Improved Detection of Ochratoxin A by Marine Bioluminescent Bacteria *V. harveyi* BA

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Abstract

Wang W., Zhang M., Fang J., Zhang L., Zou X., Wang X. (2013): **Improved detection of Ochratoxin A by marine bioluminescent bacteria *V. harveyi* BA**. Czech J. Food Sci., **31**: 88–93.

We applicate the bioluminescent assay system for evaluating the toxicity of Ochratoxin A (OTA). The optimum conditions for the growth and bioluminescence of *V. harveyi* BA were investigated, including NaCl concentration and pH in the medium, incubation temperature, and OTA action time. The growth and luminescence reached the perfect phase with the NaCl concentration in the range of 1% to 2%, pH 8–9, incubation temperature 25–30°C, and OTA acting for1 hour. Based on these optimum conditions for bioluminescence, the inhibitory effect of OTA on luminosity was pursued. When OTA concentration fell into the range of 0.1–1.0 μg/l, bioluminescence inhibition followed a linear pattern with a good correlation coefficient (R^2 = 0.944). The calculated recovery percentages fell into the range of 81–102% within the spiking range of 20–200 μg/kg. This system provided a screening method for the measurement of toxic OTA by monitoring the changes in luminescence.

Keywords: bioluminescence; toxicity; OTA; *V. harveyi* BA

Ochratoxin A (OTA), a fungal secondary metabolite, is one of the most common and dangerous mycotoxins in foods and feeds, naturally produced by three main species of mould, *Aspergillus ochraceus*, *Penicillium verrucosum,* and *Aspergillus* section Nigri, especially *Aspergillus carbonarius* (van der Merwe *et al*. 1965; Eskolar *et al.* 2001). OTA has been extensively documented as a global contaminant of a wide variety of foods such as cereals and cereal products, pulses, coffee, beer, grape juice, dried vine fruits, wine, cocoa products, and nuts as well as spices (Visconti *et al*. 1999; Blesa *et al*. 2004).

OTA has been described as nephrotoxic, carcinogenic, teratogenic, immunotoxic, and hepatotoxic to several species of animals (Koller *et al*. 2004). It is also considered as responsible for Balkan Endemic Nephropathy and urothelial tumours in humans. The interest into OTA increased when it was classified as 2B possible human carcinogen by the International Agency for Research on Cancer, based on sufficient evidence of carcinogenicity in experimental studies on animals (IARC 1993). Due to these findings, many countries have set limits on OTA levels in food, typically in the range of 1 and 10 ppb depending on the type and quality of the foodstuff (Commission regulation (EC) No. 123/2005).

Although sensitive and accurate, most of the chromatographic methods developed are laborious, expensive, time-consuming, and unsuitable for the analysis of a great number of samples in a short time. They also require sophisticated equipment and extensive clean-up procedures. The past decade, however, has witnessed considerable progress

Supported by the Grant of Zhejiang Provincial Natural Science Foundation for Basic Research No. Y3090599; Program of Key Scientific and Technological Innovation Team of Zhejiang Province (No. 2010R50028).

in biological determination of mycotoxins. A few immunological methods such as ELISA and biosensors have been developed for the estimation of mycotoxins (Schneider *et al*. 1995; van der Gaag *et al.* 2003). In recent years, the bioluminescent assays have gained increased attention for highly sensitive and specific analytical purposes (Girotti *et al*. 2001). They have become user-friendly in terms of simplification, on-site screening, and long-term stability. A bioluminescence (BL) inhibition assay is often chosen as the first screening method in a test battery, based on the speed and cost considerations (Kratasyuk *et al*. 2001).

The aim of this study was to determine the essential factors for optimising the bacteria growth and bioluminescent intensity. The recovery test was carried out to explore the possibility of using the device for the detection of OTA in real samples. This research proposes an experimental system that can be used as a reliable tool for monitoring OTA toxicity by means of recording the changes in bioluminescent intensity in batch culture.

MATERIAL AND METHODS

Chemicals. Ochratoxin A of HPLC grade was purchased from Sigma (St. Louis, USA).

