Innovative Food Science and Emerging Technologies 26 (2014) 375–380

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

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

Bioluminescence system assisted by NAD(P)H conversion to increase the sensitivity of quantitative bacterial cell assay

Huihui Liu [a], Hong Lin [a], Quangui Mu [b], Xiaodong Lu [a], Jingxue Wang [a,*], Muhammad Naseem Khan [a,c]

[a] College of Food Science and Engineering, Ocean University of China, Qingdao 266003, PR China
[b] National R&D Branch Center For Kelp Processing (Weihai), Shandong Haizhibao Ocean Science and Technology Co. Ltd, PR China
[c] Microbiological Analytical Centre, FMRRC, PCSIR Labs. Complex, Karachi 75280, Pakistan

ARTICLE INFO

Article history:
Received 2 May 2014
Accepted 29 August 2014
Available online 8 September 2014

Editor Proof Receive Date 16 September 2014

Chemical compounds studied in this article:
NADH (PubChem CID: 439153)
NADPH (PubChem CID: 5884)
NAD^+ (PubChem CID: 5892)
$NADP^+$ (PubChem CID: 5886)
CTAB (PubChem CID: 5974)
TCA (PubChem CID: 6421)
DMSO (PubChem CID: 679)
Dodecyl aldehyde (PubChem CID:8194)
Dehydrated alcohol (PubChem CID:702)
$FMNH_2$ (PubChem CID:44229161)

Keywords:
NAD(P)H conversion
Bacterial bioluminescence
Rapid detection
Total viable count

ABSTRACT

The NADH luminescence assay is a rapid, sensitive and easy-to-perform bacterial detection method. However, the detection limit is approximately 10^7 CFU/mL, which is inadequate for many applications. The purpose of this study is to amplify luminescence assay signals by converting $NAD(P)^+$ to NAD(P)H to provide a more sensitive method for the detection of bacteria. Under optimal conditions, the luminescence intensity correlated well with the bacterial count ($R^2 = 0.98$) and the detection limit was 1.05×10^5 CFU/mL. The sensitivity of this novel bioluminescence enzymatic cycling method is nearly 10^2 times higher than previous bioluminescence methods. Thus, this improved method can be used to rapidly determine the total viable count with higher sensitivity.

Industrial relevance: This improved method can be used to rapidly determine the total viable count with higher sensitivity in food. This study lays the foundation for the future development of a fast detector using bacterial bioluminescence.

1. Introduction

The total viable count (TVC) is a key indicator for controlling the quality of food processing. It is not only useful for monitoring food products but also helpful for the production environment, including raw materials, water, surfaces and air. Moreover, TVC beyond the critical control point or standard limit is helpful to analyze potential food spoilage (Fung, 1994). Traditional count methods such as standard plate count (SPC) and turbidimetric measurement (TM) are widely used to estimate the bacterial count; however both methods have limitations. SPC requires incubation periods of 24–48 h, whereas TM cannot distinguish between live and dead cells. Therefore, the development of rapid and sensitive methods to determine the total bacterial count is imperative. Recently researchers have developed several new assays for detecting bacteria. These methods include polymerase chain reaction-based methods, enzyme-linked immunosorbent assays, immunofluorescence assays, impedimetry, bioluminescence detection and biosensor (Gracias & Mckillip, 2004; Lazcka, Campo, & Munoz, 2007; Picard, Ponsonnet, Paget, Nesme, & Simonet, 1992; Swaminatham, 1994).

The bioluminescence assay is a rapid, sensitive and easy-to-perform method. The firefly luciferase bioluminescence is routinely used for the detection of bacterial contamination via the detection of ATP that is present in all actively metabolizing cells (Satoh, Kato, Takiguchi,

Abbreviations: NADH, nicotinamide adenine dinucleotide(reduced form); NADPH, nicotinamide adenine dinucleotide phosphate(reduced form); NAD^+, nicotinamide adenine dinucleotide (oxidized form); $NADP^+$, nicotinamide adenine dinucleotide phosphate (oxidized form); ATP, adenosine 5′-triphosphate; FMN, flavin mononucleotide; G6PD, glucose-6-phosphate dehydrogenase; LDH, lactate dehydrogenase; ADH, alcohol dehydrogenase; GR, glutathione reductase; Tris, Tris(hydroxymethyl) aminomethane; TCA, trichloroacetic acid; DMSO, dimethyl sulfoxide; CTAB, cetyltrimethyl ammonium bromide; CFU, colony-forming units; PBS, phosphate buffer.

