



Nanohybrid electrochemical enzyme sensor for xanthine determination in fish samples

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Received: 27 November 2020 / Accepted: 10 March 2021
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Abstract

An amperometric biosensor for xanthine was designed, based on covalent immobilization of xanthine oxidase (XO) of *Bacillus pumilus* RL-2d onto a screen-printed multi-walled carbon nanotubes gold nanoparticle-based electrodes (Nano-Au/c-MWCNT). The carboxyl groups at the electrode surface were activated by the use of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS). The working electrode was then coated with 6 μL of xanthine oxidase (0.273 U/mg protein). The cyclic voltammetry (CV) study was done for the characterization of the sensor using $[\text{K}_3\text{Fe}(\text{CN})_6]$ as an artificial electron donor. The sensitivity (S) and the limit of detection (LOD) of the biosensor were $2388.88 \mu\text{A}/\text{cm}^2/\text{nM}$ ($2.388 \mu\text{A}/\text{cm}^2/\mu\text{M}$) and 1.14 nM, respectively. The developed biosensor was used for determination of fish meat freshness.

Keywords Amperometric biosensor · Xanthine oxidase (XO) · *Bacillus pumilus* RL-2d

Introduction

Development of biosensors is the most important area of research with applications in encompassing medical diagnostics, environmental monitoring, and food analysis (Mousty 2010). Xanthine oxidase (XO) catalysis and inhibition has been linked with both food and pharmaceutical industries (Saleheh et al. 2018). Meat and fish are among the most consumed food of the human diet and quality of food products is a major concern to the consumers. The freshness of meat and their products is the most important criterion in the quality control for their consumption. The complex microbiological, chemical as well as physical processes lead to the loss of freshness and subsequently cause spoilage (Alomirah et al. 1998). Biochemical activities of microorganisms generally lead to loss of meat freshness. Rapid degradation of nucleotides results in the evaluation of hypoxanthine which is often used as an index of freshness of meat in the food industries. When a fish is caught and sacrificed,

it loses freshness during autolysis, and the breakdown of ATP (adenosine-5-triphosphate) in the fish produces ADP (adenosine-5-di phosphate) and further decaying products, such as AMP (adenosine-5-monophosphate), IMP (inosine-5-monophosphate), HxR (inosine), Hx (hypoxanthine), X (xanthine) and U (uric acid). One of the major factors to the pleasant flavour of fresh fish is IMP. After fish dies, hypoxanthine and xanthine are accumulated in the fish meat and imparts a bitter “offtaste” (Masoud et al. 2018). Therefore, their quantification can be as an indication of the fish freshness and the conversion of hypoxanthine to xanthine through the catalytic effect of xanthine oxidase (XO) together with the production of H_2O_2 and reaction of O_2 was found to be the rate determining step in the overall reaction sequence in fish muscle (Lawal and Adeloju 2008).

Various methods have been used for the determination of xanthine and its metabolites, such as spectrophotometry (Khajehsharif and Pourbasheer 2011), mass spectrometry (Rashed et al. 2005), high-performance liquid chromatography (HPLC) (Coopera et al. 2006), capillary electrophoresis (Mu et al. 2012), chemiluminescence based fiber-optic biosensor (Hlavay et al. 1994) and electrochemiluminescence biosensor (Lin et al. 2008), but these methods are costly and taking more time to get the results. However, the enzyme-based electrochemical sensors are one of the most important research topics in this field due to simplicity of operation,

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high selectivity and sensitivity (Shin et al. 2013; Basniwal et al. 2013; Sharma et al. 2012). Various methods, such as adsorption, covalent linkage, and fixing via intermediate linker molecule, have been used to immobilize enzymes onto solid substrates (Yang et al. 2006; Devi et al. 2013) but in the past few years, use of nanoparticles has revealed significant potential in biosensor's performance in bioanalysis. The construction of biosensor uses various mediators, such as gold nanoparticles (Pingarron et al. 2008), colloidal gold (Liu et al. 2004), Prussian blue (Teng et al. 2010), cobalt phthalocyanine (Kilinc et al. 1998), Co-doped CeO₂ nanoparticles (Lavanya et al. 2016), carbon nanotubes/graphene complexes (Si et al. 2018) and ferrocene (Arslan et al. 2006).

