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Zinc finger peptide based optic sensor for detection of zinc ions

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ABSTRACT

In the present work, polyacrylamide gel has been used as a matrix for the immobilization of zinc finger peptide and fluorescent dye acrydine orange on the micro well plate to fabricate the fluorescence based biosensor for the detection of zinc ions in milk samples. The fluorescent dye moves in the hydrophobic groove formed after folding of the peptide in the presence of zinc ions. Under optimized conditions, linear range was observed between 0.001 μg/l to 10 μg/l of Zinc ions, with a lowest detection limit of 0.001 μg/l and response time of 5 min. Presented biosensor has shown 20% decrease in fluorescent intensity values after 5 regenerations and stable for more than one month, stored at 4 °C. Interference study with other metal ions like lead, cadmium and copper showed a negligible change in fluorescence intensity in comparison to zinc ions. Developed bio sensing system was found to be novel, quick, reliable, miniaturized, stable, reproducible and repeatable and specific for zinc ion, which has been applied to various milk samples.

1. Introduction

Zinc (Zn) metal acts as a micronutrient which is essential to our body and has a range of biological roles but when its amount exceeds in the body than normal, this metal proves to be lethal. Both acute and chronic forms of zinc can be toxic (Osredkar and Sustar, 2011). Increase in zinc level in the body than normal, results in severe health damages like anemia, nausea, vomiting, skin problems, and stomach aches. Higher zinc content, destructs the pancreas by disturbing the protein metabolism causing arteriosclerosis. Exposure to the air containing zinc metal can result in to a metal fever by causing flu like symptoms (Moyo, 2014). Higher concentration of unnatural zinc in the environment is due to industrial sources such as mining, coal and waste combustion, steel processing, toxic waste sites, and other human activities. Symptoms of airway irritation and inflammation are caused by airway exposure to zinc dust and zinc-containing ambient particulates (Gu and Lin, 2010). In drinking water, zinc concentration is magnified, when it is stored in metal tanks. In natural surface water, concentration of zinc is usually below 10 μg/l. In ground water, its concentration is 10–40 μg/l. In tap water, the zinc level can be much higher as a result of the leaching of zinc from piping and fittings (World Health Organization guidelines for drinking water quality 2013).

Zinc metal can also enter in to dairy and milk products through grazing of dairy animals in the soil containing un-natural zinc, which could affect the health of infants and adults. Mother's milk containing large amount of zinc can be a lethal to unborn and newly born children (Moyo, 2014). Due to 150–450 mg of zinc per day intake, altered iron function, reduced immune function, genitourinary defects, copper deficiency and reduced levels of high-density lipoproteins can occur (Johnson et al., 2007).

Zinc concentration should not exceed more than 20 mg/day in healthy individuals (Maret and Sandstead, 2006). The Recommended Dietary Allowance (RDA) for women is 8 mg/day and for men it is 11 mg/day (Connie and Christine, 2009). The Permissible limit for Zinc in milk is 0.3–1 mg/l (According to Codex Alimentarius Commision).

Due to the above mentioned problems, there is a foremost requirement to develop greatly selective, rapid, sensitive and reliable method to analyse the concentration of zinc metal ions in various samples which are causing severe problems by entering in to the ecosystem more than the required level through different sources. As milk is the main source of daily diet for infants and adults, so milk was selected for this particular study. Zinc can be transferred from animal feed to food produced by animals like milk and other dairy products and enters the food chain results in severe health effects to the consumers.

Analysis of a the metals could be done by using several conventional techniques like Atomic absorption spectrophotometry (AAS), Differential pulse polarography, Electrothermal AAS (ETAAS), Differential pulse cathodic stripping voltammetry

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(DPCSV), Inductively coupled plasma mass spectrometry (ICP-MS), Dynamic reaction cell inductively coupled plasma mass spectrometry (DRC-ICP-MS) and Electrochemical metal analyzer (Verma and Singh, 2005). To perform these techniques, there is a requirement of highly experienced and trained lab operators. Sample pre- treatment causing the process to be very much expensive and time consuming (Moyo, 2014). Biosensors are gaining importance and popularity over conventional analytical techniques because of specificity, low cost, miniaturization of sample, fast response time, portable ease of use. Biosensors are analytical tools consisting of an immobilized biological material (enzyme, antibody, whole cell and organelle) in intimate contact with transducing material which converts biochemical signal in to quantifiable electronic signal proportional to the concentration of the analyte present. These are considered to be useful and reliable tools for clinical and food analysis of compounds within short time (Verma et al., 2007).

