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Whole cell based disposable biosensor for Cadmium detection in milk

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ABSTRACT

In the current work authors report the development of a biosensor for the detection of cadmium and its application in milk. Bacillus badius cells were immobilized onto the nylon membranes using sol-gel approach with alcohol and TEOS. The cells were mixed with sol-gel and poured onto nylon membrane and then dried. Hydrosol-gel immobilized cells were used as a biocomponent for the study. Ammonium ISE used as transducer with bioassay principle based on inhibition of urease activity. The lowest detection limit achieved was 1.0 µg/l of Cd. The prepared biosensor was applied to natural and fortified, acid extracted milk samples. The linear range of detection is between 10µg–1mg/l for Cd. Storage stability of the developed biosensor is 65 days when stored at 4^0C in 10% glycerol. A good correlation of results was obtained with spiked samples.

Keywords: Bacillus badius, whole cell Biosensor, Milk, Cadmium, Sol-gel, Urease.

INTRODUCTION

Cadmium is the seventh most toxic substance according to the list of toxic substances named as "Top 20 hazardous substances" released by Agency for Toxic Substances and Disease Registry, a part of U.S. Department of Health and Human Service. Cadmium is toxic to a wide range of organs and tissues. Target organs of cadmium include almost all important parts of the body e.g. liver, placenta, kidneys, lungs, brain and bones. Chronic exposure to cadmium is known to cause anemia, arthritis, learning disorders, migraines, growth impairment, emphysema, osteoporosis, loss of taste and smell, poor appetite and cardiovascular disease as well as toxic effect on reproductive system. Other Cd toxicity symptoms in human include hypertension, cancer and immune disorders [1].

Cadmium acts as a cancer promoter through mutagenic effects on gene expression and produces malignant tumors in experimental animals [2]. Cadmium may enter the body through food, water, air or absorption through the skin however food and smoking are the main source of exposure in the non-occupationally exposed population [3]. Concentrations of cadmium and lead

in human milk in the Greater Accra region of Ghana were 0.0246±0.0116µg/l (range 0.0085-0.0500µg/l) and 0.0329 ±0.1263µg/l (range 0.0122-0.0644µg/l) in Accra and Tema respectively while the mean for lead levels was 2.476 ± 1.097µg/l (range 0.0456 - 5.224µg/l) and 3.367 ±1.131µg/l (range1.375 -5.890µg/l) in Accra and Tema respectively. Level of both metals was found significantly increased in breast milk of women passively exposed to smoking compared to non exposed ones [4].

Studies suggest that bioavailability of cadmium is different for different food source. Marginal deficiencies of essential nutrients like Zn and Fe enhance the Cd absorption as much as tenfold from the diets containing low Cd concentrations similar to that consumed by populations. As it has been studied that cadmium absorption is increased with an increase of fat and protein content, milk being rich in fat and protein and usually devoid of iron and zinc, is a potential contender for Cd exposure to humans [5-6]. Milk being inevitable component of daily food may become a potential source of cadmium exposure; hence milk was taken as a candidate for cadmium detection in present study. Survey of heavy metal contents of the food crops (cassava mash and maize grains) reports Cd concentration of 0.210-0.410µg/g [7]. As far food of animal origin is concerned bioaccumulation of cadmium in the different fish species has been reported by different workers, accumulation of cadmium is reported to be second highest after copper [8-9]. Since heavy metals are not metabolized they have a tendency to be carried over from the animal feeds to food of animal origin (meat, organs, milk & eggs etc.), cadmium enters the milk from animal feed via blood [10].

The statewide survey in California had shown that mean concentration of cadmium in 320 raw milk samples was 6µg/kg [11]. Studies by Alonso *et al.,* suggest that dairy cattle may be more susceptible to the accumulation of Cd and Pb than beef cattle [12]. The average cadmium content in milk from different farm was found to be 0-20µg/l [13]. Cadmium being nontoxic to plants has the greatest potential for transmission through the food chain [14](Gupta, 2006) unlike other metals that are phytotoxic and experience a plant barrier which limits their transmission through food chain.

Concentrations of Cd in complete dairy rations were found closest to US maximum acceptable concentration [15]. Ayer *et.al*, found the concentration of Cd in different milk and dairy products to be ranging from 9µg/Kg in whey powder and yogurt to 91µg/Kg in tulum cheese in middle Anatolia, Turkey [16].

The standard techniques for trace heavy-metal analysis in milk include Atomic Absorption Spectrometry (AAS) and Inductively Coupled Plasma-Mass Spectrometry (ICPMS). Differential pulse polarographic determination of cadmium, lead and copper in milk has also been carried out [17]. Virtually all of these methods involve complicated and time-consuming sample treatment and pre-concentration steps that can be carried out only by trained professionals. This prohibits screening for heavy metals at various stages of food production and hinders the objective of preventing heavy metal contamination as early as possible in the production chain.

