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Development of cadmium specific recombinant biosensor and its application in milk samples

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ABSTRACT

A novel and highly specific recombinant biosensor has been investigated for detection of highly toxic metal ion cadmium (Cd(II)) based on *Escherichia coli* DH5α (pNV12). The working principle of biosensor is based on the expression of *gfp* gene under the control of *cad* promoter and the *cadC* gene of *Staphylococcus aureus* plasmid pI258. *Escherichia coli* DH5α (pNV12) was tested for its response in the presence of different combinations of Pb(II), Cd(II) and Zn(II) ions. The investigation resulted in the development of a cadmium specific recombinant whole cell biosensor with a response time as low as 15 min. A good linear range for cadmium ion concentrations of 10–50 μg/l with R^2 0.9946 was obtained and the detection limit of 10 μg/l. Further, we reduced the detection limit up to 5 μg/l by increasing the incubation time from 15 to 30 min. High throughput microarray techniques for testing of 48 samples simultaneously has also been developed with reaction volume miniaturized from 2000 μl to 200 μl. The developed biosensor was applied to detect cadmium ion concentration in milk samples collected from different areas of Punjab, India. Two samples collected from industrial areas, shown to have cadmium concentration above permissible limits (10 μg/l).

1. Introduction

Some heavy metals nickel, cobalt and zinc are essential in trace amounts for the growth of living beings, whereas others for example cadmium and lead which have no known beneficial roles and are toxic at higher concentrations [1]. There are certain microbes that have developed different mechanisms e.g. precipitation, metal efflux, suppression of influx and enzyme detoxification etc. to deal with metal concentrations and grow in metal contaminated zone [2]. For conventional detection of cadmium metal ion atomic absorption (AA) spectroscopy, inductively coupled plasma (ICP) optical emission spectrometry and inductively coupled plasma mass spectrometry (ICPMS) are widely used [3]. Although, these methods are sensitive and allow discrimination of different metal ions however, they require tedious sample pretreatments (e.g., desalting, filtration and concentration) and are time consuming. Therefore, it is necessary to develop a rapid, simple and specific detection method to assured the presence of metal ions.

A whole-cell bioassay has been developed for total toxicity testing of liquid samples. The method is based on the induction of the bioluminescent activity of genetically manipulated mammalian cells. For that purpose, a DNA sensing element that responds to chemical stress agents (heavy metals, genotoxic agents, and endocrine-disrupting chemicals) was transfected in HeLa cells. Such element was designed to direct the expression of a reporting gene (firefly luciferase) through the activation of *Drosophila melanogaster hsp22* promoter [4]. Although, there is many other stress causing reasons such as genotoxicity, endocrine disruption etc. to induce the *luc* gene expression, therefore this approach is not suitable in the term of specific detection of cadmium metal ion.

A new type of membrane containing immobilized whole cell microalgae was used for development of cadmium biosensor. In this the *Chlorella vulgaris* alkaline phosphatase activity (APA) was inhibited in the presence of cadmium ion and the detection limit achieved was 1 ppb [5]. Silwana et al. [23] was reported an amperometric biosensor based on enzyme horseradish paroxidase immobilized in polymeric material on platinum electrode. The enzyme showed inhibition in the presence of cadmium, lead and mercury metal ions with detection limit 8×10^4 ppb for cadmium metal ion. Qu et al. [24] have used ssDNA/Au probe for the detection of various heavy metal ion including cadmium. Although they achieved a very low detection limit up to 0.3×10^{-3} ppb, but selec-

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tivity for cadmium metal ion has not been quoted clearly. Present study is an effort to develop a cadmium specific biosensor with a lower detection limit by expressing GFP variant under the control of cadmium specific promoter.

Recently, Matsuura and coworkers [6] have developed a recombinant biosensor for the detection of cadmium and other toxic metals in environmental samples. They targeted the gene which expresses enzyme phytochelatine synthase (PCS) for which heavy metals act as cofactor. They have engineered PCS gene into yeast cell and the whole recombinant cell were used to construct a recombinant biosensor for the detection of cadmium. In the same way, a specific and sensitive biosensor for cadmium ion was developed [7]. In this approach two promoter sequences CadR-TC10 and CadR-TC21 were redesigned by truncating 10 and 21 amino acids from the C-terminal extension of CadR, respectively. Genes of CadR, CadR-TC10 and CadRTC21were used to construct the sensing element of the GFP based *E. coli* biosensors. A comparison of various biosensors for the detection of cadmium metal is listed in Table 2. Most of the developed biosensors are not specific for cadmium and have an interference of other metals *e.g.* Pb(II), Zn(II) etc. hence the study was carried out to develop a biosensor with specificity towards cadmium and application of the same to detect cadmium in milk.

Some of mechanisms are very specific and precisely regulated by genetically encoded resistance. The resistance mechanism against cadmium in *Staphylococcus aureus* due to plasmid pI258 was identified by Novick & Roth [8], and Endo & Silver [9], *cad*A operon from *Staphylococcus aureus* plasmid pI258 consist of two genes i.e. *cad*A and *cad*C in which *cad*A gene encodes for an energy-dependent ion pump for cadmium efflux [10] while *cad*C gene encodes for the regulatory protein. Contamination of milk with toxic heavy metals ions is considered most alarming as it is largely being consumed by infant and children [27]. The main source of cadmium in milk is through food chain, as plant and grasses grown in contaminated soil are fed by cattle [28,29]. Many studies have been carried out for estimation of cadmium in milk samples [30–32]. This sort of studies give an alarm that milk can serve as a potential source of cadmium for human community, and could be more toxic even at lower concentration in many cases. As it has also been reported that cadmium absorption increases with increase in fat and protein contents and milk is rich in fat and protein [33].