The electrophoretic analysis (Bory et al. 1996), GC/MS (Chabard et al. 1980), HPLC (Yamamoto et al. 1996), chemiluminescence (Hlavay et al. 1994) and UV spectrophotometry (Kito et al. 1983) are the most common methods for detecting and quantifying purines. However, these methods taking more time, involve costly equipment, and are labor-demanding in terms of sample preparation. On the other hand, electrochemical methods offer portability, ease, selectivity, and high sensitivity and can be applied to samples without any pretreatment. Number of chemically modified electrodes have been used for the detection of xanthine, such as polymer films (Kalimuthu and John 2009), pretreated carbon paste (Cai et al. 1994), nanoporous carbon fiber (Kathiwala et al. 2008), pre-anodized nontronite-coated carbon (Zen et al. 2002) and multi-wall carbon nanotube composite (Wang 2011)-modified electrodes. The main interfering substances, such as uric acid, ascorbic acid, glucose, and sodium benzoate, which are widely used in preservation of meat and meat products show negligible effect and Dervisevic et al. 2017 showed that the selectivity of the biosensor towards xanthine, the amperometric response to interferants is negligible.

In the present communication, an amperometric biosensor developed using screen-printed multi-walled carbon nanotubes gold nanoparticle (Nano-Au/c-MWCNT) treated with a mixture of cross-linker, i.e. NHS (N-hydroxysuccinimide) and EDC (1-Ethyl-3-(3-dimethylaminopropyl carbodiimide) and coupled with xanthine oxidase is being reported which is highly selective, sensitive and cost-effective for determination of xanthine in fish meat samples.

Materials and methods

Chemicals and reagents

1-Ethyl-3-(3-dimethylaminopropyl carbodiimide) (EDC), N-hydroxysuccinimide (NHS), potassium ferricyanide K₃[Fe(CN)₆] were procured from Sigma Company, USA. Xanthine was procured from HiMedia. The molecular

biology-grade chemicals were used in the study. The Milli-Q water was used for the preparation of all reagents. Screen-printed carbon nanotubes gold nanoparticle-based electrodes (Nano-Au/c-MWCNT) and specific connector were purchased from DropSens, Spain. The NOVA software was used for electrochemical measurements which were carried out on μ AutoLab, The Netherlands.

Fabrication of biosensor

The fabrication of enzyme-based sensor was carried out using the three-electrode system-based screen-printed electrode (nano-Au/c-MWCNT). The autoclaved Milli-Q water and PBS buffer (0.1 M, pH 7) was used for washing the electrode, respectively, and was then dried well at room temperature. After this, the carboxyl group on the nano-Au/c-MWCNT electrode surface was activated by treating with a mixture of 10 mM EDC and 10 mM NHS [(EDC, NHS acts as cross-linker, (1:1, v/v in PBS, pH 7)] for 1.5 h (Gupta et al. 2016). The PBS (pH 7) buffer was used to remove the excess of reagent on electrode surface. Then, the electrode was allowed to dry at room temperature. The working electrode was then coated with 6 μ L of xanthine oxidase (0.273 U/mg protein) and incubate for 5 h at 4 °C. The xanthine oxidase activity was determined by Nitroblue tetrazolium (NBT)-based method (Monika et al. 2019). After incubation, the working electrode was washed with PBS buffer (pH 7). 1 mM potassium ferricyanide acted as an artificial electron donor and was used in the characterization of the sensor using cyclic voltammetry (CV). Scanning electron microscopy (SEM) images were taken from Hitachi-make model JSM6100 for bare as well as xanthine oxidase immobilized electrode.

Binding and characterization of biosensor with xanthine

The xanthine (substrate) of varying concentrations (10^{-7} μ M to 10 μ M) after heating at 50 °C was hybridized onto the surface of xanthine oxidase-fabricated Nano-Au/c-MWCNT. Electrochemical studies were then characterized by cyclic voltammetry and hybridization time was standardized as 10 min, followed by electrode surface washing using PBS (pH 7.4) to remove the unbound xanthine. A standard curve of varying xanthine concentrations vs. change in relative peak current (μ A) reading was made. The start potential and stop potential parameters used in CV analysis are given in Table 1.