* Corresponding author. Tel.: +86 53282032389.
E-mail address: snow@ouc.edu.cn (J. Wang).

http://dx.doi.org/10.1016/j.ifset.2014.08.007
1466-8564/

Ohtake, & Kuroda, 2004; Squirrell, Price, & Murphy, 2002). Approximately 10^4 colony-forming units (CFU) can be detected by ATP luminescence method (Beckers & Lang, 1983; Davidson, Griffith, Peters, & Fielding, 1999). However, fireflies were obtained from artificial farming, which have certain limitations, including long production time, high cost and restricted geographical location. Luminous bacteria are ubiquitous in marine environments (Zhang, Wu, & Zhou, 2001). Similar to the firefly luciferase, bacterial luciferase also produces light during the catalytic oxidation of NADH in the presence of flavin mononucleotide (FMN) reductase, FMN-Na_2, dodecyl aldehyde and oxygen (Schmid, Schimz, & Sahm, 1989). Therefore, bacterial luciferase bioluminescence may be more suitable to determine bacterial count on the basis of NAD(P)H detection.

$$\text{NADH or NADPH} + \text{FMN} \xrightarrow{\text{FMN reductase}} \text{NAD}^+ \text{ or } \text{NADP}^+ + \text{FMNH}_2$$

$$\text{FMNH}_2 + \text{RCHO} + \text{O}_2 \xrightarrow{\text{luciferase}} \text{FMN} + \text{RCOOH} + \text{photo}$$

The combination of bacterial bioluminescence and NADH produced by the enzymatic reaction provides a highly sensitive analytical system and has been used as a rapid way to monitor various chemicals and enzymes since the 1970s (Haggerty, Jablonski, Stav, & DeLuca, 1978). Our laboratory has established the bacterial NADH bioluminescence system that is stable and suitable for estimating the viable count. By using this system, bacterial count had a good linear relationship with the luminescence intensity by detecting the *E. coli*, *Klebsiella* and *Vibrio parahaemolyticus* for the first time and the detection limit was approximately 10^7 CFU/mL (Mei, Wang, Lin, & Wang, 2009). Because of the low levels of cellular NADH, previous bacterial bioluminescence assay was not sensitive enough for many industrial applications.

Pyridine nucleotides, including NAD(P)H and $NAD(P)^+$, are fundamental mediators of various biological processes such as energy metabolism, calcium homeostasis, antioxidation, gene expression and mitochondrial functions (Ying, 2008). They are not present in dead cells (Lipton & Wetzel, 2002; Ying, 2008). The universal and constant presence of pyridine nucleotides in all living cells (Fuhrer, Kubicek, & Rohr, 1980; Karl, 1980) makes these compounds excellent indicators of the presence of bacteria. The luciferase-FMN reductase system can utilize NAD(P)H to produce light, but it cannot use $NAD(P)^+$ as substrates. For $NAD(P)^+$, specific enzymes could be used to transform them into NAD(P)H. Previous studies showed that NAD^+ was enzymatically converted to NADH by the addition of alcohol dehydrogenase (Karp, Raunio, & Lovgren, 1983), and $NADP^+$ was reduced to NADPH by glucose-6-phosphate dehydrogenase (Lane & Nadeau, 1988). Thus, we used the bacterial bioluminescence system assisted by NAD(P)H conversion to detect the total viable count.

The NADH bioluminescence assay is a rapid, sensitive and easy-to-perform bioanalytical method suitable for the detection of foodborne pathogens. The purpose of this study is to improve the sensitivity of this bioluminescence assay for determining total viable count with the concomitant use of enzymes. The luminescence signal was enhanced by combining enzymatic reactions in which the oxidized nucleotides (NAD^+ or $NADP^+$) are converted to the reduced forms (NADH or NADPH). The improved assay was applied to detect bacteria and the detection limit was found to be 1.05×10^5 CFU/mL. The combination of enzymatic amplification and bacterial bioluminescence will find many applications in the detection of food pathogens.