Electrochemical methods are seen as complementary to the aforementioned techniques and are especially attractive because they allow the possibility of creating inexpensive and portable instrumentation (e.g. PalmSens for differential pulse voltammetry). In the present study an electrochemical biosensor for the detection of cadmium in milk has been developed and tested

with spiked milk samples. Present biosensor is different in its novel source of biocomponent *i.e. Bacillus badius* that has got different enzyme kinetics for urease and rather specificity towards Cd, lower detection limit, application in milk samples, detailed comparison with previously made biosensors is made in discussion part.

MATERIALS AND METHODS

Reagents

All chemicals used were of analytical grade. The microorganism used in the study (*i.e. B. badius)* was an isolate of lab itself.

Strain and Culture conditions

B. badius, MTCC 8082 had been isolated in the Biosensor Technology Lab, Dept. of Biotechnology, Punjabi University Patiala. The novel isolate *i.e. B. badius* is a urease producing micro-organism, has been isolated from urea rich soil near National Fertilizer Limited (NFL), Nangal, India, cultured at 37^0C and 200 rpm, in a nutrient medium containing Beef Extract 10g, Peptone 10g Sodium Chloride 5g and Urea at a concentration of 25g/l, pH of the medium was kept 7.0–7.5. Culture was harvested and a 10%glycerol stock of *B. badius* was prepared with an average OD of 1.0 at 600nm for further study.

Kinetic characterization

Kinetic characterization of the enzyme was performed in absence and presence of Cd^{2+}. Micromoles of ammonia released due to enzyme activity were estimated with Nessler's method. Km and Vmax values were determined accordingly.

Calibration of NH_4^+ISE (Ion Selective Electrode)

The transducer was a potentiometer (Cyberscan-2500) in conjunction with a NH_4^+ ion selective electrode that detects the electrode potential developed across the membrane of the electrode when it comes in contact with ammonium ions released as a result of urea hydrolysis. For calibration of ion selective electrode (ISE), stock solution of ammonium chloride was prepared with a concentration of 0.55×10^{-1} mol/l, made different dilutions with the concentration varying 10 folds each time till 0.55×10^{-5} mol/l to have NH_4^+ ion standards. 5 mol/l NaCl was used as ionic strength adjuster (ISA); 2 ml of ISA was added to every 100 ml of standard solution to maintain a background ionic strength of 0.1 mol/l. Effect of Cd^{+2} ions on ISE was also studied by adding solution of different Cd concentrations to NH_4^+ standard.

Construction of Biosensor

Biomass of 21 h grown *B. badius* was centrifuged at 5000rpm, for 10min at 4^0C, suspended in 250µl of 10% glycerol and OD was set to be 1.0 at 600nm. Sol-gel was prepared by mixing 570µl ethanol, 50µl Tetra Ethyl Ortho Silicate (TEOS), 10µl NaOH (5mM) and 60µl water, incubated at 4^0C for 1 h. 100µl of this preparation was mixed with 50µl of suspended biomass and poured onto the nylon membrane, dried and stored at 4^0C for further application. The immobilized biomass is brought in close proximity to ISE electrode with O ring and dipped into the substrate solution with and without cadmium ions. An already optimized urea concentration of 100 mM was used for the study. Urea hydrolysis was studiedc with ISE in the absence and presence of different Cd^{2+} concentration (1µg-1mg/l) by noting change in potential (ΔmV/min;

Fig-1). The response time for hydrolysis was also determined. The biocomponent together with the transducer form a portable system giving a continuous real time analysis.

Application of the developed whole cell biosensor

The developed biosensor was used to monitor Cd^{2+} in raw milk and spiked milk samples. Milk samples were procured from Verka milk booth and spiked with different concentrations of cadmium. Acid extraction of Cd^{2+} from milk samples was achieved by adding 2-3 drops of HNO_3 acid in 10 ml of milk sample followed by centrifugation at 3400 g for 20 min at 4^0C.

The supernatant was taken; pH was brought back to 7, added urea to have a final concentration of 0.1 mol l^{-1} and used for the study. For the study immobilized biocomponent was coupled with ISE and dipped into 10 ml of the synthetic solution/milk sample taken in a cell with 100µl of ISA (5 mol/ l NaCl), the change in potential was noted after10 min. The linear range of detection was determined by using deferent concentration of Cd^{2+} ranging from 1 µg/l to 1 mg/l. The effect of other metal ions on inhibition of urease activity was studied with a concentration of 100 µg/l for each metal. Storage stability of the biocomponent part of biosensor when stored at 4^0C in 10% glycerol was also checked.

