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RESEARCH ARTICLE

Development of low-cost paper-based biosensor of polyphenol oxidase for detection of phenolic contaminants in water and clinical samples

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Abstract

In the present work, polyphenol oxidase (PPO) enzyme was purified from potato peel using three-phase partitioning (TPP). In this method, ammonium sulfate and t-butanol were added to precipitate the protein/enzyme from the crude aqueous extract. The PPO enzyme precipitated as an interfacial layer between the upper organic solvent phase and lower aqueous phase. Different purification parameters such as crude extract to t-butanol ratio, ammonium sulfate concentration, temperature, and pH were optimized for TPP. About 69% PPO enzyme activity was recovered in a single step of TPP with 9.2-fold purification. The sodium dodecyl sulfate–polyacrylamide gel electrophoresis profile of partially purified PPO enzyme showed molecular weight in the range of about 30–40 kDa. The PPO enzyme was then investigated for the fabrication of a portable, cost-effective, and disposable colorimetric paper biosensor or colorimetric "test strips" for detection of phenolic contaminants. PPO and a chromophore reagent (3-methyl-2-benzothiazolinone hydrazine) generated a range of color in the presence of phenolic compounds (catechol, phenol, p-cresol, 4-methyl catechol) within 15 min, and limit of detection was found to be 0.5 μM. The biosensor worked in a broad range of pH from 3 to 11 and showed good storage stability at 25 °C and 4 °C for 30 days with no significant loss of activity. The biosensor was also applied on environmental water and urine sample to show reliability of biosensor.

Keywords Polyphenol oxidase · Three-phase partitioning · Biosensor · Phenols · Catechol · Pollutants

Introduction

Polyphenol oxidases (PPO) (EC 1.14.18.1) are metalloenzymes containing copper which catalyzes the oxidation of mono-, di-, and polyhydric phenols to quinones; hence, they are bi-functional in nature (Robb 1984). The product o-quinones formed gets polymerized further, which results in the formation of black, brown, or red pigments such as melanin. These enzymes are responsible for browning of cut fruits and vegetables, and are found in microorganisms also (Burton 1994). PPO is commercially demanded as its application ranges from bioremediation of phenolic effluents, detection of phenolic content, and synthesis of 3,4-dihydroxy phenylalanine (L-DOPA) by biotransformation of L-tyrosine (Atlow et al. 1984; Tillyer and Gobin 1991). So far, this enzyme has been extracted and purified using different purification techniques from various sources such as apple (Ni Eidhin et al. 2006), apricot (Derardja et al. 2017), potato (Mukherjee et al. 2012), walnut (Zekiri et al. 2014), banana (Yang et al. 2000), tomato (Saeidian 2013), *Boletus erythropus* (Özel et al. 2010), and *Duranta plumieri* (Roy et al. 2002). PPO enzyme has been purified earlier also using three-phase partitioning (TPP) from different sources like wild mushroom (Saki et al. 2018), borage (Alici and Arabaci 2016), and potato (Niphadkar and Rathod 2015).

TPP is non-chromatographic bio-separation technique which has gained popularity due to its simplicity, cost-effectiveness, high throughput of desired product, and easy scalability (Gagaoua et al. 2017; Ketnawa et al. 2017). Apart from protein or enzyme, many biomolecules such as oils, pigments, and inhibitors have been extracted and purified from this technique (Chew et al. 2019). Researchers have employed this methodology to purify various enzymes like pectinase (Sharma and Gupta 2001), peroxidase (Narayan et al. 2008), cucumisin (Gagaoua et al. 2017), green fluorescent protein

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(Jain et al. 2004), invertase (Duman and Kaya 2014), and also soybean oil (Sharma et al. 2002). In this method, water-miscible aliphatic alcohol along with salt is added to slurry of proteins, thus forming phases as follows: alcohol-rich upper phase containing lipids, pigments, and hydrophobic materials; intermediate precipitate containing mostly protein of interest and lower salt-rich phase containing polar components (Roy et al. 2004).