2. Materials and methods

2.1. Bacterial strains and chemicals

The luminous bacterium *Photobacterium leiognathi* YL was isolated from the coast of Qingdao, China. The strain was preserved at the China Center for Type Culture Collection and deposited under the number M 206139. *E. coli* (ATCC 25922) was used as test organism. Bass (*Lateolabrax japonicus*) was purchased alive from Jusco supermarket (Qingdao, China).

Chemicals and enzymes used in this study, including NAD(P)H, $NAD(P)^+$, dodecyl aldehyde, FMN-Na_2, alcohol dehydrogenase ADH (Catalog No. A7011), lactic dehydrogenase LDH (Catalog No. L2500), glucose-6-phosphate dehydrogenase G6PD (Catalog No. G4134) and glutathione reductase GR (Catalog No. G3664), were either analytical grade or the highest purity available from Sigma-Aldrich Chemicals. Dehydrated alcohol (≥99.7%, analytical reagent) was obtained from Sinopharm Group Co. Ltd.

Crude enzymes were prepared as described previously (Mei et al., 2009). *P. leiognathi* YL were inoculated into 2216E liquid medium and cultured at 25 °C overnight with continuous rotation (150 rpm). The bioluminescence intensity of *P. leiognathi* reached nearly 3,000,000. The cells were collected by centrifugation and suspended in phosphate-buffered saline (PBS) buffer (the ratio of cells to buffer was 1:4). After ultrasonic disruption, the bioluminescence intensity of the cells decreased to 100,000. The supernatant was collected by centrifugation and the crude lysate was treated with ammonium sulfate (the fraction precipitating between 40 and 80% of saturation). After centrifugation to obtain the insoluble fraction, the precipitate was subjected to dialysis against PBS buffer at 4 °C for 24 h. Crude enzymes containing both luciferase and FMN reductase were collected and the luminescence intensity reached 3,000,000. The average protein content was 4.8 mg/mL.

The ADH system consisted of 1 mL PBS (0.01 M, pH 9.5), 100 μL dehydrated alcohol (0.1 M), and 30 μL ADH (3 kU/mL). The G6PD system contained 1 mL PBS (0.01 M, pH 7.5), 100 μL glucose-6-phosphate (5 mM), and 40 μL G6PD (10 U/mL). The GR system contained 1 mL PBS (0.01 M, pH 8.5), 100 μL glutathione (oxidized form, 50 μM), and 30 μL GR (5 U/mL). The LDH system contained 1 mL PBS (0.01 M, pH 7.0), 100 μL pyruvate (1 mM), and 30 μL LDH (11.6 U/mL).

2.2. Luminescence measurement

The bioluminescence reagent included 1 mL crude enzymes, 100 μL dodecyl aldehyde (27 mM) and 2.5 μL FMN-Na_2 (10 mM). Bioluminescence assays were performed by adding 300 μL sample to the bioluminescence reagent; the light emission was measured immediately with a luminescence analyzer (Institute of Biophysics, Beijing, China). Intensity values were measured for a period of 600 s at 1 s intervals.

2.3. Specificity of FMN reductase

The specificity of the FMN reductase with regard to pyridine nucleotides is very important. Briefly, 300 μL of the respective pyridine nucleotide was added to the bioluminescence reagent to assess whether the FMN reductase can utilize it. The luminescence intensity values were measured immediately with a luminescence analyzer.

2.4. Enzymatic conversion reaction (from $NAD(P)^+$ to NAD(P)H)

In the experimental group for NAD^+, 100 μL of 0.14 mM NAD^+ was added to the ADH system and the bioluminescence intensity was measured after adding 300 μL of the reaction solution to the bioluminescence reagent. In the blank group for NAD^+, ADH was replaced by PBS buffer. In the experimental group for $NADP^+$, 100 μL of 0.14 mM $NADP^+$ was added to the G6DH system, and the bioluminescence intensity was measured after adding 300 μL of the reaction solution to the bioluminescence reagent. In the blank group for $NADP^+$, G6PD was substituted for PBS buffer.