In the present study *cad* promoter and *cadC* gene was amplified from plasmid pI258 and ligated to the upstream to a modified promoter-less gene encoding a variant of green fluorescent protein in pAD123. A new plasmid pNV12 has been constructed, and used as sensing element for cadmium metal. The specificity and sensitivity of the developed biosensor was also evaluated. The system tested in real and spiked milk sample and validation of the sensor response was done using F-AAS.

2. Experimental

2.1. Materials

Tryptone, Yeast Extract, ampicillin, chloramphenicol, C-TAB, Tris-HCL, Na_2EDTA, LiCl, NaCl, $CdCl_2$, $ZnCl_2$ and $Pb(NO_3)_2$ were procured from Himedia Labs Pvt. Ltd. Mumbai, India. Chloroform, Isoamyl alcohol and Isopropanol were procured from Qualigen fine chemicals Mumbai, India. Restriction enzymes *Eco*RI and *Bam*HI, DNA-polymerase, primers used for PCR amplifications were procured from B. Genei. MSB Vario Cleanup PCR purification Kit (Cat. No. 10202302) was procured from Invitek, Germany while Hipura Agarose gel DNA extraction kit (Cat. No. MB-511-50PR) was purchased from Himedia. Skim milk powder, BCR certified reference material grade was procured from Sigma-Aldrich, Mumbai, India.

2.2. Bacterial strains and plasmids

Bacterial strains and plasmids used in the study are *Staphylococcus aureus* (pI258), *Escherichia coli* (pAD123) and *Escherichia coli* DH5α. *Staphylococcus aureus* (pI258)—NCTC 50581 was procured from National Collection of Type Cultures, Colindale, London, UK. *Escherichia coli* (pAD123) and *Escherichia coli* DH5 α were provided as a generous gift by Bacillus Genetic Stock Centre (BGSC), Department of Biochemistry, Ohio State University. Plasmids containing bacterial strains were maintained on L-agar (LA) plates.

2.3. Construction of plasmid pNV12

The strategy for the construction of recombinant plasmid pNV12 has been depicted in Fig. 1. *cad* promoter and *cad*C of pI258 were amplified through PCR and cloned into pAD123 upstream to promoter-less gene encoding a variant of green fluorescent protein in a promoter-trap plasmid, pAD123 [11]. Plasmid pNV12 was constructed employing standard recombinant-DNA techniques [12]. Plasmid pI258 was isolated from *S. aureus* (NCTC 50581) following Atashpaz et al. [13] with some modifications and used as a template for polymerase chain reaction (PCR) using primers 5′-ATAT**GAATTC***GTGTATTTTTTAATAAATTATTTTACTT*-3′ at the beginning of the *cad*C gene and 5′-TTAA**GGATCC**CCTT*TCAGACATTGACCTTCAC*-3′ at the end of the *cad*C gene [14] to generate a fragment containing *cad*C gene and promoter/operator of the *cadA* operon with *Bam*HI and *Eco*RI restriction sites at ends. In Primer sequence restriction sites of *Eco*RI and *Bam*HI have been bolded and bases in italics correspond to the *cadC* gene. DNA was amplified using RT-PCR miniopticon (Bio-Rad) with an initial Denaturation step of 3 min, followed by 20 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min. After 20 cycles, sample was kept for 5 min at 72 °C for a final extension followed by incubation at 4 °C for 5 min.

The resulting PCR product of 572 bp was purified with MSB Vario cleanup PCR purification kit (Invitek Germany) and digested with *Eco*RI and *Bam*HI and repurified from an agarose gel by Hipura agarose Gel DNA extraction kit (Himedia). The resulted fragment was ligated to *Eco*RI and *Bam*HI sites of double digested plasmid pAD123 with the same restriction enzymes, thus generated plasmid was named pNV12 was transformed into *Escherichia coli* DH5α through competent cell method [15].

2.4. Cultivation of microbes and fluorescence measurement

Staphylococcus aureus (pI258) was cultured in LB medium containing erythromycin (50 μg/ml) at 37 °C and 200 rpm while *Escherichia coli* (pAD123) and *Escherichia coli* (pNV12) were cultured in LB-medium (10 g Tryptone, 5 g Yeast extract, 5 g NaCl, and Distilled water 1 L, pH 7.0) supplemented with ampicillin (100 μg/ml) and chloramphenicol (10 μg/ml) at 37 °C and 200 rpm.

For the assay a single colony of *E. coli* harboring pNV12 was grown overnight in LB media supplemented with ampicillin, chloramphenicol at a concentration of 100 μg/ml and 10 μg/ml respectively at 37 °C, 200 rpm. Next day the overnight culture was diluted 100-fold in fresh LB medium supplemented with ampicillin, chloramphenicol and incubated at 37 °C, 200 rpm until the optical density at 600 nm (OD600) reached 0.6. Thus prepared culture was used for the bioassay.

2.5. Biosensor assay route

For biosensor assay, 400 μl of diluted milk sample (200 μl sterile DI water + 200 μl milk (from reference material)) was added to 1.6 ml of fresh culture of *E. coli* harboring pNV12 (OD = 0.6 at