Bacteria strain and medium. *V. harveyi* BA was kindly provided by Ocean University of China and grown in a medium containing distilled water, peptone (5.0 g/l), NaCl (19.5 g/l), yeast extract (1.0 g/l), $MgCl_2$ (6.0 g/l), Na_2SO_4 (3.2 g/l), $CaCl_2$ (1.8 g/l), KCl (0.55 g/l) and other trace salts for marine bacteria culture.

Determination of bioluminescence spectrum. The single colony was inoculated into 25 ml liquid medium and cultured for 15 hours. Bioluminescence spectrum was measured with a Shimadzu Fluorescence Spectrophotometer (RF-5301PC; Shimadzu Scientific Instruments, Columbia, USA), which records the light between 260 nm to 700 nm.

Growth of batch culture. Cultures (25 ml) in 250 ml conical flasks were incubated at 30°C in an orbital shaker at 200 rpm after inoculation with 1ml of brightly glowing culture observed in the dark. The effects of different NaCl concentration (0–8%) in the medium, pH (6–10), and temperature (15–35°C) were evaluated to determine optimum conditions for bioluminescence and cell growth of the strain.

Luminosity and growth measurement. A Fluorescence Spectrophotometer (RF-5301PC) was used to measure the bioluminescence of the batch culture. The optical cell density at 610 nm was applied to the growth measurement by a UV spectrometer (UV-2501PC; Shimadzu Scientific Instruments, Columbia, USA).

Detection of OTA toxicity. The optimum OTA treatment time was investigated by adding OTA to the bacteria suspensions (1 μg/l) and measuring bioluminescence every 0.5 h of incubation. Then the inhibitory effect of different OTA concentrations (0–20 μg/l) was determined as mentioned above. Ultra pure water was incubated as control.

Recovery test. Spiked sorghum rice, barley, and wheat samples in the range of 20–200 μg/kg were prepared by adding appropriate concentrations of OTA to 2 g of powered samples. The samples (2 g) were weighed into a 50-ml centrifuge tubes, vortex-mixed with 10 ml of 75% methanol for 3 min, and then centrifuged at 4000 rpm for 10 minutes. The supernatant was then diluted with 0.01M PBS (pH 7.4) in the ratio of 1/20. The inhibition rate was determined as mentioned above after the action time of 1 hour.

RESULTS AND DISCUSSION

Luminescence spectrum obtained from *V. harveyi* BA is shown in Figure 1. The wavelength of maximum emission was 470 nm. Scheerer *et al*. (2006) examined the light emission of a *V. fischeri* strain at 490 nm. The λ_{max} for *P. phosphoreum* was 478 nm. Most marine luminescence has maximal emission between 440 and 505 nm (Nicol *et al*. 1958; Widder *et al*. 1983; Latz *et al*. 1988). Bioluminescence plays important roles in the sea, because it has evolved in nearly every group of marine organisms (Harvey 1952). Sophisticated physiological and behavioural mechanisms for

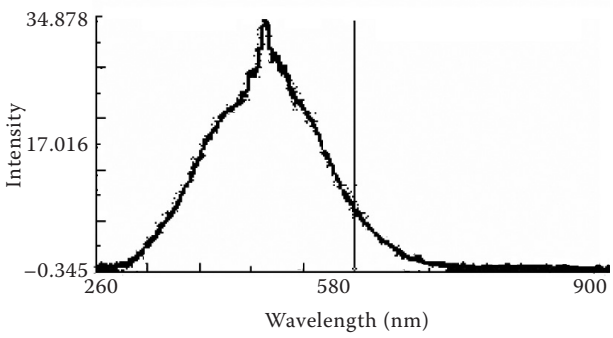

Figure 1. Bioluminescence emission spectra of *V. harveyi* BA tested

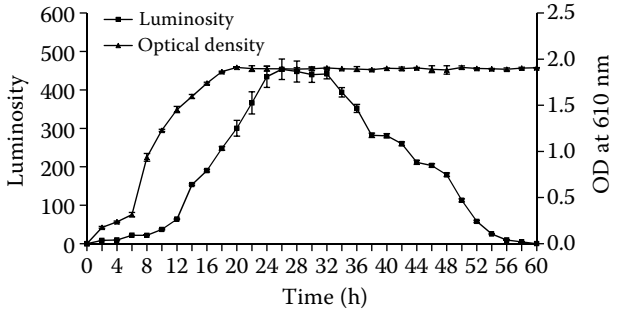

Figure 2. Changes in bioluminescence during continuous culture

controlling luminescence are also suggestive of its importance. The wavelength is an aspect of luminescence that is subject to great variations. It is important because it can determine how far the light travels through the water and how well organisms can perceive a luminous signal. But it was believed that the wavelength of maximum emission did not vary between the measurements of the individual specimen or species (Haddock *et al*. 1999).

