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# A novel *in vivo* β carotene biosensor for heavy metals detection

**Ling Shing Wong,*, Bei Ru Lee, Chia En Koh, Yun Qiu Ong and Chieh Wean Choong**
Faculty of Science, Technology, Engineering and Mathematics, INTI International University
Persiaran Perdana BBN, Putra Nilai, 71800 Negeri Sembilan, Malaysia
*Corresponding Author E-mail: lingshing79@yahoo.com.sg

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**Abstract**

A novel whole cell-based biosensor was constructed using *Daucus carota* cell as a biological component, with *in vivo* β-carotene as a reporter group. In this biosensor, the cells were immobilized using agarose gel, and change in *in vivo* β-carotene, before and after exposure of cells to heavy metals was noted using spectrophotometer at λ = 450 nm. Biosensor was found to operate at its optimum condition using cells from day-14 culture with density of 8 x $10^5$ cells/cuvette, and immobilized with 0.5 % agarose at 45°C. The performance of biosensor was affected by pH with the best response produced at pH 7.5. Pb and Cu tests showed that biosensor was able to detect the presence of both heavy metals within the range of 0.01 ppm – 10.00 ppm. The performance of biosensor decreased in stability test with prolonged storage of 40 days, with a stable performance obtained after 10 days of storage. The results showed that β-carotene contain in naturally available cell *D. carota* responded well to the presence of heavy metals. It is a good indication that biosensor designed is a good candidate to be used for environmental toxicity assessment.

**Key words**

Biosensor, β-carotene, Copper, *Daucus carota*, Lead,

## Introduction

Biosensors are analytical tools which transduce the biochemical responses to readable electronic signals. The biological responses such as fluorescence from photosynthetic pigments (Wong *et al.* 2013a), changes in carotenoids content (Wong and Choong, 2014), activities of enzymes (Ghica *et al.* 2013), and expression of certain reporter proteins (Raja and Selvam, 2011; Yagur-Kroll and Belkin, 2011) have been utilized in biosensors.

In the development of whole cell-based biosensor, chlorophyll has been extensively studied. The nature of green pigment to fluorescence has been well utilized in construction of several bionsensors (Nguyen-Ngoc and Tran-Minh, 2007; Rashkov *et al.* 2012; Wong *et al.* 2013b). Environmental pollutants like heavy metals and pesticides which inhibit photosynthetic pathway lead to increase in fluorescence emission- a response to diffuse light energy accumulated. On the other hand, reduction in oxygen production due to inhibition of photosynthesis has been used in development of biosensors that measures change in oxygen concentration (Chay *et al.* 2005; Rashkov, *et al.*, 2012; Wong *et al.* 2008).

While chlorophyll has drawn much attention of researchers, another widely available natural pigment in plant-carotenoids which is responsible for producing yellow-to-orange colour in plants is nor studied much studied. As β-carotene play role as antioxidant in plants, presence of heavy metals which increase oxidative stress in plants which triggers synthesis of pigment (Azevedo and Azevedo, 2006; McElroy and Kopsell, 2009). Yoshida *et al.* (2008) reported that carotenoids were utilized in transgenic bacteria for arsenic detection, while Wong and Choong (2012) confirmed that the concentration of β-carotene in *D. carota* cells in suspension changed due to the presence of Cu, Zn and Pb. Rahman *et al.* (2011) stated that carotenoids in cyanobacteria are good candidate for biosensor application. However, to date, the pigments in natural cells has not been reported in biosensor construction.

In the present study, *D. carota* cell-based biosensor was developed using naturally occurring β-carotene as bioreporter. Detection was done with spectrometric approach in simple one-step detection.

## Materials and Methods

***D. carota* cell culture and determination of cell growth :** *D. carota* taproot was peeled into discs with thickness of approximately 1 cm. Then, taproot was sterilized in 70% ethanol and sterized (0.525% NaOCl, 0.05% Triton X-100) for 30 sec and 25 min. Taproot was then washed in distilled water and cultured on MS medium (Murashige and Skoog, 1962) with 25 g $l^{-1}$ sucrose and supplemented with 1 mg $ml^{-1}$ 2,4-dichlorophenoxyacetic acid at 25°C, under dark condition, for 60 days.

*D. carota* cells were sub-cultured after 60 days onto the same medium overlaid with MS broth medium, followed by incubation at 25°C in dark condition, for 30 days. After that, the resulting liquid with cell suspension was filtered through a sterile mesh filter and centrifuged at 500 g for 10 min at 25°C to collect the cells. Homogenous *D. carota* was selected and cultured in MS medium and the culture was kept at room temperature under dark condition, with agitation at 120 revolution/ minute using automatic shaker (Green Sseriker II, Vision) to avoid clumping of cells. Subculture was carried out every 20 days.

