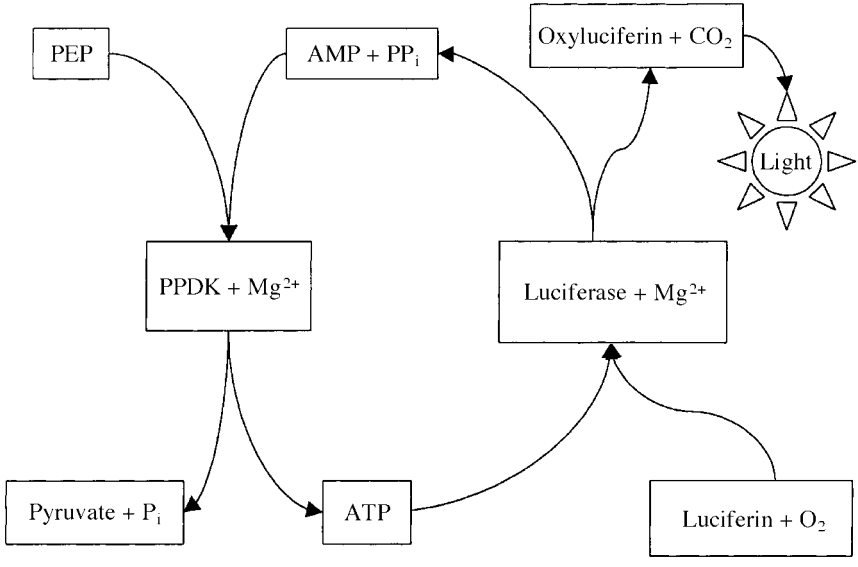

Fig. 8.3 Reaction scheme for bioluminescence cycling assay for ATP and AMP.

that AK could be used not only as a bacterial cell marker as proposed by Squirrell and Murphy (1995), but as a marker of food residues as well. The technique is particularly applicable to the meat and vegetable industry and to certain dairy products (milk, yogurt, cottage cheese). Further research is required to implement this method in a fruit processing environment.

8.6 The use of assays to detect particular pathogens

Despite the fact that ATP bioluminescence has been used for bacterial detection for a long time, it is only recently that this technique has been adapted for detection of specific pathogens. The developed protocols include, as a first step, the recognition of the target bacteria followed by its lysis and ATP assay. Recognition of the bacteria can be achieved by specific antibodies or by specific bacteriophages.

8.6.1 Antibody-based bioluminescent methods for detection of bacterial pathogens

Most of the traditional methods for pathogen detection that are based on the recognition of specific bacterial antigens by antibodies are able to detect the presence of target organisms in complex mixtures. However, on average, these systems detect from 10^4 to 10^6 cells per ml (Niroomand and Lord, 1994; Huang and Chang, 1996). Even the introduction of firefly luciferase, one of the most sensitive labels, as a label for the secondary antibody did not improve the sensitivity of Bioluminescent Enzyme ImmunoAssay (BLEIA) significantly (Fukuda *et al*., 2000). As a result, in order to detect 50 cfu/ml of, for example, *Staph. aureus* a 5 h enrichment step is necessary. The introduction of a step involving specific concentration of bacteria using a biosorbent substantially decreases the analysis time (Blais *et al*., 1997). One such method, immunomagnetic separation (IMS) is based on the use of magnetic beads coated with a target cell-specific antibody for capturing the cells. Methods incorporating IMS have gained widespread acceptance for the detection of foodborne pathogens in recent years. In addition to concentrating target bacteria in a small volume and, thus, avoiding a long enrichment step, biosorbents also permit removal of all food components from the sample which otherwise could interfere with analysis. Coupling of IMS with ATP bioluminescence resulted in the development of methods for enumeration of *E. coli* O157:H7 (Tu *et al*., 2000; Aytac *et al*., 2001). It was shown that addition of glucose to the solution increased the ATP content of free as well as captured cells (Tu *et al*., 1999), providing a better assay sensitivity. According to the developed experimental protocol, beef hamburger patties spiked with different levels of *E. coli* O157:H7 were incubated for 6 h in specific media at 37ºC. After incubation *E. coli* cells were captured by IMS, washed and resuspended in glucose-containing buffers. Since the meat samples might contain extracellular ATP from damaged beef

