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Development of a whole cell biosensor for the detection of inorganic mercury

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Abstract

A whole cell bacterial biosensor was developed by modification of chromosomal DNA of an environmental bacterial isolate *Sphingobium* SA2. The sensing element contained green fluorescence protein gene *gfp* fused to short segment of *merA* gene of *Sphingobium* SA2. The sensing element was introduced into electro-competent cells of *Sphingobium* SA2, where it integrated into the bacterial chromosomal DNA due to homologous recombination. The transformed cells were able to produce green fluorescence in 5 h in the presence of nano-molar concentrations of mercury. A linear positive correlation was observed between 0 – 40 nm Hg and fluorescence intensity.

Key words: bioavailable metal, *gfp*, detection, toxicity, *Sphingobium*,

1. Introduction

It is important to have an appropriate monitoring system which measures the bioavailable fraction of a pollutant in the environment. A number of classical analytical methods are available for the detection and quantification of Hg from environmental and biological samples such as atomic absorption spectrophotometry (AAS), cold-vapour atomic flame absorption spectroscopy (CVAFS) (BáStockwell and TáCorns, 1995), atomic emission spectroscopy (AES) (Jamoussi et al., 1995) and inductively coupled plasma mass spectrometry (ICP-MS) (Hintelmann et al., 2000). These methods are highly sensitive and characterized by low detection limits however, the equipment are very expensive, require trained operators and laborious sample preparation procedures. Furthermore the equipment are not suitable for their use in the field (Bontidean et al., 2004). Some of the alternatives to these analytical techniques include electrochemical methods (Turyan and Mandler, 1993) such as ion selective electrodes (IES), anodic stripping voltammetry (ASV), potentiometric stripping analysis (PSA), current stripping chronopotentiometry (CSP) and differential pulse voltammetry (DPV). The disadvantage of all these methods is that they cannot differentiate between bioavailable fraction which is responsible for risk and the unavailable fraction of Hg.

Detection of bioavailable Hg is important because this is the fraction that causes toxicity to plants and animals and also is the substrate for biotic methylation and reduction processes. Biosensors have been used as tools for detection and quantification of bioavailable portion of

Hg in environmental samples. A biosensor combines a biological recognition element (biochemical receptor) and a suitable transduction element that can provide specific quantitative and semi-quantitative analytical information about the bioavailable metal. The recognition element can be an enzyme, whole bacterial cell, DNA or antibody and the transducer may be electrical, optical or thermal (Turdean, 2011). For the detection of Hg, several bacterial biosensors have been constructed by fusing *mer* gene and a reporter gene (Corbisier et al., 1994; Rasmussen et al., 2000). These gene fusions are expressed in the cytoplasm of the bio-sensing bacteria and these respond to intracellular levels of Hg^{2+} or organomercury. Since the transformation reactions of Hg occur in the cytoplasm it has been established that biosensors detect the concentration of Hg available for transformation (Barkay and Wagner-Döbler, 2005). The MerR protein is another common sensing element in Hg biosensors. The reporting elements can be bacterial luminescence (*lux),* green fluorescence protein (*gfp*), β-galactosidase (*lacZ*) or firefly luciferase (*lucFF)* (Hakkila et al., 2004; Hansen and Sørensen, 2000).

So far, a number of approaches have been used to construct whole cell biosensors to detect and quantify Hg. For example, a recombinant strain of *E. coli* MC1061 containing *mer-lucFF* gene fusion could detect Hg from soil sediment samples within a 2 h incubation period followed by 30 min settling time (Lappalainen et al., 2000). A recombinant *E. coli* (Hakkila et al., 2002) containing *merR* and *luxCDABE* from *Photorhabdus luminescence* could detect Hg from 0.001 mg/L to 0.03mg/L (Ivask et al., 2007). A number of green fluorescent protein (*gfp*) based Hg biosensors have been reported (Hakkila et al., 2002; Priyadarshi et al., 2012). A *E.coli* construct DH5α was made with the *merR* gene derived from the Pdu1358 plasmid of *Serratia marcescens* and the *gfp* gene from plasmid Pdb402 which responded to 100-1700 nM (0.02 – 0.36 mg/L) concentration of Hg^{2+} in 5 h, and was stable at very high concentrations of Hg (Priyadarshi et al., 2012). Another GFP based whole cell *E. coli* sensor could detect 0.001 – 0.12 mg/L Hg in 16 h which could simultaneously respond to Zn and Cd (Gireesh-Babu and Chaudhari, 2012).