The absorbance at 610 nm indicating bacteria growth was correlated to bioluminescence (Figure 2). A slow increase in bioluminescence of *V. harveyi* BA was observed in the early log phase, followed by a rapid increase with increasing cell density. Luminescence reached its maximum in 24–32 h after inoculation, and began to decrease thereafter. The luminescence peak appeared at the end of the bacteria logarithmic growth, and sustained 8 h during the bacteria stationary phase. Thus maximum luminescence intensity (24 h after inoculation) was used to determine the sensitivity of the factors that contribute to the stress and to test OTA toxicity.

Different conditions for bacteria growth, such as NaCl, pH, temperature, and toxin treatment time were tested by modifying the medium and environmental factors. The effect of NaCl concentration on bioluminescence is shown in Figure 3a. The luminescence response of *V. harveyi* BA indicated it as being of marine halotolerant origin. At zero NaCl concentration, bioluminescence was observed and the light density gradually increased until NaCl concentration reached a level of 1–2% with the maximum luminescence of about 18. At higher NaCl concentrations (4% and 8%), the bacteria growth was inhibited and luminescence value decreased. This result is in agreement with that obtained with *P. phosphoreum* (Hassan & Oh 2010), since the halotolerant microorganisms can survive and grow even in relatively high concentrations of salt but prefer living in the absence of it, whereas the moderate halophiles achieve their optimal growth at salt concentrations from 0.5M (25 g/l) to 2.5M (150 g/l) and the extreme halophiles at salt concentrations over 2.5M to saturation (340 g/l) (Joo & Kim 2005; Ventosa *et al*. 1998).

The results indicated that pH played a significant role in the maintenance of sustained biolumines-