In the present work, we have made an effort to develop specific biosensors which can detect zinc ions in water and milk samples by using zinc fingers as a biocomponent, immobilized in a sort of semisolid matrix, which can provide a better environment for proper folding of the peptide in the presence of zinc ions. Keeping this view in mind, polyacrylamide gel along with a fluorescent indicator acridine orange has been used in the present study. Zinc finger peptide does not has inherent signal transduction machinery; hence, a hydrophobic fluorescent dye has been incorporated to get the signal, which fluoresces in the hydrophobic core formed after folding of the peptide in the presence of zinc ions. To best of our knowledge, following zinc finger with a specific sequence has not been used for the zinc biosensor. The strategy for immobilization in polyacrylamide gel matrix of this particular bio component along with a fluorescent dye has not been experimented yet for sensing of zinc ions in milk samples. The main purpose of our work is to fabricate a specific biosensor for zinc ion detection, with very low detection limits, longer storage stability and lower response time. This would further be useful to monitor zinc ions concentration in various food samples.

2. Experimental works

2.1. Material used

All the chemicals and reagents used in the study were of analytical grade. Zinc fingers peptide was procured from Link Biotech New Delhi, immobilized on to the micro well plates (48-wells) from Axygen. Fluorescent measurements were done by using mini-opticon with a fluorescent indicator (acridine orange) in a micro-well plate.

2.2. Biocomponent

Purified synthetic Cys_2His_2 Zinc Finger Peptide (Apo form) having sequence with 26 amino acids **(Ac-PYKC-PECGKSFSQSSNLQKHQRTHTG-Am)** (Fig. 1A) is used as a bio component (Yana et al., 2010). Acetyl group is attached at N terminal of the sequence and amide group is attached to the C-terminal of the sequence. The main zinc-binding residues shown in green are conserved cysteines, which contain thiol, and in blue are conserved histidines, which contain imidazole. The orange color residues showing phenylalanine and leucine form a small hydrophobic core which coordinate Zinc metal ion (Zn^{2+}) and drive the folding of the polypeptide chain.

Bioassay principle relies on the change in fluorescent count as a result of folding of zinc finger peptide in the presence of zinc ions. Acridine orange, a hydrophobic dye, used in the assay, moves in to the vicinity of hydrophobic core of the peptide formed after folding and dye becomes strong emitter in the hydrophobic environment (Fig. 1B).

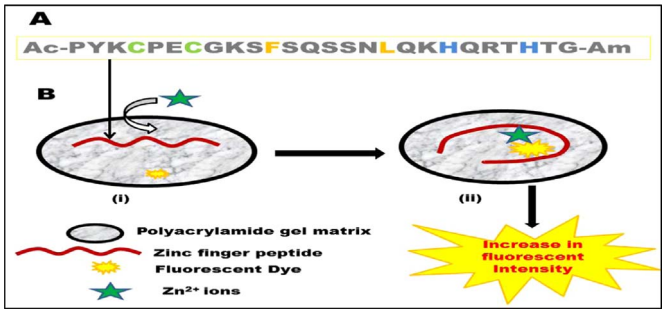

Fig. 1. (A) Zinc Finger Peptide sequence. (B) Schematic diagram showing the sensing mechanism of the presented biosensor (i) Unfolded Zinc finger peptide immobilized in polyacrylamide gel along with fluorescent dye. (ii) Folded zinc finger peptide in the presence of zinc ions forms a hydrophobic core and dye becomes a strong emitter causing increase in fluorescent intensity.