muscles and other bacteria, luciferin-luciferase mixture was added to the sample before the extractant solution in order to consume non-bacterial ATP in the bioluminescent reaction. After that bacterial extractant was added, only the intracellular ATP associated with bacterial cells was detected. The presence of less than 1 cfu of *E. coli* O157:H7 per ml of beef hamburger suspension could be detected after 6 h enrichment. Commercial magnetic beads coated with antibodies against *Salmonella* spp., *E. coli* O157:H7 and *Listeria* spp. are available from Dynal Inc. (Oslo, Norway); however no method has been published describing coupling of IMS with ATP bioluminescence for detection of *Salmonella* and *Listeria*. The sensitivity and specificity of bioluminescent IMS assays for particular pathogens depend to a great extent on the specificity and affinity of the antibody towards the live bacterial cell. The efficiency of bacterial capture is a function of antibody properties as well as environmental conditions, such as pH, media composition, osmomolarity and water activity. Screening of available antibodies and optimal conditions for cell capturing is an essential part of IMS method development. Either ATP bioluminescence or *in vivo* bioluminescence can be used as a tool for assessing the efficiency of IMS (Sun *et al*., 2002). The sensitivity of detection of captured cells by ATP bioluminescence was improved by one log unit when this approach was used to optimize IMS capture of *E. coli* O157:H7.

8.6.2 Phage-based bioluminescent assays

Phage-based biosorbents are an attractive alternative to immunosorbents due to their greater specificity and faster binding. Bacteriophages are also cheaper to mass-produce than antibodies. A phage-based biosorbent consisting of a *Salmonella*-specific phage passively immobilized on a polysterene membrane has been used to specifically separate *Salmonella* from a food matrix (Bennet *et al*., 1997). However, the efficiency of cell capture was poor. Biotinylation of phage head protein and construction of biosorbents by coating magnetic beads with phage via the biotin-streptavidin interaction significantly increased capturing efficiency (Sun *et al*., 2001). Another advantage of using bacteriophage as a biosorbent for bacterial pathogens is that it allows combination of both concentration and detection steps. The target bacterial cells can be captured by phage-based biosorbent and the resulting cell lysis can then be detected by ATP bioluminescence (Sanders, 1994a, b), providing a way to enumerate captured bacteria. Using the AK bioluminescent assay instead of ATP in phage-based pathogen detection assays was proposed by Blasco *et al*. (1998). It was expected that sensitivity would be improved significantly due to the higher sensitivity of the AK-based bacterial enumeration assay (see Section 8.2). However, this approach allowed only about 10^4 cfu/ml of *E. coli* and *Salmonella* to be detected within 1 and 2 h, respectively. Evidently phage-mediated AK release was not optimized and did not reach a maximum level. It has been shown that the multiplicity of infection (MOI) influenced the amount of AK released by lysis and this consequently affected the sensitivity of the whole assay (Wu *et al*., 2001). At

optimal MOI (10–100), the amount of phage-released AK reached almost 100 per cent and allowed specific detection of *E. coli* and *Salmonella* in mixed culture with a sensitivity of approximately 10^3 cfu/ml for both cultures, corresponding to detection of 25 cells per 25 μl sample within 2 h.

Combination of IMS and phage-mediated AK release was used by Squirrell *et al*. (2002) for rapid and specific detection of bacteria. The obtained sensitivity using bioluminescent AK assay as a detection method was around 10^2 cells per ml for *E. coli* O157:H7. Depending on the level of specificity, the assay took from five minutes to just under an hour.