In the present research, we have constructed a *merA – gfp* based Hg bio-sensing element which was successfully inserted into chromosomal DNA of a Hg resistant bacterial strain. The strain was able to detect nM concentration of Hg in solution within two to five hours.

2. Materials and methods

2.1. Bacterial strains and plasmids

A mercury resistant strain *Sphingobium* SA2 containing *merA* gene (Accession KJ866415) was used as the host strain. The isolate was previously isolated from Hg contaminated soil (Mahbub et al., 2016). A vector plasmid pR2GK (pRSET-B inserted with EmGFP gene and KanR gene) harbouring the reporter gene for the green fluorescent protein (*gfp*), and antibiotic resistance genes *kanR* and *AmpR* was collected from the microbiology laboratory in CERAR, UniSA.

2.2. Construction of sensor plasmid

The pR2GK plasmid was transformed into *E. coli* DH5α competent cells (Biolone) following heat shock procedure (Sambrook and Russell, 2001) and plated on to antibiotic resistant LB agar plate (containing 1 µg/ml mpicillin) and incubated overnight. Single colonies were picked and inoculated into 100 ml of LB medium with antibiotic (ampicillin 1 ug/ml) and plasmids were isolated from overnight culture using a plasmid mini-prep kit (Bioline). The plasmid was purified and digested with BGLII and PstI restriction enzymes. The digested plasmid of 4.5kb was purified from agarose gel using QIAquick gel extraction kit (Qiagen).

DNA was extracted from *Sphingobium* SA2 using genomic DNA extraction kit (Bioline). The primers AF1 5- ATGAACGACTGTTGCAACCG-3 and FR1 5- CGGGGTACCTCACCCGGCGCAGCAAGAC-3 were designed to amplify *merA* gene of 1439 bp size. The PCR conditions in the MyCycler thermal cycler (Bio-Rad) for amplification of *merA* gene was as follows: initial denaturation at 95 °C for 5 min, 38 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extending at 72 °C for 1.5 min. A final extension step was included at 72 °C for 10 min.

Another set of primers were designed to amplify the 340 bp long segment from *merA* gene of *Sphingobium* SA2. The forward sequence BS1 was 5 – GGAAGATCTCGCAGGCCTCACTGAAACTACA – 3 (BGLII site underlined) and the reverse BS2 sequence was 5 – AACTGCAGTCACCCGGCGCAGCAAGACAG – 3 (PstI site underlined). This short segment of *merA* gene was amplified by using PCR conditions as follows: initial denaturation at 95 °C for 5 min, 36 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extending at 72 °C for 1.5 min. A final extension step was

included at 72 °C for 10 min. All the PCR reactions were performed in a 50 µL reaction mixture containing 10 ng template, 10 µM primers, and 25 µL PCR buffer (Mango mix, Bioline) which comprised Taq polymerase, $MgCl_2$ and dNTP. The yield of genomic DNA and all PCR products were checked by gel electrophoresis using 1% agarose and fragments resolved at 80 V for 40 minutes. The specific bands were visualized with a UV trans-illuminator (Bio-Rad, USA) after staining with ethidium bromide. Sizes of the DNA bands were estimated by comparing them to a 1 kb weight molecular marker (Bioline). The PCR products were cut from the gel and purified using a gel extraction Kit (Qiagen).

The 340 bp long segment of *merA* was cloned into pGEMT easy vector (Bioline) and transformed into *E. coli* DH5α competent cells (Biolone) following the manufacturer's protocol. The transformant mixture was plated onto LB agar plate with antibiotic (1 ug/ml ampicillin). The plates were incubated at 37 ºC overnight and single colony was picked and the pGEMT easy vector containing 340 bp long segment of *merA* was purified and digested by BGLII and PstI restriction enzymes to get sticky ends. Now the digested pR2GK plasmid containing *gfp* gene and digested pGEMT easy vector (inserted with *merA* segment) were ligated which resulted in the sensor plasmid construct (pR2GK-merA). The cloned sensor plasmid pR2GK-merA was then transformed into *E. coli* DH5α competent cells (Biolone) by heat shock procedure (Sambrook and Russell, 2001). Large volume of pR2GK-merA plasmid were purified by Plasmid mini-prep kit (Bioline) for the next step.