Cell density of the culture was determined through cell count using light microscope (Eclipse E100, Nikon) and haemocytometer (Marienfield Superior, Neubauer). Growth curve of *D. carota* was determined by cell count for 20 days.

**Design of biosensor :** *D. carota* cell was immobilized with agarose gel in a clear side of a polystyrene cuvette (4.5 ml, 10 mm light path). For immobilization, 0.5 ml of culture was added into cuvette followed by 0.5 ml of agarose solution. The mixture was then left to dry on clear side of the cuvette at room temperature. The layer of agarose gel formed entrapped the cells while hold the cells to the cuvette. The design of biosensor is illustrated in Fig. 1.

Detection of heavy metal was based on OD change at 450 nm before exposure to heavy metal and 75 min after exposure, by spectrophotometer (Libra 12, Biochrom). Change of OD in percentage was calculated following the method the present study, Krause and Weis (1991).

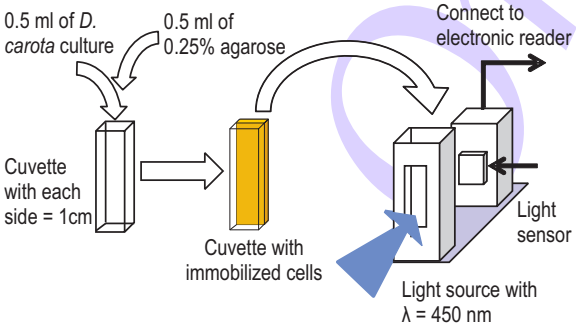


**Fig. 1 :** The design of biosensor

In the present study, all the tests were performed with 2 ml of analytes and in triplicates, unless indicated otherwise. The exposure was carried out in room condition with ambient light. Analysis of data like mean value, standard deviation and graphs were produced using Microsoft Excel 2010.

**Optimizing immobilization condition :** *D. carota* cells were immobilized using agarose gel with the concentrations of 0.25 %, 0.50 %, and 1.00 %. The immobilization was carried out by adding 0.5 ml of agarose solution to 0.5 ml of culture e.g. adding 0.5 ml of 2 % agarose solution to 0.5 ml of culture to make up the immobilization of cell with 1 % of agarose. The immobilization with different concentrations of agarose was conducted in three different temperatures- 40°C, 45°C, and 50°C and the response of the cell were tested using 5 ppm of Cu to determine the optimum agarose concentration and immobilizing temperature for the cell. Immobilization with 0.5 % of agarose at 45°C was selected as the optimum condition of immobilization.

**Effect of cell age and cell number on biosensor :** To study the effect of cell age on biosensor, 5 day, 10 day, and 14 day old cells were immobilized with 0.5 % agarose at 45°C. Biosensor was tested for seeing the response of 1 ppm, 5 ppm and 10 ppm Cu 14 day old cells were selected for biosensor.

To learn the effect of cell number on biosensor, biosensor with $1.0 \times 10^5$, $2.5 \times 10^5$, $5.0 \times 10^5$, $8.0 \times 10^5$, and $1.0 \times 10^6$ cells per cuvette was prepared. Cells were taken from day-14 culture with immobilization carried out with 0.5 % agarose at 45°C. Biosensor with different cell densities was tested on Cu solution with 1 ppm, 5 ppm and 10 ppm. Cell density of $8.0 \times 10^5$ per cuvette was chosen to be used in the biosensor.

**Effect of pH on biosensor :** Cu solutions with different pH were prepared by adding NaOH or HCl to their respective solutions. Biosensor with cells taken from day-14 culture, with cell density of $8.0 \times 10^5$ cells per cuvette and immobilization with 0.5 % of agarose gel at 45 °C was prepared. Biosensor was tested with 1 ppm, 5 ppm and 10 ppm of Cu at pH 6.0, 6.5, 7.0, 7.5 and 8.0 respectively.

**Biosensor tests on different heavy metals :** For Cu and Pb test solution of heavy metal was maintained at pH 7.5 using NaOH. Biosensor was tested with different concentrations of Cu 0.01 ppm, 0.10 ppm, 1.00 ppm and 10.00 ppm with the optimized condition. The test was repeated using Pb.

**Stability of biosensor :** Biosensor was produced at all optimized conditions and stored room temperature in dark. It was tested with 1 ppm of Cu for 30 days at 5 day interval.

## Results and Discussion

The culture of *D. carota* in MS medium is well documented (Luchakivskaya *et al.*, 2011; Wong and Choong, 2012). The lag phase of *D. carota* lasted from day-0 to day-4,

exponential phase from day-4 to day-12, and stationary phase from day-12 to day-16, respectively (Fig. 2). The sigmoid growth pattern was similar to growth of few other plant cells (Bertin, 2005; Lin and Varner, 1991).

