LWT - Food Science and Technology 56 (2014) 484–493

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

High hydrostatic pressure effects on bacterial bioluminescence

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ARTICLE INFO

Article history:
Received 10 December 2012
Received in revised form
25 April 2013
Accepted 10 December 2013

Keywords:
High hydrostatic pressure
Proteins
Denaturation
Bioluminescence

ABSTRACT

The mechanism that leads to microbial inactivation by high hydrostatic pressure remains elusive. In this study, a high-pressure system interfaced with a photomultiplier tube (PMT) was developed to monitor cellular metabolism *in situ* using bioluminescent bacterial strains. Preliminary characterization of the system was performed using *Pseudomonas fluorescens* 5RL expressing lux proteins from *Vibrio fischeri*. Stepwise increases in pressure at 34 MPa and above resulted in decreased bioluminescence. Square wave exposure to pressures of 69, 103 and 138 MPa showed bioluminescence reductions greater than 95%, but when cells were returned to ambient pressure bioluminescence returned to 51, 38, and 4% of initial bioluminescence values, respectively. An *Escherichia coli* strain expressing lux proteins from *V. fischeri* was constructed to determine whether this reversible effect could be observed in another bacterial genus. Square wave perturbations of 69, 103 and 138 MPa resulted in bioluminescence reductions of about 94% at the highest pressure treatment. Upon decompression, bioluminescence returned to 74, 58 and 30% of the initial bioluminescence values for cells treated at 69, 103 and 138 MPa, respectively. These results suggest that square wave exposure to pressure up to 138 MPa induces reversible cell damage in *P. fluorescens* 5RL and *E. coli* VF lux.

1. Introduction

High hydrostatic pressure (HHP), also known as high pressure processing (HPP) or ultra high pressure (UHP), has emerged as an alternative to thermal processes for food preservation. It consists on the application of pressure exerted by a fluid (usually water) on an object (Rivalain, Roquain, & Demazeau, 2010). Pressure levels up to 800 MPa are required to effectively inactivate the vegetative form of pathogenic and spoilage microorganisms (Considine, Kelly, Fitzgerald, Hill, & Sleator, 2008). However, microbial response to high hydrostatic pressure is strain-specific, while environmental factors such as medium composition and pressure treatment conditions can also affect the response. Specific targets within the cell that lead to microbial inactivation by hydrostatic pressure have not been fully elucidated. Several reports suggest that pressure treatments below 100 MPa cause sub-lethal damage altering bacterial cell metabolism and physiology whereas pressures levels above 200 MPa may have a lethal effect (Abe, 2007). Bacterial inactivation by pressure has been related to the loss of cell wall and membrane integrity (Mackey & Manas, 2008; Pagan & Mackey, 2000), denaturation of key proteins and enzymes (Meersman & Heremans, 2008), and impairment of transcriptional and translational processes (Bowman, Bittencourt, & Ross, 2008; Manas & Mackey, 2004). A recent study showed that loss of viability occurred prior to membrane damage in bacterial cells subjected to high pressure. Pressure lethal effect was related to denaturation of membrane bound-proteins (Ananta & Knorr, 2009). Protein denaturation by pressure is characterized by disruption of weak non-covalent bonds (hydrophobic interactions) altering tertiary and quaternary structures of proteins and therefore affecting protein functionality (Follonier, Panke, & Zinn, 2012; Rivalain et al., 2010).

In this work, bioluminescent reporters were used to better understand the effect of moderate pressure on bioluminescence in *Pseudomonas fluorescens* 5RL (King et al., 1990) and *Escherichia coli* VF lux. Bioluminescence refers to the emission of photons of visible light in living organisms due to the reaction of organic compounds and oxygen in the presence of catalytic enzymes (Alloush, Lewis, & Salisbury, 2006). An updated version of the biochemical pathway for bacterial bioluminescence is shown in Fig. 1 (Heitzer et al., 1998). Bioluminescence occurs when a reduced riboflavin ($FMNH_2$) and a long chain aliphatic aldehyde (tetradecanal) are oxidized in the presence of luciferases (Meighen, 1991). The products from the enzyme-catalyzed reaction are oxidized flavin

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0023-6438/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.lwt.2013.12.017

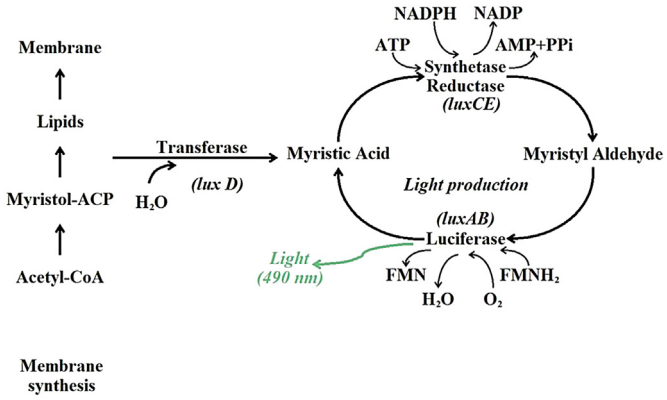

Fig. 1. Biochemical pathway for bacterial bioluminescence. The enzyme complexes transferase, and synthetase/reductase catalyze the conversion of myristic acid into myristyl aldehyde. Later, the enzyme luciferase catalyzes the oxidation of myristyl aldehyde and reduced riboflavin ($FMNH_2$). The end products of this reaction are oxidized flavin mononucleotide (FMN) and energy that is released in the form of blue-green light that can be detected at 490 nm.

mononucleotide (FMN), and energy that is released in the form of blue-green light that can be detected at 490 nm. Subunits of bacterial luciferases are encoded by *luxA* and *luxB* genes. Aldehyde synthesis entails the catalytic reaction of transferase, synthetase and reductase, which are encoded by *luxD*, *luxE* and *luxC* genes, respectively (Meighen, 1991). Cloning and expression of *luxCDABE* operon represents a highly sensitive molecular tool to determine bacterial viability, since light emission depends on the cells being metabolically active (Contag & Bachmann, 2002). Disruption of metabolic processes is usually accompanied by a decrease in bioluminescence (Alloush et al., 2006).