2.3. Association of reaction components

The reaction mixture was miniaturized to 40 μl, having 20 μl of acridine orange dye (0.2 μg/μl), 10 μl of Zinc finger peptide (0.1 μg/μl) and 10 μl of immobilized matrix (0.2 μg/μl) after optimization of experimental variables, (i) choice of suitable matrix, (ii) effect of concentration of matrix, (iii) incubation time, and effect of concentration of zinc ions were also investigated by incubating the reaction mixture at 30° C in the micro well plate and change in fluorescent intensity was measured by using mini opticon as a transducer.

3. Results and discussion

A fluorescent based biosensor has been developed for zinc ion detection, in the present study. Characterization of bioassay principle is explained in Section 3.1. Optimization of various parameters affecting the fluorescent response is discussed in Section 3.2. Regeneration properties and stability of the zinc finger peptide in the biosensing system is investigated in Section 3.3. Selectivity of biosensor over other metal ions has been checked in Section 3.4. and application of the developed biosensor in milk samples is described in Section 3.5.

3.1. Characterization of bioassay principle

In the microwell plate, the zinc finger peptide (0.1 ug/μl), acridine orange dye (0.2 ug/μl) and zinc ions (0.001–1 ug/l) were added in the ratio of 1:2:1. The fluorescent intensity was noted down at different concentration of zinc ions to confirm the bioassay principle. It was investigated that increase in fluorescent response is related to the increase in folding of zinc finger peptide, proportional to the zinc ion concentration from 0.001 to 1 ug/l (Fig. S1). Therefore, based on this characteristic, we were able to develop a fluorescent based biosensor. The folding of zinc finger peptide in the presence of zinc ions is well explained before (Yana et al., 2010; Jiang and Guo, 2004).

3.2. Optimization of variables affecting experimental parameters

Effect of different experimenting variables has been studied to optimize the reaction conditions and fluorescent response was measured.