RESULTS

Kinetic characterization

Kinetic characterization of enzyme studied in the presence and absence of Cd. Enzyme activity is inhibited non-competitively with a Km value of 2.0 m mol/l. V_{max} is 86.96µ mol/l/min in absence of inhibitor and decreased to 60.24, 51.28 and 44.44 µ mol/l/min in the presence of 100 µg/l, 1 mg/l and 2 mg/l Cd respectively (Fig.-3). *B. badius* is a novel isolate and there seems to be strain improvement through chemostat process. A comparison of kinetics for urea hydrolysis as shown in Table-1 reveals that K_m value of urease of *B. badius* is low in comparison with other micro-organism. The activity of urease by different metals is inhibited in order of Cd^{2+}> Ni^{2+}> Cu^{2+}> Zn^{2+}> Co^{2+}> Fe^{3+}> Pb^{2+}. The inhibition orders of urease activity previously reported in literature are Ag^{+}> Hg^{2+}> Cu^{2+}> Cd^{2+}> Co^{2+} > Ni^{2+} > Mn^{2+} with Pb^{2+} unassigned but less than Cu^{2+} [18], Ag^{+} = Hg^{2+}> Cu^{2+}> Cd^{2+}> Co^{2+} > Ba^{2+} > Zn^{2+} > Ni^{2+} > Fe^{2+} > Cr^{3+} > Mn^{2+} > Sr^{2+} > Pb^{2+}> Al^{3+} [19] and Hg^{2+}> Cu^{2+}> Zn^{2+}> Cd^{2+}> Ni^{2+}> Pb^{2+}> Co^{2+}> Fe^{3+}> As^{3+} [20]. Hence urease from the isolated micro-organism in the study is showing more affinity towards Cd.

Calibration curve of Ammonium ISE

The ammonium ion electrode was calibrated using standards of NH_4Cl solutions in the concentration range of $0.55x\ 10^{-1}$ mol/l to $0.55x\ 10^{-5}$ mol/l. The calibration curve is shown in fig. 2, slope value is 57.22. There was no effect of addition of Cd^{2+} in NH_4^{+} standard on ISE reading.

Whole cell biosensor

Urea is hydrolyzed to give a reasonable change in potential (Δ mV) within10 min thus considered as response time which is quite less in comparison to other workers [37-40]. Urease activity has been inhibited in the presence of cadmium ions as low as 1.0 ppb. There is a decrease in Δ mV with increase in cadmium concentration (Fig.-4). No inhibition of urease activity was observed below 200 µg$l^{-1}$of lead ion under similar conditions. The lowest detection limit is 1.0 µg/l of Cd *i.e.* lower than biosenosrs developed by other workers [41-45]

Application of the developed whole cell biosensor

The developed biosensor was used to monitor Cd in natural and spiked milk samples. The Cd equivalent concentration was found to be 6 ppb in the Verka milk sample which is safe, within the permissible limit defined by EPA (1975) i.e. 0.010 mg l^{-1} for drinking water. Permissible limit for Cd ion in milk sample has not been defined, not found in the literature. The reliability of the developed biosensor was checked by spiked samples. A good correlation of results was obtained with spiked samples (Table-2). Reliability ranges from 85.71% to 99.94% for 6 ppb to 1ppm Cd spiked in milk samples (Fig-4b). The storage stability of the biocomponent was found to be 65 d when stored at 4^0C in 10 % glycerol.

DISCUSSION

Tauriainen *et al.*, constructed a recombinant plasmid by inserting the regulation unit from *cad*A determinant of plasmid pI258 to control the expression of firefly luciferase. The resultant plasmid was expressed in two different strains *Staphylococcus aureus* strain RN4220 and *Bacillus subtilis* strain BR 151 thus produced luminescent bacterial sensor for cadmium and lead. Strain BR 151 responded to cadmium at 3.3 n mol l^{-1} while Strain RN4220 responded at 10nM; the results were obtained with 2-3 hrs incubation [37]. Present biosensor is faster in response and comparatively sensitive. May and Russell developed a biosensor based on changes in structure of urease enzyme after binding with cadmium being the basis of surface plasmon resonance biosensing system. The enzyme was modified with N-succinimidyl 3-(2-pyridylthiol) propionate (SPDP) to facilitate the formation of a self assembled monolayer of urease on the gold coated glass SPR sensor disk. It is this change of enzyme monolayer measured by SPR, which has been related to the cadmium ion concentration in the range of 0-10 mg l^{-1}[41]. Current study is novel and significant for its source of enzyme *i.e.* urease, bio-sensing system with a quick response time; having a significantly low detection limit (1µgl^{-1}).