The presence of contaminants such as phenols and its derivatives in water is the major cause of toxicity to aquatic as well as human life. These organic impurities are the by-products of various industries that are discharged in water bodies, and thus bioaccumulate in the environment (Alkasir et al. 2012). Due to the high level of toxicity, most phenolic compounds have been added to the list of major pollutants by the US Environmental Protection Agency (EPA). The maximum concentrations allowed in drinking water for total and individual phenols are 0.5 and 0.1 mg/l (Mazhari et al. 2017).

Catecholamines (like dopamine, adrenaline, and noradrenaline, etc.) have a phenolic structure and act as a neurotransmitter (Manbohi and Ahmadi 2019; Rozet et al. 2006). They play a significant pharmacological role in the body and control motor activity, blood pressure, peripheral circulation, arteriole contraction, and the cardiovascular system (Burghardt et al. 2012; Ghasemi et al. 2016). Catecholamine acts as diagnostic marker; their abnormal concentration in blood plasma or urine specifies clinical diagnosis of tumors of the neural crest cell (neuroblastoma, pheochromocytoma), schizophrenia, Alzheimer, adrenocortical carcinoma, pituitary adenoma, and multiple sclerosis (Ghasemi et al. 2016; Jafarinejad et al. 2017). The expected range of compounds, dopamine, adrenaline, noradrenaline, homovanillic acid (HVA), vanillylmandelic acid (VMA), metadrenaline, and normetadrenaline are < 1, < 1, < 3, < 50, < 50, < 5, and < 5 μM, respectively, in normal human urine (Hee An et al. 2016; Tillyer and Gobin 1991). Many analytical techniques have been developed to determine and monitor the presence of phenol, substituted phenols, and catechol in environmental and pathological samples; however, all of them are less sensitive, more complex, and non-economical in nature (Grouzmann and Lamine 2013).

Hence, a new type of simple, rapid, specific, economical, and easy to use colorimetric paper-based biosensor is gaining popularity for on-site monitoring of phenols. Enzymes are used as biosensing agents and can be immobilized on paper or any matrix to form a biosensor (Alkasir et al. 2012). Immobilization is a process in which enzymes are fixed to or within solid supports, creating a heterogeneous immobilized enzyme system. The immobilized form of enzymes mimics their natural mode in living cells, where most of them are attached to cellular membranes and organelle structures. The solid support systems generally stabilize the structure of the enzymes and, as a consequence, maintain their activities. Thus, compared with free enzymes in solution, immobilized enzymes are more robust and more resistant to environmental changes (Homaei et al. 2013). Generally, the immobilization enhances chemical, thermal, and operational stability. Biosensors based on paper support have been reported in literature (Alkasir et al. 2012; Oktem et al. 2012; Şenyurt et al. 2015). Nanoparticle-based paper biosensors have been developed such as ceria nanoparticles in conjugation with glucose oxidase co-immobilized onto filter paper with a detection limit of 0.5 mM for the monitoring of glucose in human serum (Ornatska et al. 2011). A visual fluorescence paper biosensor was developed using carbon quantum dots (QDs) for on-site monitoring of 2,4,6-trinitrotoluene (TNT) in groundwater; the fluorescence of QDs was selectively quenched by TNT through a photo-induced electron transfer effect with a detection limit of 0.213 μmol/l (Tian et al. 2017). β-Galactosidase (β-GAL)-based colorimetric paper sensor was fabricated by inkjet printing of sol-gel–based bioinks for rapid determination of a range of heavy metals such as mercury, copper, cadmium, lead, chromium, and nickel (Hossain and Brennan 2011). A bioactive paper biosensor was developed using acetylcholinesterase (AChE) entrapped between two layers of silica sol-gel to detect AChE inhibitors (paraoxon and aflatoxin) rapidly with a detection limit of 30 nM (Hossain et al. 2009). So, there is a need to develop cost-effective and simple methods/protocols to fabricate a paper biosensor.

In the present work, PPO has been partially purified by simple and rapid TPP method using biowaste (potato peels). This partially purified enzyme is immobilized on paper by a simple process of adsorption for construction of a paper-based colorimetric biosensor. Furthermore, the paper strips were used for detection of phenolic compounds in river water samples and urine samples.