Another approach describing how bioluminescence can be used for detection of specific bacteria was first suggested by Ulitzur and Kuhn (1987, 1989). They put the genes encoding a bacterial luciferase (*lux* genes) into phages specific for *E. coli.* The phages are not able to express the genes, so they remain dark. However, when the phage infects the host cell, the luciferase is synthesized and the *E. coli* cells light up and can be detected with a luminometer. This is represented schematically in Fig. 8.4. Bioluminescent reporter bacteriophages were constructed for detection of *Enterobactereaceae* (Kodicara *et al*., 1991), *Listeria monocytogenes* (Loessner *et al*., 1996, 1997), *Salmonella* (Stewart and Williams, 1992; Turpin *et al*., 1993; Chen and Griffiths 1996), and *Mycobacterium tuberculosis* (Riska *et al*., 1997). Bioluminescent phage-based methods for enumeration of pathogens were tested for detection of *Salmonella* in eggs (Chen and Griffiths, 1996) and environmental samples such as water, soil and sewage sludge (Turpin *et al*., 1993). Pre-incubation for a short period of time (6 h) resulted in a reliable detection of as low as 10 cfu of *Salmonella* per ml of original sample. The system allowed *Salmonella* cells to be detected in artificially contaminated whole eggs. After incubation for 24 h, eggs inoculated with 10^2–10^3 cfu per egg became luminescent (Fig. 8.5) (Chen and Griffiths, 1996). A phage-based bioluminescent test for *Listeria* identified viable pathogen cells in ricotta cheese, chocolate pudding and cabbage at a level of less then one cell per g of food after a 20 h pre-incubation (Loessner *et al*., 1996). In foods having a large and complex microbial background flora, such as minced meat and soft cheese, at least ten *Listeria* cells per g of product were necessary to produce a positive bioluminescent signal. An additional advantage of recombinant phage technology for pathogen detection is that it can be performed under field conditions using a simple photographic film-based device for light detection (Riska *et al*., 1999).

In the earlier publications mentioned above, insertion of *lux* genes into the phage genome was performed by transposition using a transposon with a selectable marker such as antibiotic resistance. This method is rapid but rather hit or miss. If the phage lacks non-essential genes or packaging limits of the phage head are very strict, then the addition of several kilobases may lead to the loss of its viability. In this situation it is preferable to gain some knowledge of the molecular structure of the phage to allow non-essential sites to be identified for cloning the reporter genes into the phage DNA. Kuhn *et al*. (2002a) cloned and sequenced part of genome of Felix 01 phage that is considered the best