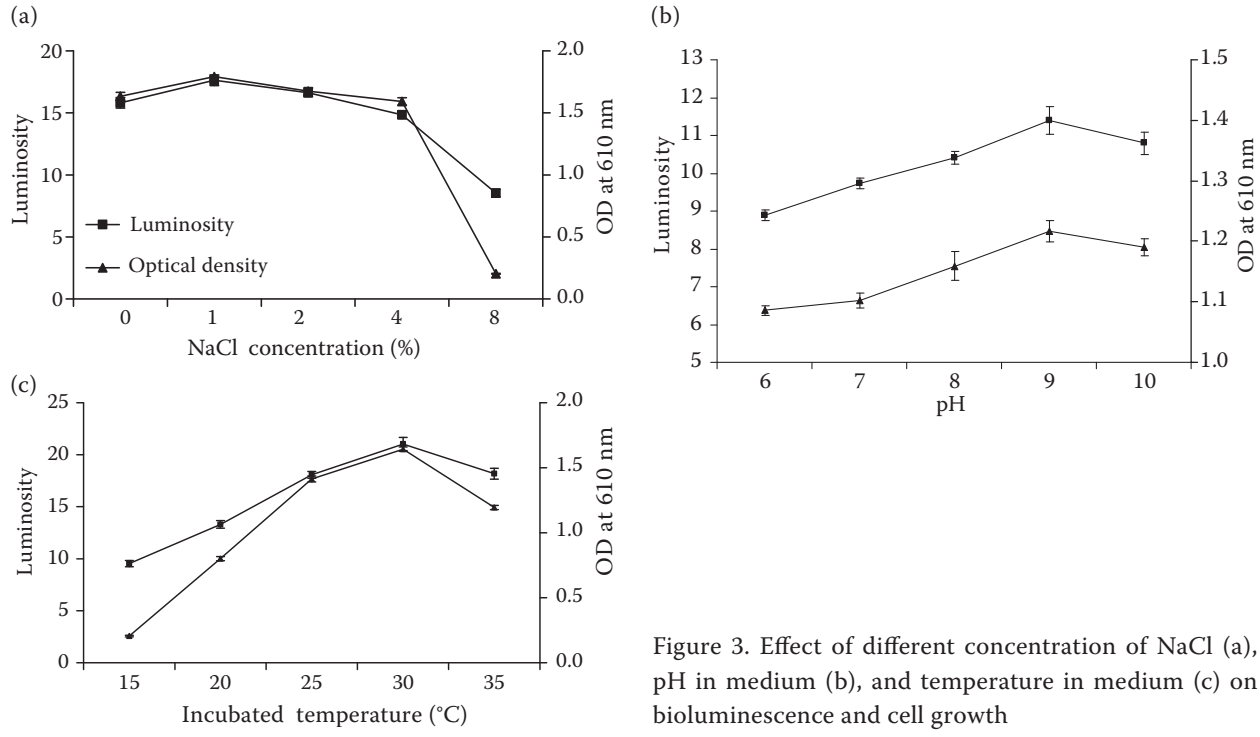

Figure 3. Effect of different concentration of NaCl (a), pH in medium (b), and temperature in medium (c) on bioluminescence and cell growth

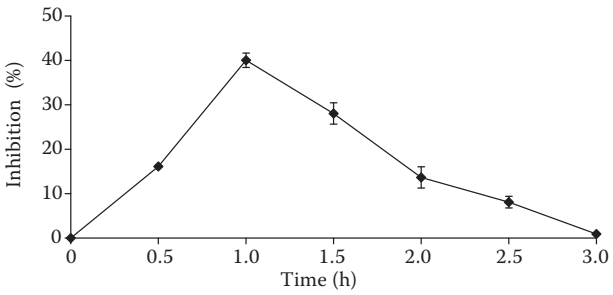

Figure 4. Effect of different OTA action time on bioluminescence and cell growth

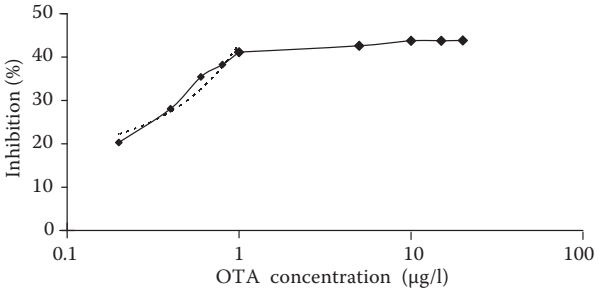

Figure 5. The curve on bioluminescent inhibition versus OTA concentration

cence; optimal bioluminescence occurred over a narrow pH range (Figure 3b). Bioluminescence increased slowly in the pH range of 6–9 and attenuated above pH 9. The higher values of cell density observed at pH 8–9 reflected well the bacteria growth. The results were in contrast to the findings by Hassan and Oh (2010), who reported that the maximum bioluminescence at pH 7.0 and 9.0 was 13 200 and 3400, respectively, for *P. phosphoreum*. Our results demonstrated the correlation between the bacteria growth and luminescence.

Temperatures of 15°C to 35°C have been tested in order to evaluate the impacts on the luminescence and cell density for each growth (Figure 3c). The shifts down to 25°C and above 30°C led to a large attenuation of luminosity. And the greater was the increment of the temperature decrease, the greater was the decrease in luminosity. Changes up to 30°C led to equivalent luminosity changes. Likewise, the temperature interval of 25°C to 30°C resulted in a better bacteria growth. However, some species could not produce considerable quantities of luminescence at incubation temperatures above 26°C.

As it can be seen in Figure 4, in all cases of the signal measurements, the value of luminosity inhibition oscillated in the range from 0% to 40.05%, depending on the time of bacteria incubation with the mycotoxin OTA solution. The inhibition rate increased with the prolonged action time of OTA, and it ranked the highest at 1 hour. Moreover, when the time of incubation was prolonged (up to 60 min), the toxic effect of OTA increased and the value of the bioluminescence inhibition dropped to 0.92%. These results were consistent with those with T2 mycotoxin which caused a decrease of BL of *V. fischeri* F1 at different periods of exposition (Nickolay *et al*. 2008). It is necessary to underline that the sensitivity to a toxin appeared to be species specific.