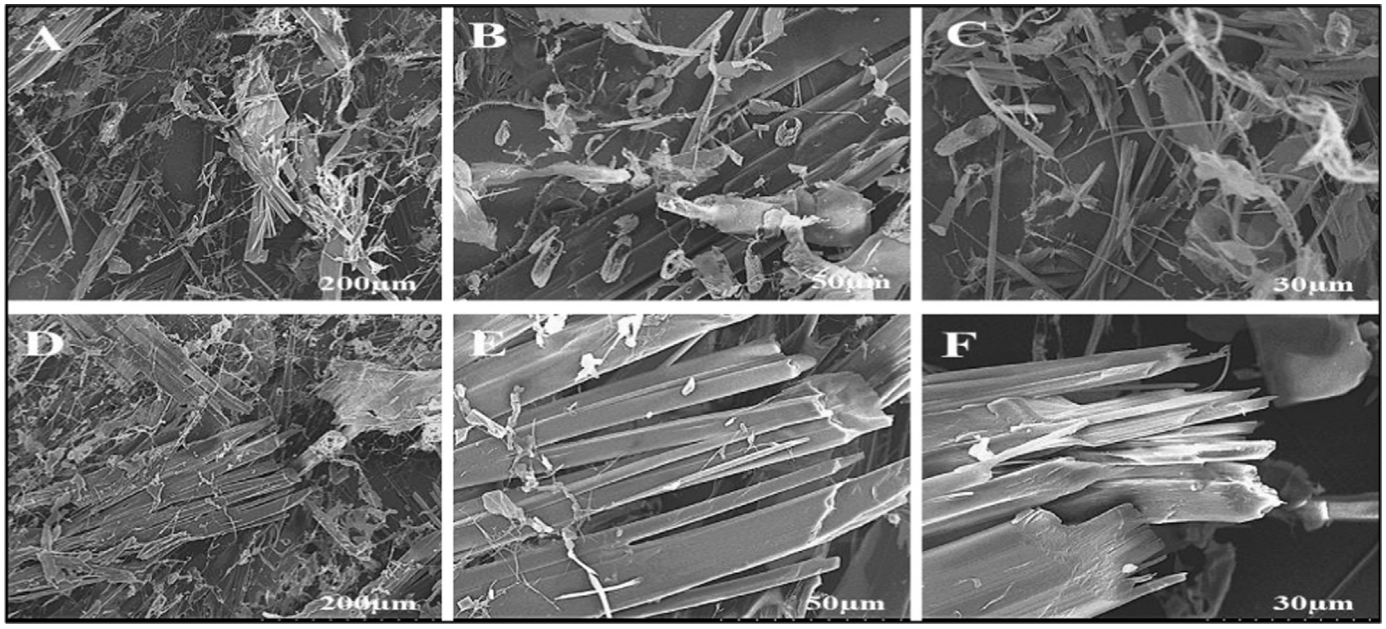

Fig. 2. SEM IMAGES (A) Polyacrylamide gel (200 μm). (B) Polyacrylamide gel (50 μm). (C) Polyacrylamide gel (30 μm) shows the absence of rod like structure, depicting peptides. (D) Polyacrylamide gel & Peptide (200 μm). (E) Polyacrylamide gel & Peptide (50 μm). (F) Polyacrylamide gel & Peptide (30 μm) shows the prominent rod like structures confirming the immobilization of peptide with the polyacrylamide gel.

3.2.1. Selection of suitable immobilization matrix

Calcium-alginate (Kumar et al., 2013), Chitosan (Verma et al., 2013), sol-gel (Verma et al., 2010), Polyacrylamide gel (Singh and Verma, 2008; Verma et al., 2015) were considered for the immobilization of bio-component Zinc finger peptide. There was a need of semi-aqueous immobilized matrix which can provide suitable environment for proper folding and unfolding of zinc finger peptide during the process. Polyacrylamide gel has shown the good semisolid environment for efficient folding of the bio-component in the presence of zinc ions in comparison to other matrices. Immobilization of Zinc Finger was done by using polyacrylamide gel for further experiments.

Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM) studies were also performed to confirm the immobilization of peptide within the polyacrylamide gel. SEM technique has been used to analyse fabrication of zinc finger peptide with the polyacrylamide gel. The three dimensional structure of polyacrylamide gel along with peptide has shown the presence of rod type structure (Fig. 2D–F) in comparison to the bare polyacrylamide gel images without peptide (Fig. 2 A–C), which confirms the immobilization scheme of zinc finger peptide within the polyacrylamide gel.

The FTIR spectrum has been generated from wave numbers (cm^{-1}) 4000 to 650, using a Perkin Elmer Frontier FTIR spectrophotometer with Attenuated Total Reflection (ATR) microscope. The different spectra were generated for the sample containing polyacrylamide gel and acridine orange (Sample A) and polyacrylamide gel loaded with zinc finger peptide and acridine orange (Sample B). The absorption peaks of sample A as shown in Fig. 3A, at wave numbers 3186 cm^{-1} confirms N–H bond in polyacrylamide gel, 2855 cm^{-1} shows C–H bond in polyacrylamide gel and acridine orange, 1319 cm^{-1} is due to C–N bond in polyacrylamide gel and 1181 cm^{-1} depicts the C–N (aromatic) in acridine orange. The peaks interpreted in sample B are shown in Fig. 3B at wavenumbers 3749 cm^{-1} confirms the O–H stretching mode in peptide containing aminoacids,1960 cm^{-1} characterise C=C stretching in histidine and band observed at 1504 cm^{-1} is a characteristic of phenyl group of the phenylalanine. Further, the shift in some peaks is observed in sample B (Fig. 3B) as compared to sample A (Fig. 3A) is due to the interaction of polyacrylamide gel with the peptide, which confirms its immobilization (Pereira et al., 2013).

3.2.2. Optimization of concentration of matrix for immobilization

Optimization of gel concentration was studied, which could efficiently able to immobilize zinc finger peptide and fluorescent dye. Different concentrations of polyacrylamide gel involving acrylamide and bisacrylamide in deionized water were considered (Table 1S). Polyacrylamide gel at concentration of acrylamide (1.6 μg/μl) and bis acrylamide (0.08 μg/μl) was found to be the best immobilizing substance for the zinc finger peptide (bio-component). Suitable immobilization was achieved by adding polyacrylamide gel, fluorescent dye and zinc finger peptide in the ratio of 1:2:1, which is selected for the rest of optimization steps. Swelling factor during sensor preparation and zinc ion sensing was nullified by subtracting the fluorescent intensity values of the prepared sensor (i.e. before adding zinc ions) from the fluorescent intensity values of the applied sensor (i.e. after adding zinc ions).