Evaluation of fish freshness

Fish was procured from local market and cut into small pieces. The small pieces of fish were homogenized in a

Table 1 The start and stop potential parameters used in CV analysis

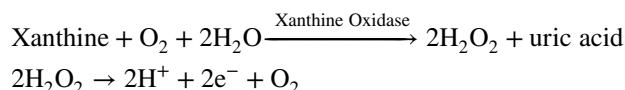
Start potential (V)	− 0.200 V
Upper vertex potential (V)	− 0.080 V
Lower vertex potential (V)	− 0.450 V
Stop potential (V)	0.8 V
Number of stop crossings	2
Step potential (V)	− 0.00244 V
Scan Rate (v/s)	0.02000

blender. The homogenized sample was mixed with double distilled water in a 1:3 (w/w) ratio. The mixture was stirred mechanically for 10 min and centrifuged at 8000 g. Then, the xanthine was extracted from homogenate by filtered through 0.2- μ m membrane filter. The xanthine content in the filtrate was estimated using the biosensor operating under standard conditions. Current change (μ A) was studied for each samples and the xanthine concentration was calculated from the standard reference plot.

Results

Development of xanthine oxidase based amperometric biosensor for xanthine determination

The fabrication of the nano-Au/c-MWCNT-based biosensor and immobilization of the xanthine oxidase is conceptually presented in Fig. 1. Binding of immobilized enzyme with xanthine and its electrochemical detection may be described by the following reaction mechanism:



SEM (scanning electron microscopy) study for the characterization of working electrode

The morphology of the bare as well as immobilized electrode was characterized by SEM micrographs. The surface of bare electrode, i.e. Nano-Au/c-MWCNT and xanthine oxidase-fabricated Nano-Au/c-MWCNT obtained by SEM was shown in Figs. 2a, b, respectively. The surface of the bare electrode was smooth, whereas the surface of electrode after xanthine oxidase fabrication shows roughness and homogeneous immobilization of enzyme on the surface of the modified Nano-Au/c-MWCNT electrode.

Cyclic voltammetry (CV) studies

Voltammetric measurements of bare nano-Au/c-MWCNT, immobilized xanthine oxidase and after interaction with substrate (xanthine) were carried out using redox indicator, i.e. potassium ferricyanide. The peak current (I_p) in cyclic voltammetry (CV) study of immobilized XO/ nano-Au/c-MWCNT and after catalysis (Fig. 3) was reduced in comparison to bare nano-Au/c-MWCNT). The observed peak current for bare nano-Au/c-MWCNT and XO/nano-Au/c-MWCNT electrode was 52 μ A and 30 μ A, respectively. For different xanthine concentrations, i.e. 0.0001, 0.001, 0.01, 0.1, 1, 10, 100, 1000, 10,000 nM, the peak currents were 32.29, 29.22, 28.11, 27.61, 27.07, 27.02, 26.78, 25.79 and

Fig. 1 Schematic diagram for the xanthine oxidase/screen printed multiwalled carbon nanotubes gold nanoparticle based electrode (XO/ nano-Au/c-MWCNT)