Table-1: Comparison of kinetics of urease from different microbial sources

Micro-organism	Km (m mol l^{-1})	References
Providencia rettgeri	10.5-71	[21]
Spirulina maxima	0.12	[22]
Arthrobacter oxydans	12.5	[23]
Aspergillus nidulans	1.33	[24]
Staphylococcus saprophyticus	7.36	[25]
Bacillus pasteurii	40-130	[26-27]
Providencia stuartii	9.3	[28-29]
Proteus mirabilis	13	[30]
Corynebacterium renales	30	[31-32]
Helicobacter pylori	0.18	[33]
Selenomonas ruminatium	2.2	[34, 26]
Brevibacterium ammoniagenes	18-72	[35]
Vibrio parahaemolyticus	35.6	[36]
B. badius	2.0	Present study

A conductometric biosensor using immobilized *Chlorella vulgaris* microalgae was used as a bienzymatic biosensor [38] with limit of detection 10 ppb for Cd after 30 min long exposure based on alkaline phosophatase, acetyl choline transferase inhibition. Liao *et al*., [39] developed a GFP based biosensor *E coli* DH5α (pVLCDI) carrying GFP under the control of *cad* promoter

and the *cadC* gene of *Staph. aureus* plasmid pI258. DH5α (pVLCDI) responded to Cadmium 0.1n mol l^{-1}being the lowest detectable concentration with 2 hr exposure. Haron and Ray developed an optical biosensor for cadmium and lead by employing the technique of total reflection at the interface between Si3N4 core and composite polyelectrolyte self-assembled (PESA) membrane containing cycloptetrachromotropylene (CTCT) as an indicator; achieved a detection limit as low as 1ppb for both the metals [44]. Present biosensor is quick with a comparable sensitivity.

Table-2: Change in Potential with varying concentration of cadmium

Conc. of Cd	Change in Potential ± SD
0	31.9mV ± 0.8
1.0 µg/l	30.1 mV ±1.2
10 µg/l	24.9mV ±1.1
50 µg/l	18.1mV ±0.9
0.1 mg/l	14.5mV ±0.6
1.0 mg/l	11.7mV ±1.1
Raw Milk Sample	26.1mV ±1.3

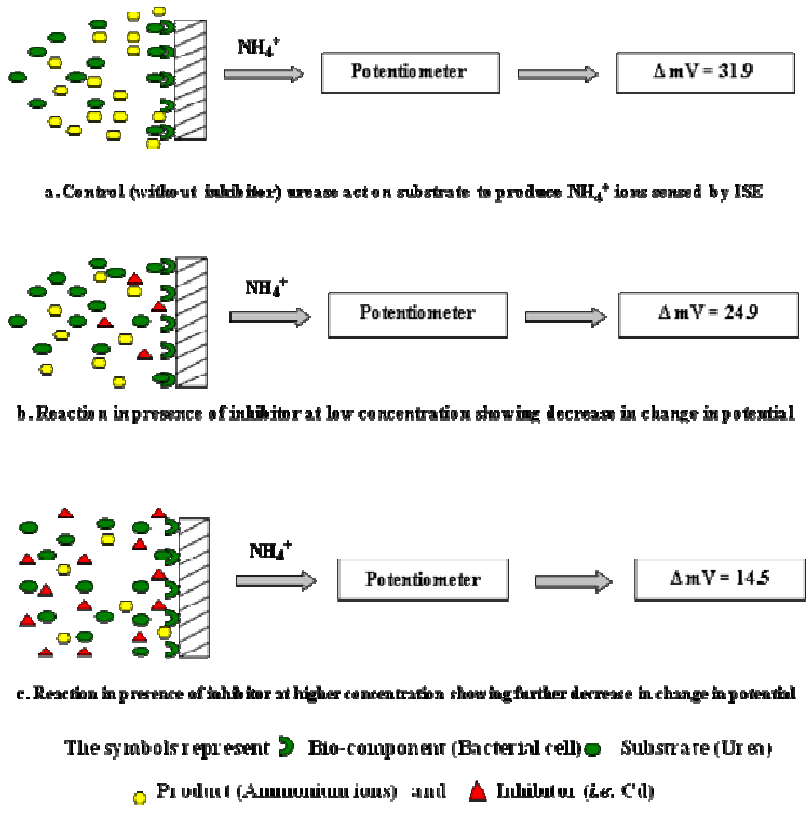


Figure 1: Schematic representation of bioassay principle how the sensor operates, production of NH_4^+ ion causes the change in potential, in presence of Cd urease change in potential (ΔmV) decrease due to inhibition of enzyme. ΔmV decreases further with increase in concentration of Cd.