2.3. Electroporation – mediated transfer of sensor plasmid to competent cells

The pR2GK-merA sensor plasmid containing short segment of *merA* and reporter gene *gfp* was transformed into *Sphingobium* SA2 by electroporation following modification of the standard procedure (Sambrook and Russell, 2001). The *Sphingobium* SA2 was allowed to grow in 50 ml LB media at 25 ˚C until OD reach 0.8 at 600 nm. The cells were then chilled in an ice bath for 20 min and were collected by centrifugation at 4000 rpm at 4 ˚C for 15 min and washed thrice with 10 ml of chilled 10% glycerol. The cells were resuspended in 10% chilled glycerol to a final volume of 100 µL and mixed with 1µg of pR2GK-merA. Electroporation was done by a Gene Pulser Xcell Electroporation system (Biorad) with a single pulse at 25 µF at 2.5 kV. Immediately after the pulse, the cells were mixed with LB and allowed to grow for 2 h at 25 ˚C. The selection of transformed colonies was done on LB agar plates containing 100 µg/L ampicillin and 20 µg/L kanamycin. These doses were

confirmed as minimal sensitive bactericidal concentrations for non – transformed *Sphingobium* SA2. The transformed colonies were expected to grow on antibiotic containing media since the antibiotic resistance genes would have been transferred to the strain's chromosomal DNA because of homologous recombination. The biosensor colony (designated as FLCA) grown on antibiotic media was further purified on a second batch of LB agar plates containing both antibiotics and 3 mg/L Hg. Transformation was further confirmed by checking the expression of a green fluorescent protein by observing fluorescent colonies and cells under the epi – fluorescence microscope using 488 nm excitation and 528 nm emission filters.

2.4. Induction and assay for detection of mercury

A stock solution of 1000 nM inorganic Hg was prepared by dissolving $HgCl_2$ in distilled water, and sterilized by 0.45 μm filters. An appropriate volume of stock solution was added to 5 ml of sterilized LB-Amp broth to get the desired final concentrations of 3, 10, 20, 40, 60, 80 and 100 nM Hg. LB-Amp broth without Hg supplementation was used as negative control. The sensor strain FLCA was grown overnight in LB-Amp media, cells were harvested by centrifugation and washed with phosphate buffer to be used as sensor inoculum in inducing media. The inoculated Hg supplemented and control media were mixed well and 300 μL aliquots of each samples were transferred to 96 well plates for fluorescence reading. The plates were kept for 5 h at 25 ˚C under constant shaking at 120 rpm and fluorescence intensity was measured after 5 h. The fluorescence was detected every 5 min for 20 nM Hg containing media. The optical density (at 600 nm) and fluorescence of all Hg supplemented media were measured after 5 h. All fluorescence measurements were done in a Bio-Tek Synergy HT Multi-Detection Microplate Reader equipped with KC4 software using a GFP filter set with excitation range 488 nm and emission range 528 nm and the optical density was measured using the same reader at 600 nm wavelength.

2.5. Statistical Analysis for biosensor experiment

Detection of mercury by sensor bacteria was done in quadruplicates. Linear regression analyses were carried out using MS Excel 2013 to establish correlation between time and fluorescence intensity, and between Hg dose and fluorescence. One way ANOVA with Tukey's post Hoc analysis was carried out to the 95% confidence interval to observe any significant difference between fluorescence in each mercury supplemented well.

3. Results and discussion

The whole cell biosensor designated as FLCA integrated with pR2GK-merA sensor plasmid in their chromosome successfully emitted green fluorescent protein after induction with 3 mg/L Hg on LB agar plates which were visualised under UV microscope (Figure 2 and 3). There was always some background fluorescence in un-induced cells (Figure 3A) compared to brighter fluorescence in induced biosensor cells (Figure 3B).

This fluorescence indicates successful cloning and expression of the BS-1A plasmid which contained the reporter *gfp* and the short segment of *merA* which was successfully integrated into the chromosome of *Sphingobium* SA2 due to homologous recombination. The present approach prevents any sudden loss of plasmid and it's accidental horizontal transfer to other bacteria because the sensing construct has been integrated into the chromosome. This is unlike most whole cell based Hg biosensors where the sensing element is integrated into the plasmid, rather than in the host chromosomal DNA. Moreover, the host cell used in this study was *Sphingobium* SA2; because it is an environmental bacterium which should provide more sustainability of the biosensor for analysing environmental samples. Previous constructs have used various *E. coli* strains which may not have appropriate physiological abilities to grow and metabolise in the conditions of an environmental sample.