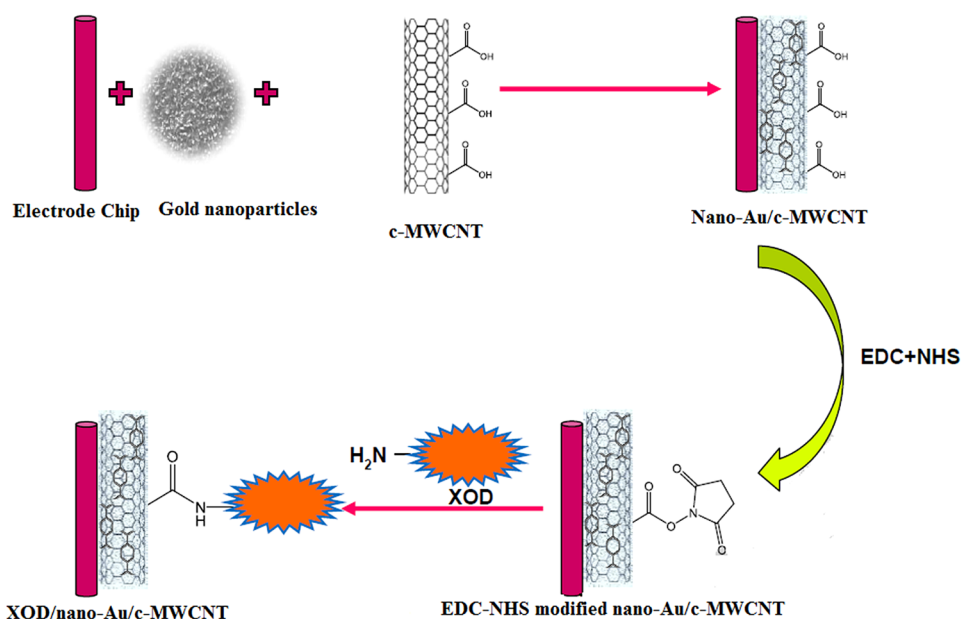


Fig. 2 **a** SEM image of bare electrode and **b** SEM image of enzyme immobilize electrode

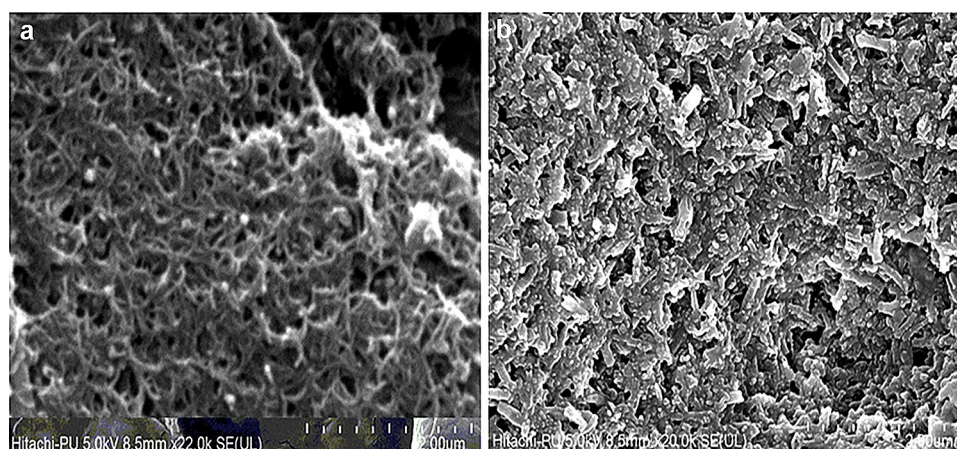
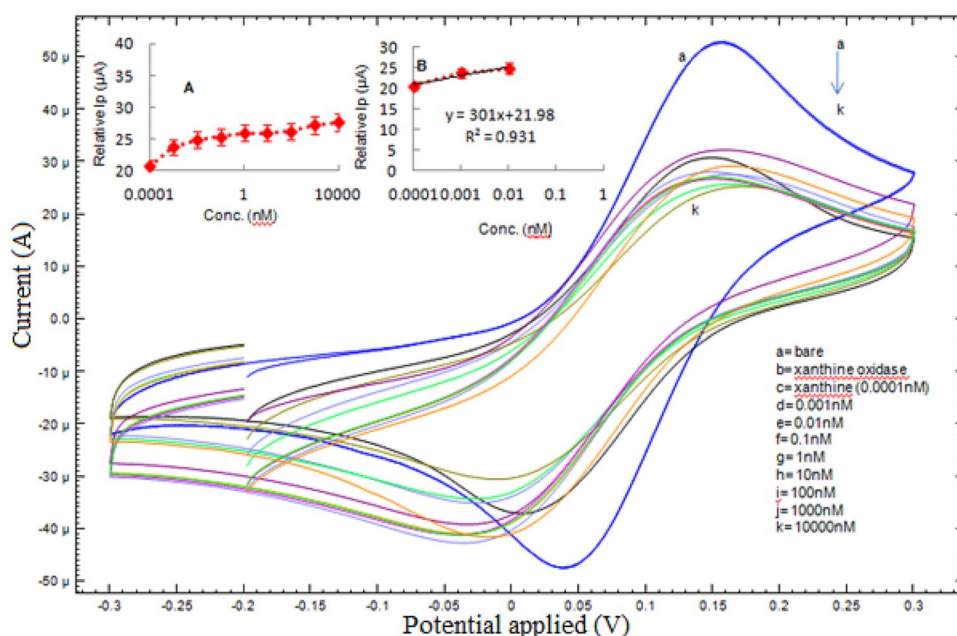