Materials and methods

Potato (*Solanum tuberosum*) peel wastes were collected from Jamia Millia Islamia (New Delhi, India) hostel campus. Catechol, 4-methyl catechol, and bovine serum albumin (BSA) were obtained from Sigma Aldrich. Ammonium sulfate [$(NH_4)_2SO_4$], t-butanol, p-cresol, disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), sodium acetate, Tris hydrochloride, sulfuric acid (H_2SO_4), sodium hydroxide (NaOH), 3-methyl-2-benzothiazolinone hydrazine (MBTH), hydrochloric acid (HCl), and phenol were purchased from Merck. Filter paper grade 2 was purchased from Whatman. All the other reagents were of analytical grade obtained from Sigma or Merck.

Experimental

Preparation of enzyme extract

Fifty grams of potato peels was taken, washed, and left to dry at room temperature. They were cut into small pieces and then homogenized in 50 ml of 0.05 M sodium phosphate buffer

(pH 7). Furthermore, the homogenate was filtered through muslin cloth and centrifuged for 15 min at 10,000 rpm and 4 °C. The supernatant (treated as crude extract) obtained was stored at 4 °C and used for further study. In a control experiment, fresh potato peels were also collected and enzyme extract was prepared as above.

Purification of polyphenol oxidase by three-phase partitioning

To carry out TPP, 45% ammonium sulfate [$(NH_4)_2SO_4$] was added to 5 ml of crude enzyme extract, vortexed gently to dissolve the salt, and then 5 ml of t-butanol in 1:1 (v/v) ratio added. The mixture was incubated at 150 rpm and 30 °C in a shaker incubator for 1 h and then centrifuged at 5000 rpm for 10 min at 10 °C. The three layers were formed, and the upper organic layer was discarded. The lower aqueous and middle layers were collected, separately. The enzyme precipitated in the middle layer was resuspended in 5 ml of 0.05 M sodium phosphate buffer. Enzyme activity and protein were determined in the dissolved precipitate and aqueous layer after they were dialyzed overnight at 4 °C against sodium phosphate buffer. TPP was also carried out at various concentrations of ammonium sulfate (20–60%) and enzyme extract to t-butanol ratio (1:0.5–1:2). The above experiment was repeated with fresh potato peel extract as control, and not much change in enzyme activity was seen compared with waste peels so further work was carried out on potato peel waste collected from the hostel campus.

Optimization of TPP

The effect of temperature on the TPP method was investigated by varying temperature in the range of 10 to 60 °C. The mixtures (containing 5 ml enzyme extract, 45% ammonium sulfate, and 5 ml t-butanol) were vortexed gently and incubated at different temperatures separately for 1 h at 150 rpm. The PPO activity and protein were estimated in interfacial layer and aqueous layer.

The TPP method was also carried out by varying pH values from 4 to 9. For this, crude enzyme extract was prepared using buffers of different pH. The buffer systems used were as follows: for pH 4–5 (sodium acetate), pH 6–7 (sodium phosphate), and pH 8–9 (Tris-HCl), while keeping other optimized conditions constant. The mixtures (containing 5 ml enzyme extract, 45% ammonium sulfate, and 5 ml t-butanol) were incubated at 30 °C for 1 h at 150 rpm. The PPO activity and protein were estimated in interfacial layer and aqueous layer.

PPO activity

The method described by Roy et al. was followed to determine PPO enzyme activity using 4-methyl catechol as a substrate. Reaction was stopped after 90 s by adding 1 ml of 10% sulfuric acid (Roy et al. 2002). The absorbance was read at 450 nm.

Protein estimation and SDS-PAGE

The protein concentrations in crude extract and TPP purified samples were determined by Bradford method (dye binding method) using standard protein as bovine serum albumin (BSA) (Bradford 1976).

According to the Laemmli method, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) experiment was performed with 10% gel on a Biorad Mini Protean electrophoresis unit (Laemmli 1970).

Fabrication of paper biosensor and its characterization

Whatman filter paper grade 2 was cut into squares of dimension 1 cm × 1 cm and autoclaved. TPP purified PPO enzyme (30 U, dissolved in sodium phosphate buffer) and 3 mM MBTH in the ratio of 1:1 (v/v) were added to autoclaved filter paper with the help of a pipette as described in literature (Şenyurt et al. 2015). Control paper was prepared without enzyme and containing MBTH only. The loaded papers were air dried and used for further studies as a paper biosensor and its characterization was done. Fourier-transform infrared (FTIR) spectra of filter paper grade 2 and paper biosensor were recorded with a Shimadzu FTIR spectrophotometer between 650 and 4000 cm^{-1}. Paper with MBTH only and paper with PPO and MBTH were directly used for the FTIR study.