The FLCA sensor successfully responded to nm concentrations of Hg within 2 – 5 h; when it showed linear increase ($R^2 = 0.97$) in GFP fluorescence against time when 20 nm Hg was present in the medium. The negative control which does not contain any Hg supplementation showed no significant increase in GFP fluorescence (Figure 4).

Furthermore, the FLCA biosensor was observed to respond to Hg^{2+} ions ranging from 3 to 100 nm concentrations (Figure 5a). One way ANOVA confirmed that the measured GFP fluorescence differed significantly ($P<0.05$) from each other at 0, 3, 10, 20 and 40 nM concentrations. The average of quadruplicate Y values were plotted against the Hg^{2+} concentrations and the regression analysis showed a linear increase ($R^2 = 0.95$) in fluorescence from 0 to 40 nm Hg (Figure 5b), beyond 40 nm concentrations the linearity was not sustained. The equation obtained from linear regression, $Y = 0.94 X + 65.5$, may be used to estimate Hg^{2+} in the range 3 to 40 nM, where Y is measured fluorescence and X is Hg concentration at nM. The cell density of the FLCA sensor strain was also monitored throughout the study which showed no significant decrease in bacterial density for any of the

experimental Hg concentrations (Figure 5). This indicates that the Hg concentrations used in the present study were not inhibitory to the genetically modified sensor strain FLCA.

The linear increase in fluorescence up to 40 nm Hg is likely to be due to the upregulation of *gfp* gene, however, beyond 60 nm concentration, the saturation of all binding sites led to the inactivation of the regulatory element (MerR). It has been reported that for some whole cell Hg biosensors, the availability of Hg^{2+} to the regulatory element of mercury operon may be reduced due to some negatively charged groups and ligands on cellular binding site (Rasmussen et al., 1997) or interference due to environmental factors such as dissolved organic carbon, salinity and pH (Barkay et al., 1997) leading to a reduction in the sensing range. Further study is required to detect lower limits because in lakes and soils, pico molar levels of Hg frequently occur and many biosensors are currently unable to detect these concentrations. This is also essential where fish have bio-augmented Hg and residual levels of Hg need to be determined (Selifonova et al., 1993). Additional studies are also needed to modify sensors to be used in the upper ranges of Hg concentrations where currently linearity has not been demonstrated.

4. Conclusion

The whole cell Hg biosensor constructed in the present study detected nano-molar concentrations of mercury within 2 – 5 h. Although the linearity of the detection was not consistent at higher concentrations, the sensor can be used for immediate qualitative detection of bioavailable inorganic mercury in a solution which would facilitate making a decision to take further steps for a quantitative approach. Moreover, the integration of the sensing genetic construct into the genome of *Sphingobium* SA2 gave more stability by reducing the chances of plasmid loss and any accidental horizontal transfer to other bacteria. Further study is required to decrease the response times, increase sensing range, and check any interference of other divalent metals and to observe the effect of cell density, pH, organic substances and storage time on the activity of the constructed sensor FLCA.