The objective of this study was to develop a laboratory scale HHP system able to monitor *in situ* bacterial bioluminescence as a function of applied pressure. Bacterial luminescence emission inside the pressure vessel was detected using a photomultiplier tube (PMT) connected to a sapphire window in the vessel. Pressure inside the vessel was monitored using a digital pressure sensor directly connected to the vessel. The bioluminescent reporter *P. fluorescens* 5RL, expressing *luxCDABE* operon from the marine bacterium *Vibrio fischeri*, which is under the control of the *sal* promoter, was initially used to monitor *in situ* lux enzyme activity when exposed to pressure levels of 69, 103 and 138 MPa. An *E. coli* expressing the same *lux* cassette from *V. fischeri* (*E. coli* VF lux) was constructed to monitor bacterial bioluminescence when exposed to the same pressures and compared to the results observed for *P. fluorescens* 5RL.

2. Materials and methods

2.1. Bioluminescent bacterial strains

P. fluorescens 5RL and *E. coli* VF lux expressing lux enzymes from *V. fischeri* were used in the present study. The *luxCDABE* gene cassette from *V. fischeri* was removed from pLJS as an *Xba* I fragment and ligated into the *Xba* I site of plasmid pCR2.1 (Applegate, Kehrmeyer, & Sayler, 1998). *E. coli* VF lux was constructed by electroporation of plasmid pCR2.1 (Invitrogen, Grand Island, NY, USA) containing the *luxCDABE* cassette (constitutively expressed from the *lac* promoter) into One Shot® TOP10 Electrocomp™ *E. coli* cells (Invitrogen, Grand Island, NY, USA).

2.2. Experimental setup

The hydrostatic pressure system consisted of a 200 cm^3 stainless steel vessel (HiP High Pressure Equipment Company, Erie, PA, USA). A diagram and a picture of the set-up are shown in Fig. 2. The pressure vessel contains a sapphire window (1.3 cm diameter) to which a Hamamatsu Photomultiplier Tube (PMT) (Hamamatsu Photonics K.K., Japan) was connected to monitor bioluminescence as photons per second. A pressure sensor, Model TJE (Honeywell Sensotec, Columbus, OH, USA), able to measure pressures up to 413 MPa was directly attached to the pressure vessel for monitoring pressure during treatments. A pressure generator Model 25-5.75-100 (HiP High Pressure Equipment Company, Erie, PA, USA) with a capacity of 4.5 cm^3/stroke and maximum pressure rating 689 MPa was used to generate pressure in the vessel using distilled water. The PMT and pressure sensor were connected to a Programmable Logic Controller (PLC). Bioluminescence and pressure were monitored by Wonderware InTouch® software and recorded in a Microsoft Excel™ spreadsheet for analysis.

2.3. Sample preparation

Stock cultures of *P. fluorescens* 5RL were grown in 250 mL Erlenmeyer flasks containing 100 mL Luria–Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract and 10 g/L sodium chloride, Difco, NJ, USA) with 50 μg/mL salicylate (Sigma–Aldrich®, St. Louis, MO, USA) and 14 μg/mL tetracycline (Fisher Scientific, Pittsburgh, PA, USA) (LBtet + sal broth), and incubated overnight at 25 °C on a rotary shaker (100 RPM). Optical density was measured until values of $OD_{600} \sim 0.5$ were obtained. Polypropylene PCR vials (volume 350 μL) were used to contain the bioluminescent bacterial strains and completely filled to eliminate any headspace with 350 μL of culture. Similarly, *E. coli* VF lux stock cultures were grown in 250 mL Erlenmeyer flasks containing 100 mL LB broth (10 g/L tryptone, 5 g/L yeast extract and 10 g/L sodium chloride, Difco, NJ, USA) with 50 μg/mL kanamycin (IBI Scientific, Peosta, IA, USA) (LBkan broth), and incubated overnight at 37 °C on a rotary shaker (100 RPM). A subculture was prepared from the overnight culture in 100 mL LBkan broth until an optical density $OD_{600} = 0.5$ was obtained. PCR vials were filled as previously described to avoid any headspace in the vial.

2.4. Pressure treatments

2.4.1. P. fluorescens 5RL exposed to a stepwise increase in pressure

A preliminary experiment in which *P. fluorescens* 5RL was exposed to a stepwise increase in pressure was conducted to determine the pressure values that had an effect on bacterial luminescence. A PCR vial containing 350 μL of *P. fluorescens* 5RL was placed inside the pressure vessel. The system was tightly closed and covered with a black cloth to avoid background light. The pressure was increased stepwise to 7, 15, 34, 69, 103 and 138 MPa and held for 5 min at each pressure level at 25 °C. At the end of the treatments, pressure was released and maintained at atmospheric pressure (0.1 MPa) for 5 min. Bioluminescence and pressure were measured and recorded every second for a period of 35 min.

2.5. E. coli VF lux exposed to a stepwise increase in pressure

An analogous experiment was performed using *E. coli* VF lux cells exposed to stepwise increase in pressure to determine whether a similar effect on bioluminescence could be observed in other bacterial genus. Pressure treatments were performed as described above for *P. fluorescens* 5RL.