According to Wong and Choong (2012), the presence of β-carotene in *D. carota* can be detected with spectrophotometer at 450 nm wavelength. In the present study, growth of *D. carota* cells was not well correlated with the spectrophotometry study of β-carotene, thus cell count by conventional haemocytometer and light microscope was preferred. Later, the effect of cell age response of biosensor was tested with cells from different growth phases.

Agarose is used as an immobilizing agent of cells in different applications (Buffi *et al.*, 2011; Eun *et al.*, 2010). For this biosensor, optimization of temperature and concentration of agarose was carried out with biosensor exposed to 5 ppm of Cu with combination of three different concentrations of agarose, respectively, at three different temperatures (Fig. 3).

The result showed that 0.5 % of agarose yielded highest response. Temperature of 40°C to 50°C were selected as agarose solidification fall within the range. With 1 % agarose, short solidification period affected the process to spread the cells evenly. On the other hand, 0.25 % of agarose was not sufficient to hold the cells effectively. Thus, 0.5 % of agarose was considered to be the most effective concentration for cell immobilization. Temperature was set at 45°C for highest response and better reproducibility.

Standardization of cell age, to be used in biosensor, is important as a cell might respond differently in different growth phases. Thus, cell age is usually defined in cell-based biosensors (Komaitis *et al.*, 2010; Li *et al.*, 2014; Wong, *et al.*, 2008). The study on the effect of cell age biosensor showed that *D. carota* cells from stationary growth phase at day-14 produced highest response to 1 ppm, 5 ppm and 10 ppm of Cu (Fig. 4). The result was different as compared to the other biosensors for heavy metal detection e.g. cyanobacteria biosensor developed by Wong *et al.* (2012), algae biosensor developed by Durrieu *et al.* (2011), or bacterium biosensor developed by Komaitis *et al.* (2010) due to difference in biosensor design and type of cell used.

The number of cells used in biosensors affect the response of biosensors, thus needed to be defined for reproducible results (Rawson *et al.*, 2011; Wong, *et al.*, 2013b). For this biosensor, 8 x $10^5$ cells per cuvette was found to produce highest response to three different concentrations of Cu (Fig. 5). The response of biosensor to three different concentrations of Cu increased with 1.0 x $10^5$ to 8.0 x $10^5$ cells per cuvette, with no significant increase observed when cell number was increased to 1.0 x $10^6$. Thus, 8.0 x $10^5$ was taken as optimized number of cells per cuvette for biosensor. The effects of high density of different types of cells to optical density were reported by Morris and Nicholas (1978) and Wong *et al.* (2012).