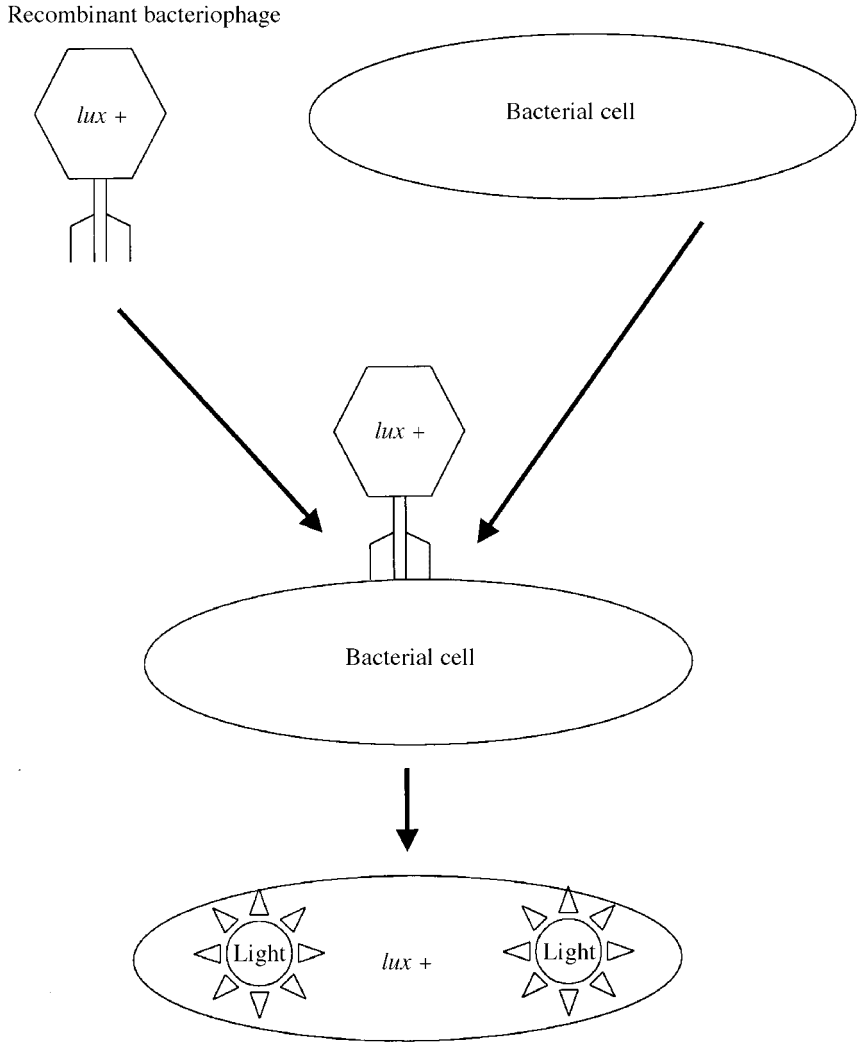

Fig. 8.4 Schematic presentation of bacteriophage-based bioluminescent method for bacteria detection.

choice with regard to *Salmonella* due to its ability to lyse almost all *Salmonella* strains. This information was used by the same authors (Kuhn *et al*., 2002b) while developing recombinant phage reagent for *Salmonella*. A non-essential gene discovered earlier allowed substitution of foreign DNA in a way that neither increased the size of the phage genome nor allowed loss of the insert by recombination. Some concerns have arisen recently regarding the possible 'release' of recombinant bacteriophages in nature. A way to avoid possible dispersal of recombinant phages was proposed by Kuhn *et al.* (2002b).

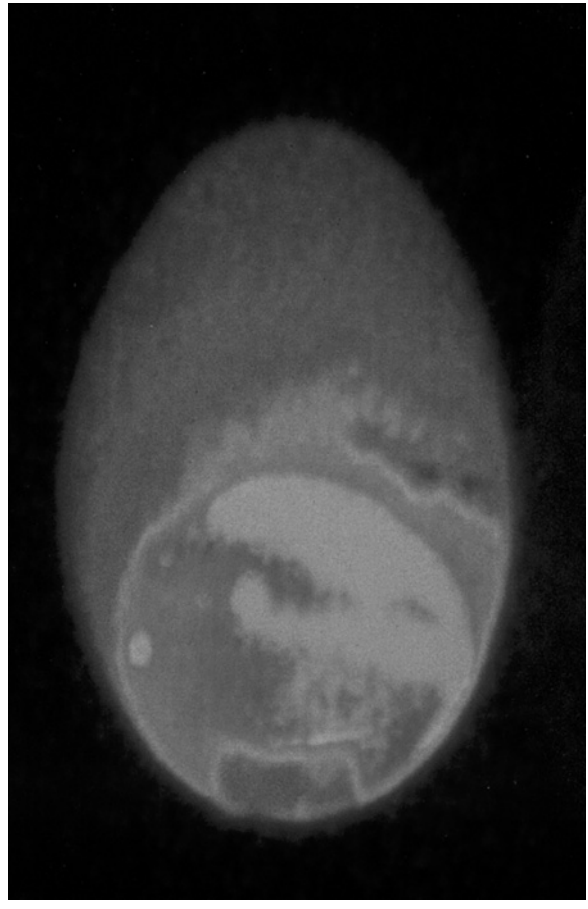

Fig. 8.5 Bioluminescent image of the egg inoculated with 10^3 cfu of *Salmonella* sp and incubated at 37ºC for 24 h. Recombinant phage was added to the egg and bioluminescence was monitored using BIQ Image Quantifier 30 min after infection.

Simultaneously with insertion of *lux* genes one of the essential phage genes responsible for phage particle assembly was removed from its genome. This did not prevent the expression of bioluminescent proteins upon infection of target bacteria, but at the same time the phage cannot multiplicate. To propagate such bacteriophage only specially constructed host bacteria strains carrying complementary plasmid with the missing essential gene should be used.