Fig. 3 Cyclic voltammetric studies of xanthine oxidase based amperometric biosensor using different xanthine concentrations ranging from 0.0001 to 10,000 nM at 50 mVs⁻¹ using 5 mM K₃[Fe(CN)₆]. The inset A shows curve from 0.0001 to 10,000 nM with linear peak current (Ip). Inset B shows the linear plot from 0.0001 to 0.01 nM xanthine concentration for the calculation of sensitivity and LOD



25.36 μA , respectively. The plot as well as linear equation [I_p (μA) = 301($\mu\text{A}/\text{nM}$) $\times$ xanthine concentration (nM) + 21.98] with regression coefficient (R^2) 0.931 was shown in Fig. 3 inset B. The Sensitivity (S) of the biosensor was calculated using the formula $S = m/A$ where, m is the slope of the linear equation and A is the area of the working gold (0.126 cm²) electrode and was calculated as 2388.88 $\mu\text{A}/\text{cm}^2/\text{nM}$ (2.388 $\mu\text{A}/\text{cm}^2/\mu\text{M}$). The limit of detection (LOD) was calculated using the formula $\text{LOD} = 3(\sigma/S)$ where σ is the standard deviation and S is the sensitivity and found approximately 1.14 nM.

Determination of Fish freshness and sensor stability

To evaluate the analytical reliability of the method, the biosensor was employed to detect xanthine in the fish samples by cyclic voltammetry studies. The observed peak current for

bare nano-Au/c-MWCNT and XOD/ nano-Au/c-MWCNT was 52 μA and 30 μA , respectively. After the addition of the fresh fish extract sample and the five-day-old fish extract sample, the peak currents observed were 29 μA (0.01 nM) and 27 μA (10 nM), respectively (Fig. 4). The stability of the developed nano-hybrid enzyme sensor was studied on storage at 4 $^{\circ}\text{C}$ using CV at regular interval of 30 days. The sensor was found stable up to 4 months at 4 $^{\circ}\text{C}$ with approx. 10% loss in original CV current (Fig. 5).

Discussion

The bacterial xanthine oxidase (XO) from *B. pumilus* RL-2d was immobilized on nano-Au/c-MWCNT to construct a xanthine biosensor. The peak current (I_p) in the cyclic voltammetry (CV) study of immobilized XOD/

Fig. 4 Cyclic voltammetric studies of xanthine oxidase based bio sensor for the detection of fish freshness. Sample 1: Fresh fish extract; Sample 2: Fish extract after five days

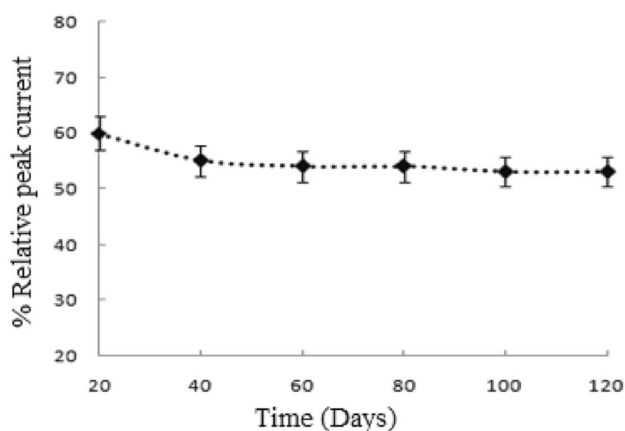
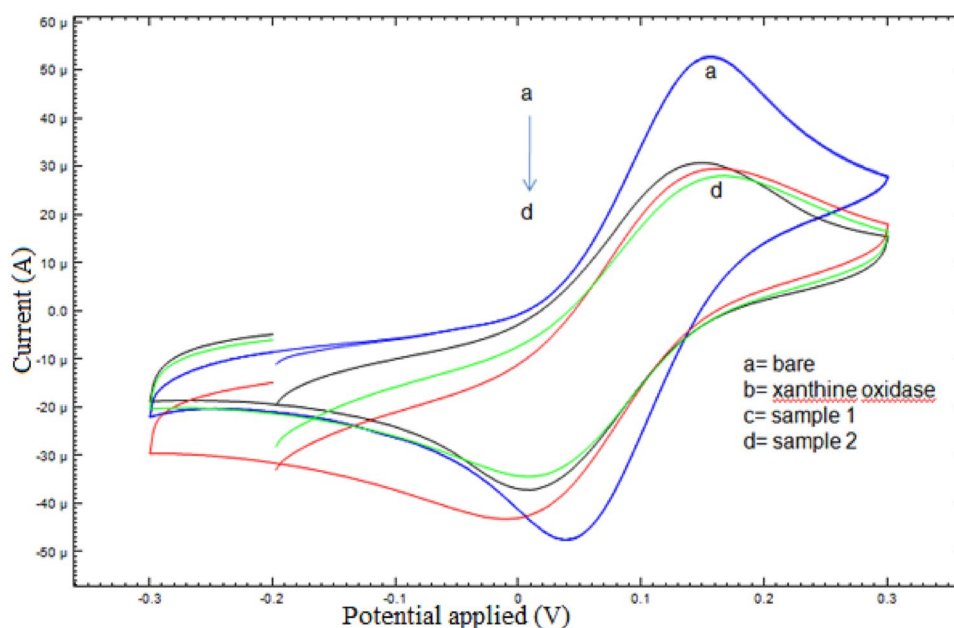