The effects of OTA on bioluminescent assay system are characterised in Figure 5. The greatest change was observed in the concentration range of 0.2–1.0 µg/l. The increase of OTA concentration caused sustained increase in the inhibition rate until the inhibitory value did not significantly differ at higher concentrations (above 1.0 µg/l). The equation describing bioluminescent inhibition as a function of OTA concentration (in the range of 0 to 1.0 µg/l) was:

$$\text{Inhibition rate} = 0.26C + 0.17$$

where:
C – concentration (µg/l)

The correlation showed a good linear relationship, with R^2 value of 0.944. If this equation is followed, the toxic effect of OTA on bioluminescent bacteria may be revealed at a concentration below 1.0 µg/l. The levels of T2 mycotoxin toxicity presented as the concentration causing 50% decrease of *V. fischeri* F1 luminescence intensity fluctuated between 7 and 19 mg/l, 3 order of magnitude lower than our results (Nickolay *et al*. 2008).

The use of aqueous methanol in various proportions has become popular for the extraction of OTA from foodstuff (Scott 2002). We used 75% methanol in water (v/v) for the extraction of cereal samples as it gave better extraction yields (Ngundi *et al*. 2005). Since this percentage of methanol in water is probably too high and inhibits the growth and luminescence of the bacteria, the dilution was performed. The calculated recoveries for OTA from non-infected samples obtained by this method were between 81–102% within the spiking range of 20–200 µg/kg. The results summarised in Table 1 show the accuracy of the method. In China, the acceptable maximum residue limit in cereal food and beans was set at 5.0 µg/kg

Table 1. Analytical recovery of OTA from spiked samples (n = 6)

Samples	Added (µg/kg)	Found[a] (µg/kg)	Recovery (%)
Sorghum rice	20	18.2 ± 0.35	91.0 ± 1.8
	60	61.4 ± 1.2	102.0 ± 2.0
	120	103.7 ± 6.8	86.4 ± 5.6
Barley	20	16.9 ± 0.7	84.5 ± 3.5
	60	57.3 ± 2.5	95.5 ± 4.2
	120	107.6 ± 4.7	89.7 ± 3.9
Wheat	20	17.3 ± 0.6	86.5 ± 3.0
	60	52.9 ± 2.2	88.2 ± 3.7
	120	123.1 ± 1.6	103.5 ± 1.3

[a]mean of duplicate determination ± SD

(CNS GB2761-2011). The fact is indicative of that the assessment of OTA toxicity should be preferred in cases where the concentration in foods is expected to be high. The fact that the bacteria respond differently to a range of toxicants, and the complexity of real samples which may be polluted with other mycotoxins, raised uncertainty of this assay. So the development of multiple toxicity bioassays is under way.

Compared to the conventional instrumental techniques, bacteria based bioassays can be usefully employed as early warning screening methods, thanks to their rapid response to biological or chemical toxins. The ease of the use of bacterial bioassays represents another advantage. Bioassays using bacterial cells have been developed just to reduce the cost and duration time of the experiments, apart from improving the sensitivity.

CONCLUSIONS

In this study, the information useful to designing a simple and sustainable bioluminescent bacteria assay was obtained through detailed research. Optimisation of the light emission necessitates a careful choice of the growth medium and culture operating conditions. NaCl concentration, incubation temperature, medium pH, and OTA action time were maintained within favourable ranges. It was shown that during the incubation for 24–32 h, toxin action time of 1 h at pH 8–9, and temperature of 25–30°C, the brightest luminescence was emitted. Under these conditions, this assay could detect OTA in the buffer in the amounts below 1.0 µg/l. Though the recoveries from spiked sorghum rice, barley, and wheat samples averaged between 81–102%, the possibility of real samples detection has to be traced further. Our results show the potential for the investigation of a variety of mycotoxins in real food samples.

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Received for publication January 16, 2012
Accepted after corrections June 7, 2012

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