8.7 Instrumentation

Three main groups of instruments capable of measuring low level luminescence are available on the market – tube luminometers, luminescent plate readers and

Table 8.1 ATP bioluminescent reagent/instrument systems available for use as hygiene monitors and microbial contamination control in food industry

Type of system	Manufacturer	Trade name and system description
Bioluminometers and self-contained swab kits	Biotrace Inc. (www.biotrace.com)	Clean-Trace® – surface hygiene test and Aqua_Trace® – water hygiene test with luminometers Uni-Lite® or Uni-Lite®XCEL
	Celsis (www.celsis.com)	Celsis-Lumac SystemSURE with Advance luminometer
	Charm Sciences (www.charm.com)	PocketSwab® – surface hygiene kit with luminometers LUM-T or Firefly
	Biocontrol Systems Inc. (www.rapidmethods.com)	IDEXX Lightning®
CCD camera and filtering system with bioluminescent reagent kit	Millipore Corporation (www.millipore.com)	MicroStar™ System for detection of microbial contamination in filterable samples
Swab-based system for hygiene monitoring	Promega Corporation (www.promega.com)	ENLITEN® total ATP Rapid Biocontamination Detection Kit (could be used with most commercial luminometers)

imaging devices. The latter are able to measure luminescence in tubes, multi-well plates or from an object of irregular shape and size such as Petri dishes, blotting membranes etc. (Berthold *et al*., 2000). The application determines the choice of a suitable instrument. Detailed surveys of commercially available instruments for bioluminescent detection are published on a regular basis by Stanley (1999, 2000).

Tube luminometers are designed to measure luminescence intensity in single tubes using mostly photomultiplier tubes (PMT) as light detectors. There are multipurpose tube luminometers that can accommodate a wide range of sample formats. Another group of tube luminometers is designed specifically for certain experimental protocols and can be used only with tubes and reagents designated for the instrument. Such portable, battery-driven luminometers have been developed for industrial microbiology applications. Examples of reagent/instrument systems for hygiene monitoring are presented in Table 8.1.

Because of the popularity of microplates, automatic plate luminometers have found a considerable market for high throughput bioluminescent analysis. Measuring luminescence from microplates presents some unique design challenges, especially in performance parameters of the plate such as so-called cross-talk between the wells (Berthold *et al*., 2000). Because of the close

proximity of neighbouring sample wells, the light transmitted from adjoining wells may add to the background signal. To reduce this cross-talk, white microplates with clear bottoms are considered the better choice. These plates show cross-talk values up to three orders of magnitude less than completely clear ones; in addition the efficiency of light detection is increased due to higher reflection from the white walls of the well. Black microplates exhibit the least cross-talk values (10 times lower than for white plates). However, they have low efficiency due to absorption of light by the black walls and are recommended only for samples with high luminescence intensity.

For particular applications, besides the luminescence intensity, information about spatial resolution is required. For this purpose semiconductor array detectors are used. Charge-coupled device (CCD) cameras dominate the market as imaging detectors. The most important technologies used for luminescent imaging are slow-scan cooled CCD cameras, intensified CCD cameras and video cameras with intensifier (Berthold *et al*., 2000). The benefits of cooled slow-scan CCDs are high image quality, sensitivity and broad dynamic range. However, they cannot operate at video rate, e.g. a chip of 512 × 512 pixels can be read out within about 5 s. Intensified CCD cameras operate at higher readout rates and are used in cases when fast image acquisition is necessary. In high-quality, commercial imaging systems, the software controls image acquisition, correction and processing, thus providing an opportunity for quantitative analysis.

Raw data in all types of luminometers are presented in relative light units (RLU). RLU is not a scientifically defined unit but rather a measure of relative light output from a sample. To compare readings for the same sample on different luminometers each instrument has to be calibrated individually using a known analyte concentration or specially calibrated light sources (O'Kane and Lee, 2000).

8.8 References

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