5. Acknowledgement

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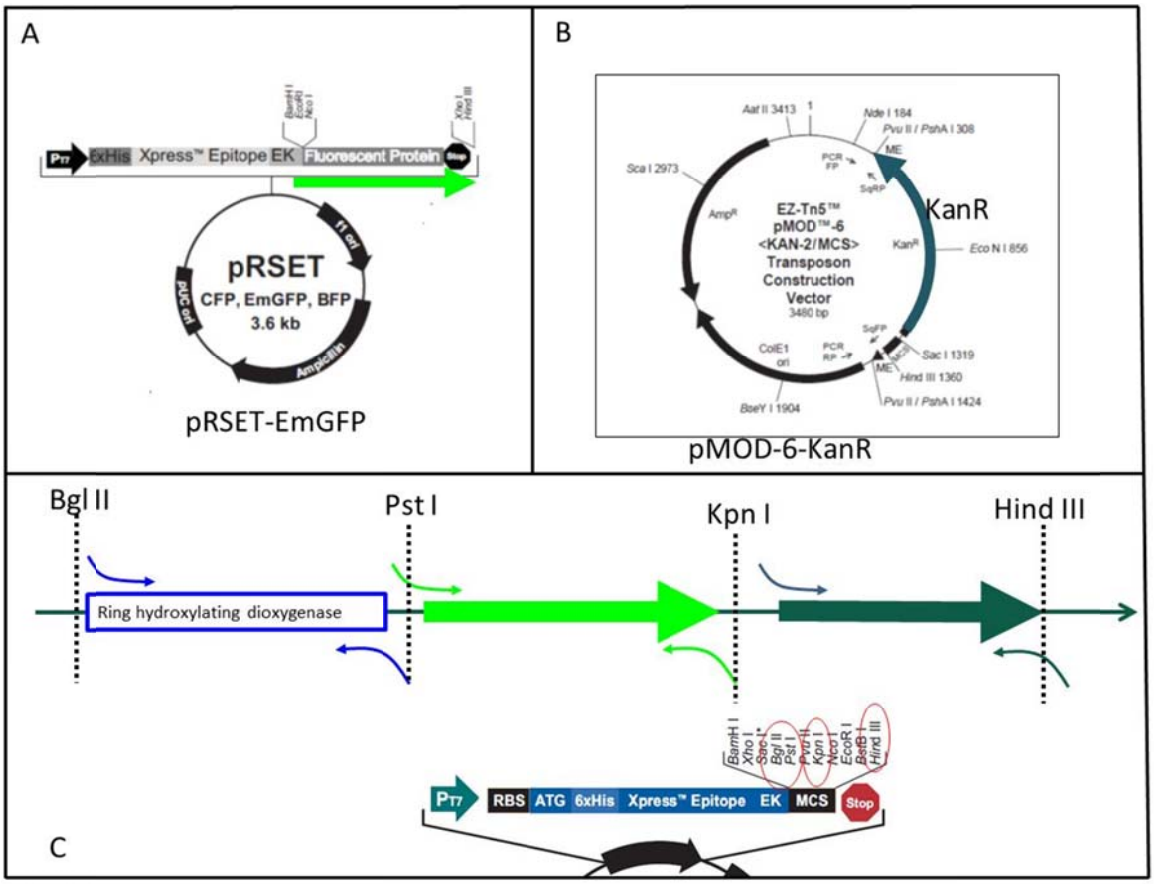

Figure 1(A) pRSET EmGFP plasmid from which EmGFP (shown by the green arrow) was amplified by PCR and used to construct the plasmid, shown in C. (B) pMOD-6-KanR plasmid from which the *KanR* gene was amplified and used in the construct shown in C. (C) Plasmid construct pR2GK.

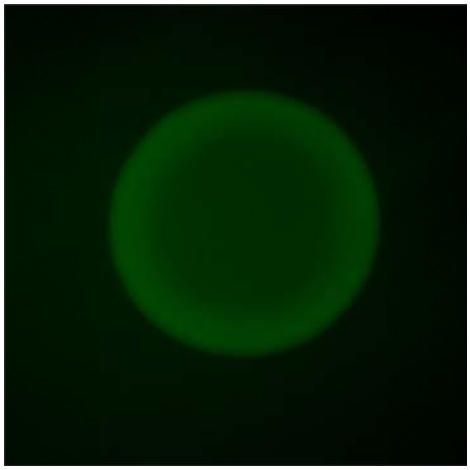

Figure 2 GFP Transformed colony with GFP fluorescence under epi-fluorescence microscope – 488 nm excitation and 528 nm emission at 10X magnification.

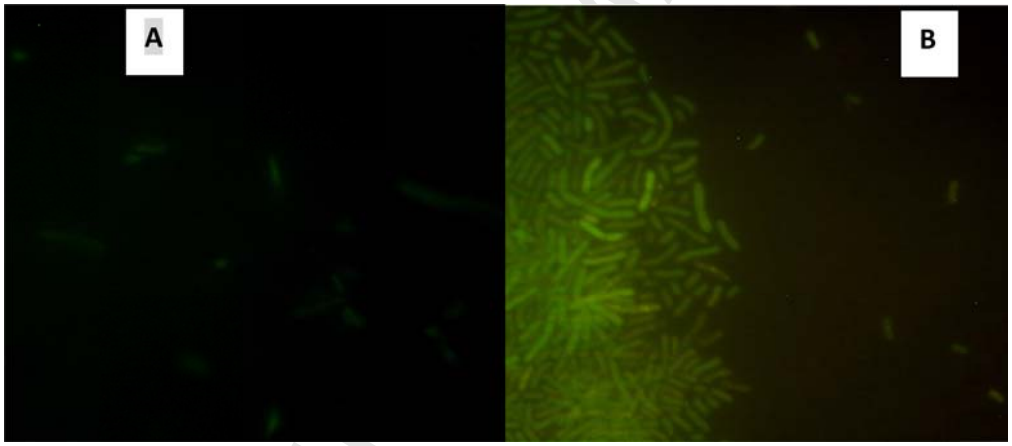

Figure 3 Fluorescent cells viewed under epi-fluorescent microscope with 488 nm excitation and 528 nm emission. A) Control cells with background fluorescence, B) $HgCl_2$ induced cells with brighter GFP fluorescence.

