Rapid luminometric technique for real-time monitoring of growth and metabolic functionality of *lux* recombinant bacteria

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Bioluminescent bacteria

Many marine microorganisms (e.g. *Vibrio, Photobacterium*) are capable of emitting light, i. e. they are bioluminescent. The light-yielding reaction is catalysed by a luciferase, and it involves the oxidation of reduced flavin mononucleotide and a long-chain aldehyde in presence of oxygen to produce a blue-green light.

Since $FMNH_2$ production depends upon functional electron transport, only live cells can produce light. Given that the production of light from bioluminescent bacteria containing the *lux* genes depends upon a functional intracellular biochemistry, it can be established that any substance or environment that impairs that biochemistry, and thus compromises cellular viability, will lead to a reduction in light emission (Stewart, 1997).

Advances in molecular genetics made possible to produce bioluminescent test organisms from food-borne bacteria. The genes responsible for bacterial bioluminescence in nature (*lux* genes) have been identified and cloned (Meighen, 1991). In some cases only the genes for luciferase are transferred and the aldehyde is supplied exogenously as it can cross the cellular membrane. These DNA recombinants become luminescent in the presence of exogenous aldehyde (Stewart *et al.,* 1988). This ability to genetically engineer „dark" microorganisms to become light emitting by introducing the *lux* genes into them has opened up a wide range of applications of bioluminescence (Baker *et al.,* 1992).

Bacterial luciferase will function also in Gram-positive organisms althought it is originally from Gram-negative organisms. The *lux* genes must be coupled to appropriate Gram-positive promoters and must be engineered to obtain stable insertion via Gram-positive

replicons (regions containing DNA necessary for the initiation of DNA replication (Stewart *et al.,* 1988; Hill *et al.,* 1994).

Examples of applications of bioluminescent recombinants

Specific lactic acid bacteria posessing a luminescent phenotype have been proposed for assessment starter culture activity in milk (Ahmad and Stewart, 1991). Levels of light emission of *luxAB* lactic acid bacteria are less than those from enteric bacteria recombinants, but it is possible to monitor concentrations of at least 10^6 bacteria per ml in „real time". The light emitting component of a starter culture can be monitored for loss of luminescence that indicates the presence of an inhibitory substance.

Bacterial bioluminescence technique allows rapid assay of biocidal efficiency (Baker *et al.*, 1992; Walker *et al.,* 1992; Andrew *et al.,* 1994). It can also be applied to study recovery of sublethally injured cells (Chen and Griffiths, 1996), or, to monitor surface attachment and biofilm formation (Hibma *et al.,* 1996)..

It was demonstrated in British studies that bioluminescence of *lux* recombinant *Listeria monocytogenes* closely correlated to its cell growth until the approach of stationary phase when bioluminescence begins to decay (Anon., 1990). Ellison et al. (1991) described the application of *in vivo* bioluminescence to monitor the recovery of *luxAB Salmonella typhimurium* from freezing injury, and explored the effect of multiple stress conditions on the recovery. Ellison *et al.,* 1994) used bioluminescence data to develop a model to describe the thermal inactivation of *Salmonella typhimurium.* Similar studies were performed by Duffy *et al.* (1995) in the presence of a competitive microflora. According to Chen and Griffiths (1996) luminescent strains of bacteria can be used to quantitatively monitor growth and survival directly in foods. Thus, they present a tool for deriving data which can be used to produce predictive models that take into account microbial interactions occurring in the food.

Bacterial bioluminescence was used as an indicator of survival of bioluminescent strains of *Listeria monocytogenes* and *Escherichia coli* in selected yogurt and cheese varieties during manufacture and storage (Hudson *et al.,* 1997; Ramsaran *et al.,*1998).

An autoluminescent (engineered to express luminescence by transformation with the entire *lux* gene casete) *Salmonella* Hadar strain was used to monitor its cell survival and viability on lactic acid-treated poultry carcasses as a function of storage temperature (Bautista *et al.,* 1998).

Luminescent phenotypes of *Salmonella enteritidis* and *Campylobacter jejuni,* resp., were effective at visualizing eggshell colonization and penetration using a photon counting charge-coupled device camera (Chen *et al.,* 1996; Allen and Griffiths, 2001).

Some own studies

We have made some preliminary studies on mixed culture growth in Don Whitley impedance broth of *Escherichia coli* and bioluminescent recombinant lactic acid bacteria obtained from the Department of Applied Biochemisty and Food Science of the University of Nottingham. The aim of our studies was to investigate how eventual interactions due to competition for nutrients, or, production of inhibitory metabolites influence the mixed culture growth. The viable cell counts on selective media, the bioluminescence activity of the lactic acid bacteria in a BioOrbit luminometer and the pH of the cultures were estimated periodically (Farkas *et al.,* 1999) Although in dilution series of their suspension the light output of bioluminescent lactobacilli cells correlated linearly over several log-cycles with their viable cell counts, the bioluminescence activity depended very much on the physiological status and perhaps their intracellular pH level, because the bioluminescence activity decreased dramatically during the stationary phase.

Luminometry was utilized in our studies as part of the EU PREMIUM project for monitoring growth of a *Listeria monocytogenes luxAB* strain in its liquid or gelified monocultures and cocultures with „acid only" *Lactococcus lactis* (Farkas et al., 2202a). At low salt concentrations in the culture medium, suppression of *L. monocytogenes* by the nisin-less strain of *Lactococcus lactis* occurred only as an early induction of the stationary state of the bioluminescent target organism. However, *Lactococcus lactis* was more sensitive than *Listeria monocytogenes* when we increased the salt concentration of the culture.medium and at 4 % NaCl level *Listeria monocytogenes* grew uninhibited to its maximal population density even in the presence of *Lactococcus lactis*.

As a rapid instrumental technique, bioluminometry is useful even at relatively low cell densities. Under our conditions, the luminometric detection limit of approx. 10^4 CFU/ml was at least two log cycles less than that of other fast but not selective techniques, e.g. turbidimetry.

For *lux*$^+$ containing bacterial spores, germination is accompanied by the emergence of bioluminescence, which provides a sensitive, „real-time" monitor of the germination and outgrowth process. We have investigated the correlation between bioluminescnce and viable cell counts as well as absorbance of the culture during incubation of vegetative population of a *luxAB* recombinant *Bac.subtilis* strain obtained also from the University of Nottingham. Kinetics of germination and outgrowth of untreated, heat-treated and irradiated spore populations of the bioluminescent *Bacillus subtilis* were also investigated luminometrically and turbidimetrically whereas phase contrast microscopic investigation were used to check cell state distributions. Both the heat treatment and the radiation dose caused approx. 90 % reduction of the number of viable spores. The kinetics of germination and the light emission during germination of radiation-inactivated spores were the same as those of untreated spores, revealing that unlike in heat treated spores, the pre-formed luciferase in irradiated spores remained intact after an irradiation dose causing 90 % lethality in their population (Farkas *et al.,* 2002b). In our recent experiments on the effects of pasteurizing levels of high hydrostatic pressure on *B. subtilis luxAB* spores, the heat sensitive luciferase pool built in during the formation of spores seemed to withstand also the pressurization without inactivation (Farkas *et al.,* 2003).

. Notwithstanding these special situations, bacterial bioluminescence systems is a sensitive tool to monitor growth or metabolic functionality of *lux*$^+$ testorganisms exposed to various environments and stresses, thus, they may find applications increasingly in the field of food microbiology. Bioluminescence can be utilized to identify critical control points and subsequently enhance the effectiveness of the hazard analysis critical control point (HACCP) system.

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