Fig. 5 Stability of the sensor measured as % relative peak current with a regular interval of 30 days for 4 months on storage at 4 °C

nano-Au/c-MWCNT and after catalysis was reduced in comparison to bare nano-Au/c-MWCNT) and may be due to the decrease in the surface area of nano-Au/c-MWCNT electrode at each step of fabrication. As the xanthine concentration increases, the current response decreases which can be attributed to their non-electrochemical activity, which blocks the electrochemical communication between the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ solution and the electrode to some extent. The present electrochemical biosensor performed in terms of applied potential and the electrocatalytic oxidation response and showed a detection limit of 1.0×10^{-13} M. On the other hand, the detection limits of xanthine-based biosensors, such as XO/layered double hydroxide (Shan et al. 2009a), XO/calcium carbonate

nanoparticles (Shan et al. 2009b), XO/laponite nanoparticles (Shan et al. 2009a, b, c), XO/ZnOx/polypyrrole (Devi et al. 2012), XO/glassy carbon paste (Villalonga et al. 2011), EPG/XDH (Kalimuthu et al. 2012), XOD/c-MWCNTs/ Fe_3O_4 /TCNQ/CHIT/GCE (Dalkiran et al. 2017) and XO/Poly(L-Asp)/MWCNT/GCE (Samira et al. 2019), were 1×10^{-7} , 2×10^{-6} , 1×10^{-8} , 8×10^{-7} , 4×10^{-6} , 2.5×10^{-10} , 2×10^{-7} and (3.5×10^{-10}) M, respectively. The sensitivity of the biosensor was $2.388 \mu\text{A}/\text{cm}^2/\mu\text{M}$ while the sensitivity of XOD/CHT/PtNPs/PANI/ Fe_3O_4 /CPE biosensor (polyaniline nanoparticles/chitosan/carbon paste electrode) was $13.58 \mu\text{A}/\mu\text{M}/\text{cm}^2$ (Sadeghi et al. 2014). The detection limit for the present biosensor was very low as compared to other reported xanthine biosensors and the fabricated biosensor was effectively used for the detection of fish and chicken meat freshness.

Conclusion

The xanthine oxidase (XO) of *B. pumilus* RL-2d was immobilized on nano-Au/c-MWCNT to construct an amperometric biosensor for detection/assay of xanthine. This electrochemical biosensor worked in terms of applied potential and the electrocatalytic oxidation and its sensitivity was $2388.88 \mu\text{A}/\text{cm}^2/\text{nM}$ ($2.388 \mu\text{A}/\text{cm}^2/\mu\text{M}$). The limit of detection (LOD) for xanthine was observed to be 1.14 nM. It was used for the analysis of fish freshness and the observed peak currents for fresh fish extract sample and the five-day-old fish extract sample were 29 μA (0.01 nM) and 27 μA (10 nM), respectively.

Acknowledgements The authors acknowledge the University Grants Commission vide F. No. 39-274/2010 (SRF) to Nirmal Kant Sharma for financial assistance. Tek Chand Bhalla greatly acknowledges UGC, New Delhi for BSR-Faculty Fellowship (F. No. 18-1/2011 (BSR)/24th Feb 2014).

Declarations

Conflict of Interest Authors declare no conflict of interests.

Ethical statement The authors declare no studies with animals or human participant.

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