3.2.3. Optimization of response time

For the optimization of response time, the reaction was run for different time intervals i.e. 2 min, 5 min, 10 min in the micro well plate at 30 °C by adding different concentrations of zinc ions to the immobilized polyacrylamide gel containing fluorescent dye and bio component in the ratio of 1:2:1. It was observed that results obtained at 5 min have shown better fluorescent intensity count for different concentration of zinc ions (0.001–1 μg/l). Thus, 5 min was considered to be appropriate response time for further steps (Fig. 4A).

3.2.4. Linear range of detection of Zn^{2+} ions

Under optimized conditions, good linearity has been observed between 0.001 and 10 μg/l, describing the equation: $y=0.113x+0.777$ where y is change in fluorescent intensity measured in arbitrary unit (AU) and x is concentration of zinc ions (log value) and $R^2=0.9586$ (Fig. 4B). The lowest limit of detection for Zinc ions was found to be 0.0010 μg/l of zinc ions which is quite low as compared to horseradish peroxidase based zinc biosensor

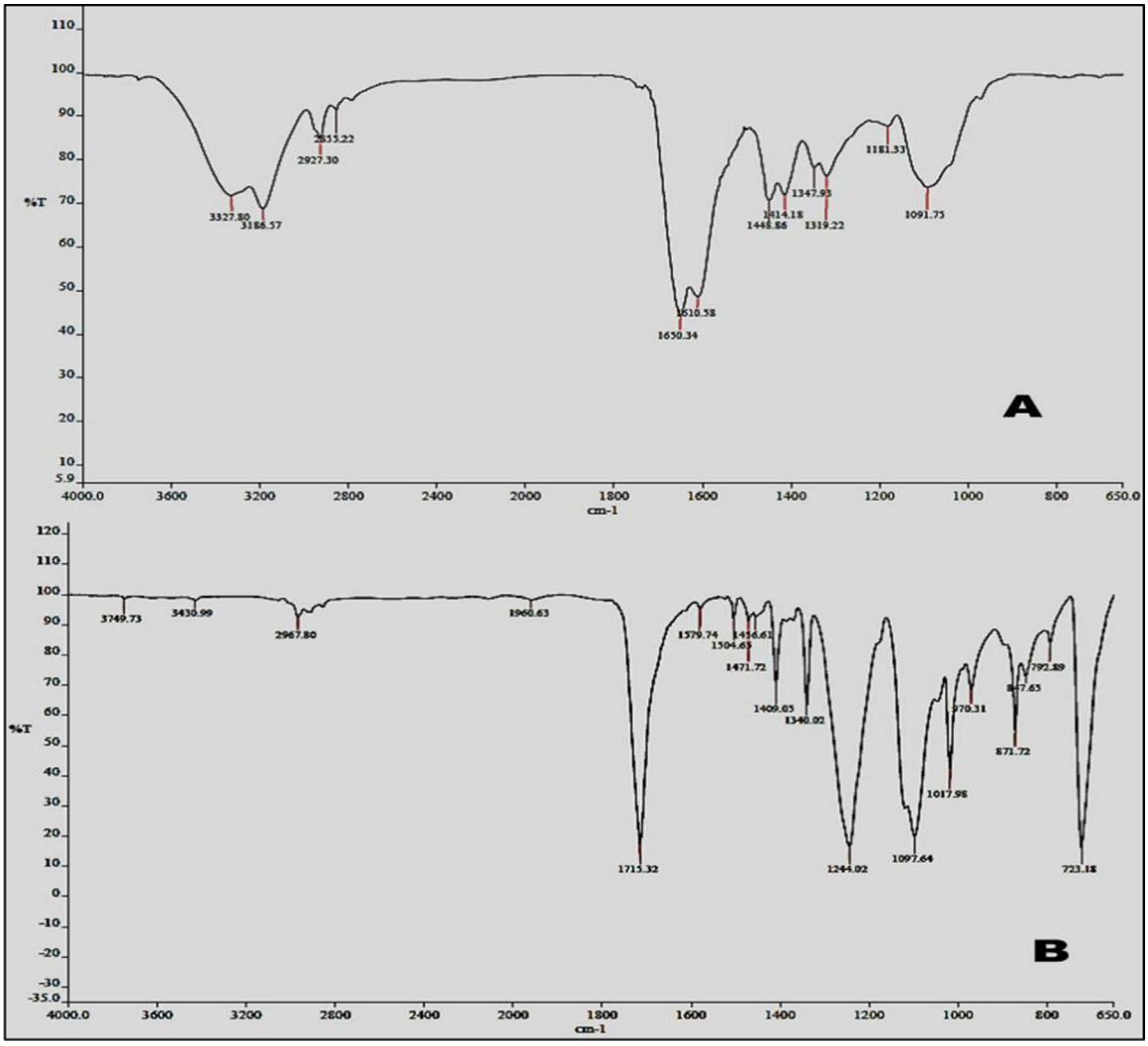

Fig. 3. FTIR-ATR spectra for confirming immobilization of peptide within the gel (A) Sample A (Polyacrylamide gel and acridine orange). (B) Sample B (Polyacrylamide gel loaded with zinc finger peptide and acridine orange).

(detection limit of 7.5 μg/l) (Moyo, 2014), and glucose oxidase based senor (detection limit of 9 μg/l for zinc) (Ghica and Brett, 2008) and almost similar to that of carbonic anhydrase based zinc biosensor (detection limit of 0.0013 μg/l) (Wang et al., 2011). The linear range and limit of detection in the present study were sufficient for the various environmental samples with an advantage of having faster response time.

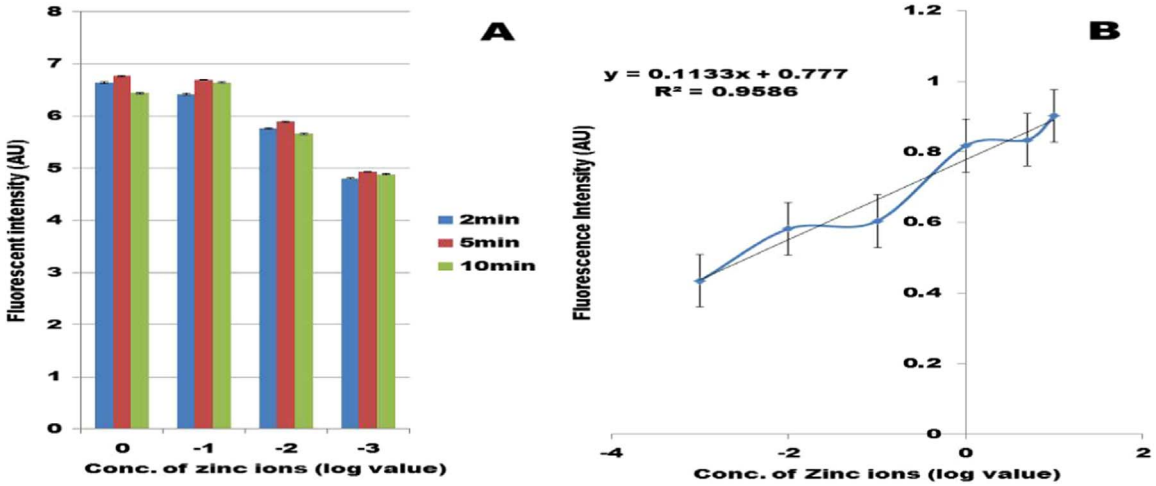

Fig. 4. (A) Optimization of response time for different concentration of zinc ions. (B) Plot of fluorescent response versus concentration of zinc ions showing logarithmic relationship (0.001–10 μg/l).