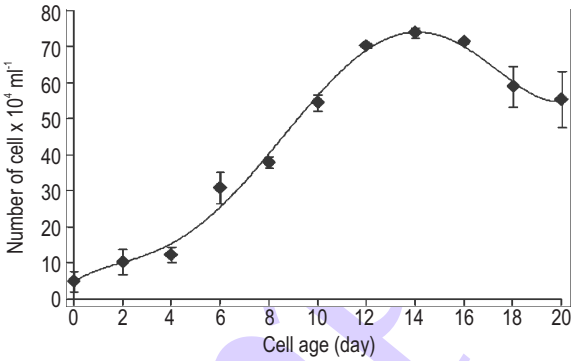


**Fig. 2 :** Growth curve of *D. carota* in after 20 days of subculture

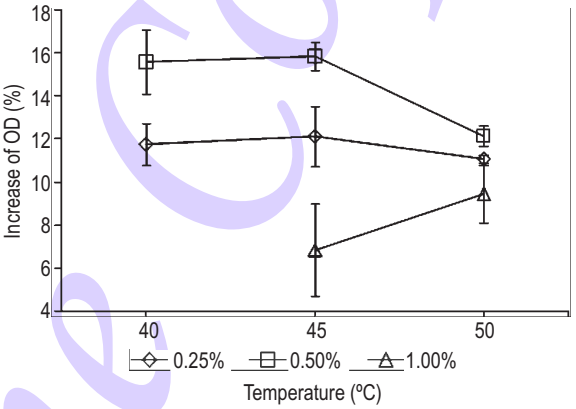


**Fig. 3 :** Effect of agarose concentration and immobilizing temperature in response of biosensor

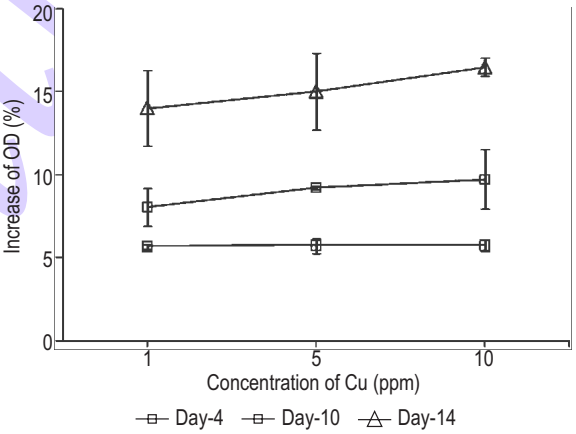


**Fig. 4 :** Response of biosensor to 5 ppm Cu with cells from different culture age

The performance of biosensors is affected by pH value (Komaitis, *et al.*, 2010; Liu *et al.* 2011; Yüce *et al.* 2010). The test of pH range from 6.0 to 8.0 on this biosensor showed that pH 7.5 was optimum (Fig. 6.). It was noted that the optimum pH for biosensor was different from the pH of MS medium (5.8).

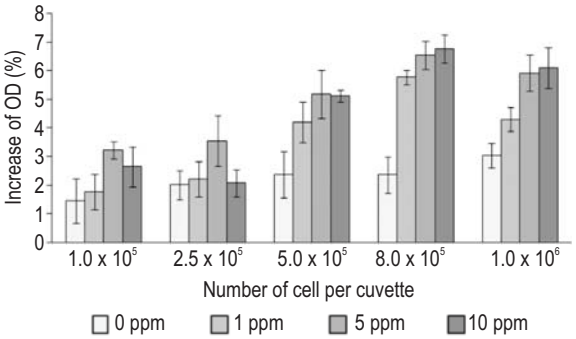


**Fig. 5 :** Response of biosensor with different number of cells to 0 ppm, 1 ppm, 5 ppm and 10 ppm Cu

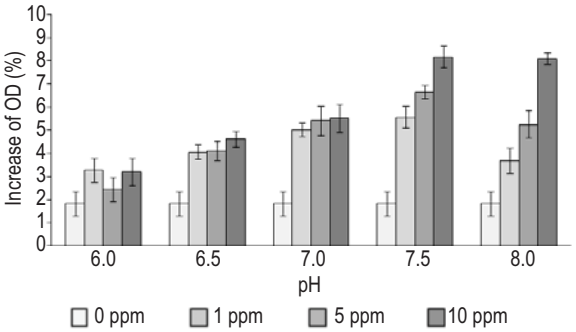


**Fig. 6 :** Effect of pH to response of biosensor to 0 ppm, 1 ppm, 5 ppm and 10 ppm Cu

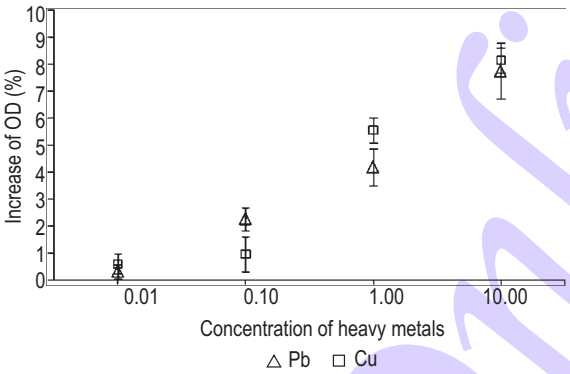


**Fig. 7 :** Response of biosensor to various concentrations of Pb and Cu

Fig. 7 depicts the response of biosensor tested on Pb and Cu. The result showed that the response increased with heavy metal concentration from 0.01 ppm to 10.00 ppm. Biosensor showed good reproducibility in Pb and Cu detection with average SD value ± 0.60 % and 0.49 % respectively. ANOVA showed that there was no significant difference ($p < 0.05$) in the response of biosensor to these two heavy metals. Biosensor confirmed the ability to assess heavy metal toxicity in water samples.

The stability of biosensor was tested with 1 ppm of Cu. From the biosensor, initial response of 5.56 % increase in OD at day-0, the performance of biosensor decreased gradually to 3.38 % at day-40. From 57.48 % of diminish in response (from 5.56 % to 3.38 %), a fast diminish of 50.87 % was observed in first 10 days of storage, followed by a slower 6.61 % decrease from day-10 to day-40 of storage. Decrease in performance might be result of deterioration of the biological component, which was agreeable with the whole cell-based biosensor using cyanobacteria *A. torulosa* as reported by Wong *et al.* (2013a).

In conclusion, *D. carota*, immobilized with agarose gel in a cuvette, responded well to Cu and Pb within 0.01 ppm to 10.00 ppm respectively. The best response of biosensor was obtained by immobilizing 8 x $10^5$ cells with 0.5 % agarose gel at 45°C, and when biosensor was operated at pH7.5. In stability test, the performance of biosensor was diminished within 40 days of storage. The results showed that biosensor could be an effective tool to detect the presence of heavy metals in aquatic environment toxicity assessment.

## Acknowledgment

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