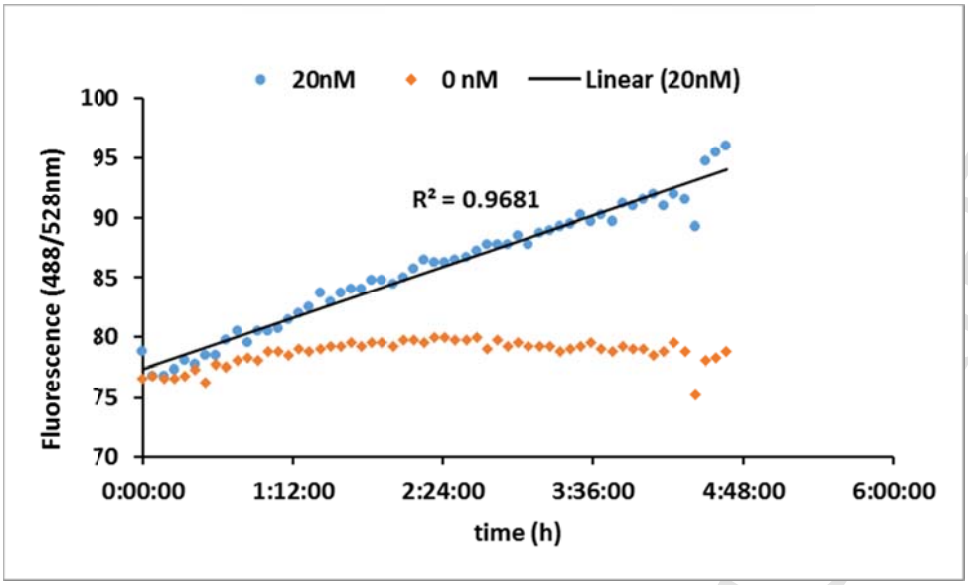

Figure 4 The induction of GFP in the presence of 20 nM mercury at 5 min intervals.

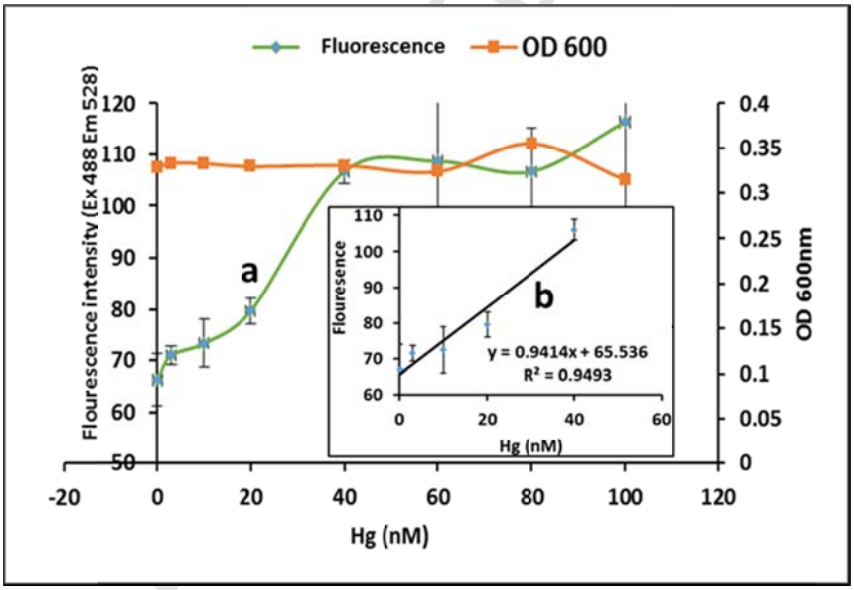

Figure 5 Graph showing fluorescence and absorbance as a function of Hg concentration after incubating biosensor for 5 h. Green line: Fluorescence vs Hg concentration and orange line: OD600 vs Hg concentration at 5 h.

Highlights

- *Gfp* based mercury sensing element was integrated into chromosomal DNA of *Sphingobium* sp. SA2.
- The sensor bacteria responded to 20 nm Hg in 2 – 5 minutes.
- Linear increase in fluorescence was observed at a range of 0 – 40 nm inorganic Hg.