Determination of detection limit and sensitivity

For detection limit and sensitivity determination, 100 μl of phenolic compounds (phenol, catechol, p-cresol, and 4-methyl catechol) in the range of 0.125 to 256 μM was added to paper biosensor dropwise and the color change was observed. Paper biosensor was also tested for mixture of phenols (phenol, catechol, p-cresol, and 4-methyl catechol) and the final concentrations of phenols in mixture were varied from (0.256 to 1 mM). The color intensity was measured according to the method described in the following section. The substrate was also applied to the filter paper containing only MBTH which was treated as a control.

Color intensity measurement of biosensor

After the colorimetric response of biosensor to phenols, the paper was dried, and the numerical data was analyzed by scanning the colored paper squares using a Hewlett-Packard Scanjet 4850. According to the procedure described by Russell and Burton, the color intensity was measured in the

Adobe Photoshop software (Russell and Burton 1999). The level of red color was measured in terms of Adobe color units which are expressed on a scale of 0–255 (where 0 is pure black and 255 is pure white). By subtracting the measured color units with the pure white value (255), the red color unit was obtained. Furthermore, in this study, this color unit was subtracted with the value of filter paper containing enzyme and MBTH and no substrate.

Detection of phenols in water samples by biosensor

Water samples were collected from three different locations (Yamuna Ghat, Okhla barrage, and Yamuna Canal) of Yamuna River (New Delhi, India). These samples along with tap water were tested on the PPO biosensor to check its functionality and the color produced was compared with those of phenolic substrates. A total of 100 μl of the water sample was added to the paper biosensor and the resulting color intensity was measured.

Preparation of urine samples spiked with catechol and its detection

According to the protocol given by Shmaefsky, artificial urine was synthesized (Shmaefsky 1990). The synthesized artificial urine sample was spiked with different concentrations of catechol (0.125–4 μM), and 100 μl of the sample from each concentration was spotted onto the paper biosensor separately. The resulting color change was observed with the naked eye and color intensity was measured.

Effect of pH on PPO biosensor response

The colorimetric response of PPO biosensor was investigated by taking phenol at various pH by adding HCl or NaOH. Phenol (256 μM) was used as a substrate in the pH range of 2–13 and was applied on the biosensor to record the color change. To study the effect of pH on PPO in solution, the method described in the "PPO activity" section was followed. PPO enzyme (30 U, dissolved in sodium phosphate buffer) was taken and phenol (256 μM) at different pH (2–13) was used as a substrate. The absorbance was read at 450 nm to determine the activity at different pH.

Storage stability of biosensor

To investigate the storage stability, PPO biosensor was kept at room temperature (25 ± 2 °C) and at 4 °C. The biosensor was sealed properly and covered with foil in order to prevent the exposure of light, moisture, and oxygen. The activity of