3.3. Regeneration, repeatability, and stability of the bio sensing system

For regeneration of developed Zinc biosensor, series of reactions were performed by converting zinc Finger peptide (folded) in to apo form (unfolded), using 0.01 M ethylene diamine tetraacetic acid (EDTA). It was observed that there was an average of 20% decrease in activity of the constructed zinc finger peptide biosensor after 5 regeneration and reuses (Table 2S). The sensor has shown good repeatability for n=3 with a relative standard deviation value of less than 2% which is less than the previously reported horseradish peroxidase enzyme based zinc biosensor, having relative standard deviation (RSD) of 5.3% (Moyo, 2014). The stability of the constructed biosensor was checked by performing zinc detection assays over a period of 20 days stored at 4 °C. The sensor showed a constant response for the first 7 days, with a gradual decrease in fluorescent intensities up to 15 days, retaining its 80% of the original fluorescent response, which almost equal to the stability (18 days at 4 °C) of the enzyme based zinc biosensor (Moyo, 2014). To evaluate the reason for the decrease in activity or response, photo stability of the fluorescent dye has also been checked, which showed a negligible change in fluorescent read even after 15 days (Table 3S). It was interpreted that reason for decrease in response may be due to the leaching of peptide after number of assays. The sensor is stable for almost one month, stored under optimum conditions at 4 °C. The stability can be improved further by sealing the immobilized gel with parafilm, stored in the refrigerator for longer period.

3.4. Selectivity of the developed sensor over other metal ions

Selectivity of the bio component is the main parameter for the detection of various heavy metal ions with biosensors. Other heavy metal ions like copper (Cu^{2+}), Lead (Pb^{2+}), Cadmium (Cd^{2+}) (each at the concentration of 10 μg/l) were selected to evaluate the interference in response of the developed biosensor for the detection of zinc ions. Pb^{2+} and Cd^{2+} metal ions have shown negligible change in fluorescent intensity compared to blank, but in the presence of Cu^{2+} ions (10 μg/l), minor response was observed, due to its cofactor property like zinc ions, causing slight folding of the peptide (Fig. 2S). At concentration less than 10 μg/l of Cu^{2+} ions, there was no interference in fluorescent response, resulting in zinc finger peptide biosensor, highly specific for Zinc ions.

The performance parameters of the developed method are tested and optimized for the improvement of detection system as compared to heavy metal sensors already reported in literature (Table 1).

3.5. Application in milk samples

Optimizing the different performance parameters, application of developed biosensor assembly has been checked for the presence of zinc ions. The standard milk sample from verka milk booth has been spiked by addition of zinc ions from 1 ppb down to 0.001 ppb (1, 0.1, 0.01, 0.001 ppb) after acid extraction. Acid extraction of milk was done by adding 200 μl of concentrated nitric acid to 10 ml of milk sample. Then centrifugation was done at 5000 rpm for 10 min. Then pH of the supernatant was maintained to neutral with 2 M sodium hydroxide (Verma et al., 2010). Reference was set with unspiked standard milk sample. Milk sample from local milk dairy, Lehal Colony Patiala, has also been analyzed to check the unknown concentration of zinc ions. In milk sample analysis the same pattern was observed with a linear range from 0.001 μgZn^{2+}/l to 1 μg Zn^{2+}/l (Table 2). The concentration of zinc ions in the unknown milk sample was calculated to be 54.9 μg/l,

Table1
Comparison of process parameters achieved by different zinc biosensors (specific/non-specific).

Bio-component	Detection limit for Zn^{2+} (μg/l)	Metal detected	Comparison with present study	Reference
Zinc finger peptide	0.0010	Zn^{2+}	Quick response time, very low detection limit, miniaturized sample volume, applied to milk samples.	Present study
HRPase	7.5	Zn^{2+}	Higher detection limit, applied in water samples only	(Moyo, 2014)
Carbonic anhydrase	0.0013	Zn^{2+}	Having similar detection limit, specific, applied to measure free Zn^{2+} in living cells.	(Wang et al., 2011)
Nitrate reductase	32.69	Cu^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}	Detection limit is higher and non-specific	(Wang et al., 2009)
Burkholderia sp (RASC C2)-/Lux	0.0017	Cu^{2+}, Zn^{2+}	Recombinant based, detection limit almost equal with the present study, non-specific	(Chinalia et al., 2008)
Glucose oxidase	9	Cu^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}	Higher detection limit and non specific	(Ghica and Brett, 2008)
Alkaline phosphatase	1000	Hg^{2+}, Cu^{2+}, Cd^{2+}, Ag^{2+}, Zn^{2+}	Quite high detection limit and common for so many metals	(Shyuan et al., 2008)
Mammalian cells	650	Hg^{2+}, Cu^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Fe^{2+}	Higher detection limit and non specific	(Liua et al., 2007)
Chlorella vulgaris	10	Cd^{2+}, Zn^{2+}	whole cell based with higher detection limit and non specific	(Chouteau et al., 2005)
Znt A and cop A promoters from *E. Coli*	0.006	Cu^{2+}, Cd^{2+}, Pb^{2+}, Hg^{2+}, Zn^{2+}	Equivalent detection limit, recombinant based and non specific	(Reither et al., 2001)