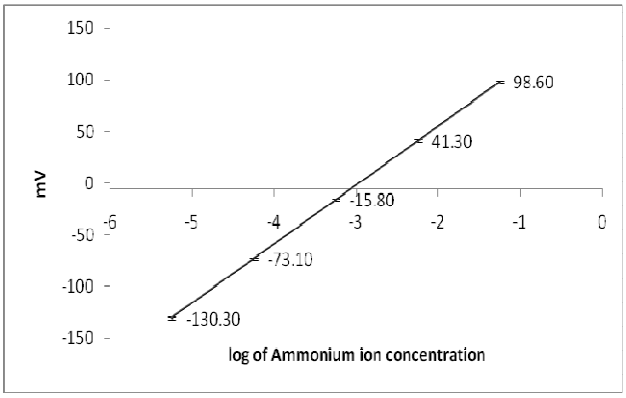


Fig- 2: Calibration curve of NH_4^+ ISE

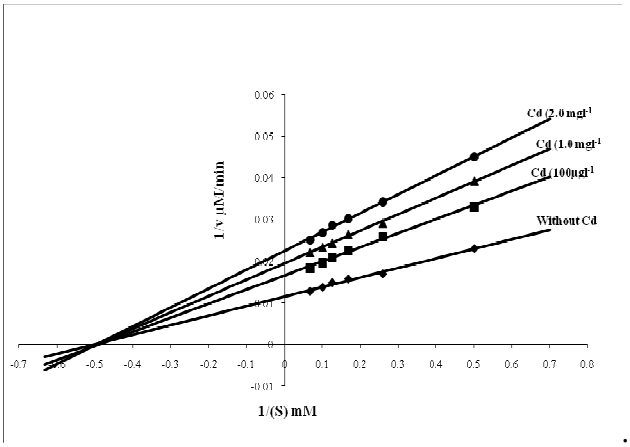
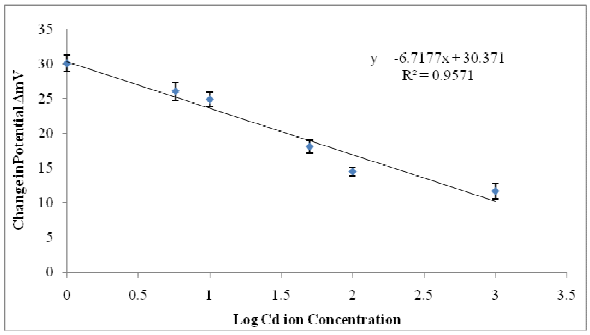


Fig- 3: Graph showing non-competitive inhibition, change in V_{max} in the presence of different concentration of Cd with no change in Km value

Chong et al., developed a whole cell biosensor on a diamond electrode. Unicellular microalgae *Chlorella vulgaris* was entrapped in the BSA membrane and immobilized directly onto the surface of a diamond electrode for heavy metal detection [45]. The cell based diamond biosensor could attain a detection limit of 0.1ppb for cadmium. Application of developed biosensor is not highlighted.

A novel transmission-based localized surface plasmon response (LSPR) fiber-optic probe has been developed to determine Cd ion concentration. The LSPR sensor was constructed by immobilizing phytochelatins (PCs), (gammaGlc-Cys) (8)-Gly, onto gold nanoparticle-modified optical fiber (NM (Au) OF); got a detection limit of 0.16ppb. The sensor retained 85% of its original activity after nine cycles of deactivation and reactivations; in addition sensor retains its activity upto 35 d at 4^0C in 5% d-(+)-trehalose [46]. Application of the developed biosensor has not been highlighted. Present biosensor is more stable up to 65 d at 4^0C in 10% glycerol.

(a)



(b)

Figure 4: (a) Bio-component (membrane with immobilized bacterial cells) in close proximity of transducer i.e. tied with ISE, immersed in sample solution (b) Change in potential (Δ mV) decreased with increasing Cd ion concentration

CONCLUSION

Conclusively the study has resulted in the development of a whole cell based biosensor to monitor cadmium equivalent level in milk. In comparison with previously developed biosensors, present biosensor has fast response, novel in its source, lower detection limit, rather specificity for Cd, higher storage stability. A good correlation of results was obtained with spiked samples. The present biosensor has the advantage of portability, simplicity, reliability and continuous real time analysis. Pre-concentration on cadmium specific column can further improve the detection limit and specificity for Cd ions in milk.

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