Table 2
Application of the developed zinc biosensor in milk samples.

Log value of Zinc ions	Conc. of zinc ions in spiked milk samples (μg/l)	Change in fluorescent intensity (AU)
–	Unspiked	0.406 (± 0.015)
−3	0.001	1.432 (± 0.010)
−2	0.01	1.522 (± 0.013)
−1	0.1	1.679 (± 0.012)
0	1	1.798 (± 0.015)
x	Milk sample (local diary)	1.290 (± 0.011)

which is safe, within the permissible limits as defined by Codex alimentarius commision (0.3–1 mg/l).

Other researchers have experimented with recombinant based microorganisms like cyanobacterium *Synechococcus* PCC 7942 (Bontidean et al., 2000), *E. Coli* and *Vibrio fischeri* (Reither et al., 2001), whole cells such as *Chlorella vulgaris* (Chouteau et al., 2005), mammalian Cells (liua et al., 2007), enzymes like glucose oxidase (Ghica and Brett, 2008), carbonic anhydrase (Wang et al., 2011), horseradish peroxidase (Moyo, 2014), plant cells like *Arabidopsis thaliana* and *Populus tremula* (Adams et al., 2011), for zinc ion analysis in combination with suitable transducers. Some scientists have investigated carbonic anhydrase enzyme along with Fluorescence resonance energy transfer (FRET) based mechanism for the zinc ion detection, as zinc acts a cofactor for the activity of carbonic anhydrase. Thompson et al. (2002) investigated the study with sulfonamide as a fluorophore and protein moieties fused with carbonic anhydrase (CA) showed the detection limit of 10 pM for zinc ions. In a similar study, arylsulfonamide has been used as a fluorescent moiety along with red fluorescent proteins attached with carbonic anhydrase (CA), achieved the detection limit of 20 pM (Wang et al., 2011). The fluorescence resonance energy transfer (FRET) based studies have difficulty in achieving proper synchronization of the fluorophore and protein moieties attached to the enzyme, signaling to the presence of zinc ions, results in poor shift of excitation and emission wavelength (Jiang and Guo, 2004).

Literature survey reveals that biosensors for zinc ions have certain disadvantage of having non-specificity. These biosensors are not specific particularly for zinc ions, rather they are common for other metal ions like Pb^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+} and Co^{2+} (Reither et al., 2001; Liua et al., 2007; Ghica and Brett, 2008; Wang et al., 2009). The applicability of most of the biosensors has been tested in water samples only. The earlier biosensors have also shown longer response time with detection limit at higher concentrations of zinc ions in comparison to the present study.

4. Conclusion

Zinc finger peptide based biosensor has been demonstrated for its ability to detect zinc ions in water and milk samples. Results have shown the novelty of the particular zinc finger peptide, immobilized in polyacrylamide gel matrix along with acrydine orange, which is highly specific to zinc ions with a lowest detection limit of 0.001 μg/l and response time of 5 min. The sensing system was reproducible and reusable after 5 regenerative cycles, repeatable, and stable for more than one month, under optimal conditions at 4 °C, with negligible interference by other heavy metal ions like lead, cadmium and copper. The sample volume has been miniaturized to 10 μl in micro well plate which enable multiple samples assay. Further, the developed approach can also be applied to juice, wine, food and soil samples. In future, improvement in stability of sensor can be achieved by immobilizing the peptide and gel on a plastic chip, with greater miniaturization and easy handling of the sensor chip.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.06.088.

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