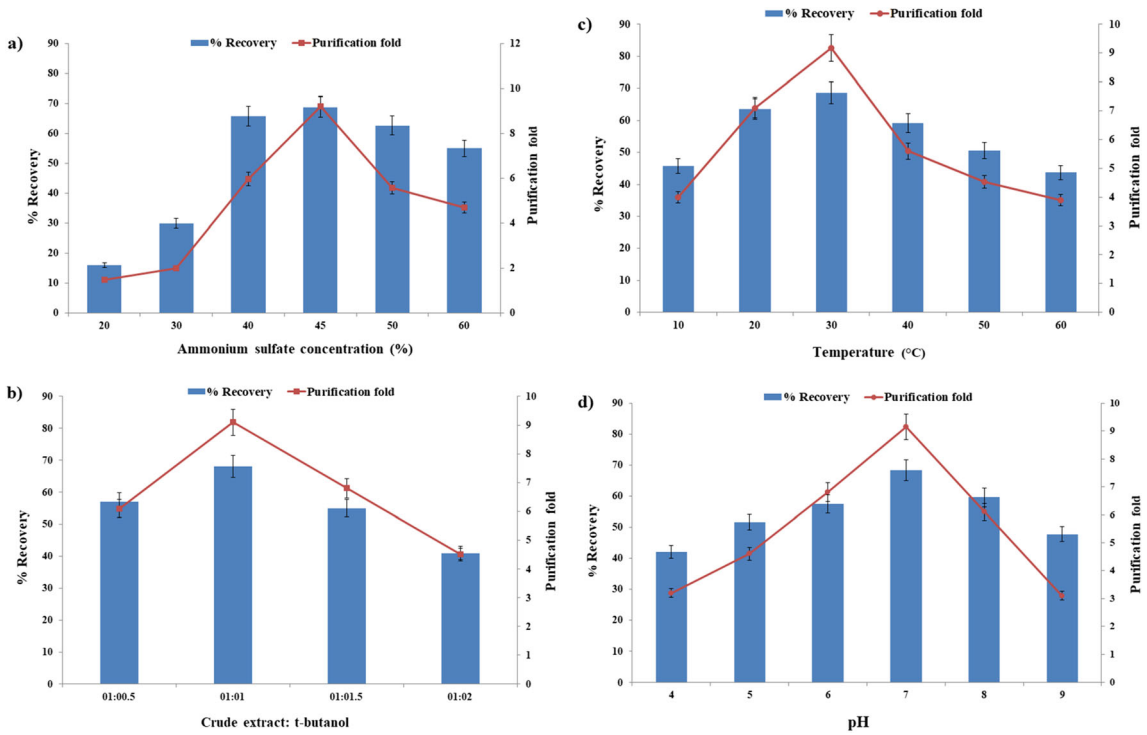

Fig. 1 **a** Ammonium sulfate concentration was varied from 20 to 60% to find out the highest recovery and purification fold. All the values are means of triplicate ($n = 3$) ± SD. ANOVA significant at $P \leq 0.05$. **b** t-Butanol concentration in crude extract:t-butanol ratio was varied as 1:0.5, 1:1, 1:1.5, 1:2, and 1, to determine the optimum condition. All the values are means of triplicate ($n = 3$) ± SD. ANOVA significant at $P \leq 0.05$. **c** Temperature from 10 to 60 °C was varied and the optimum condition was observed. All the values are means of triplicate ($n = 3$) ± SD. ANOVA significant at $P \leq 0.05$. **d** pH was varied from 4 to 9. All the values are means of triplicate ($n = 3$) ± SD. ANOVA significant at $P \leq 0.05$

Table 1 Three-phase partitioning purification and recovery profile of polyphenol oxidase from waste potato peels (*Solanum tuberosum*). Purification fold was calculated as the ratio of specific activity (units/mg) of purified sample and initial sample (crude enzyme) while % recovery is the ratio of enzymatic activity (units) of purified sample and initial sample (crude enzyme) × 100. All the values are means of triplicate ($n = 3$) ± SD. ANOVA significant at $P \leq 0.05$

Purification step	Total Activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude extract	487.33	3.33	146.34	1	100
TPP interfacial phase	335.77	0.250	1343.08	9.19	68.89
Aqueous phase	14.33	2.9	4.82	0.032	2.94

biosensor was checked every 7th day for 1 month using phenol (256 μM) as a substrate, and the reproducibility of biosensor was evaluated by calculating the residual activity. To study the storage stability of PPO in solution, the enzyme in phosphate buffer was kept at room temperature (25 ± 2 °C) and at 4 °C. The activity was determined at an interval of 7 days by following the protocol described under the "PPO activity" section using phenol (256 μM) as a substrate. The absorbance was read at 450 nm.

Statistical analysis

The results presented here are means of triplicates ($n = 3$) ± SD (standard deviation). One-way ANOVA (analysis of variation) was performed to analyze significant differences among multiple samples. Significance level was taken as $P \leq 0.05$ and the data has been presented as mean ± SD.

Results and discussions

TPP is a bio-separation technique which has been applied in the present work to extract and purify PPO enzyme from potato peels. For this, enzyme extract from potato peel was prepared by homogenization in sodium phosphate buffer (pH 7). After filtration, the supernatant obtained was subjected to TPP. For this method, ammonium sulfate and t-butanol were used. It has been reported in literature that ammonium sulfate and t-butanol are most effective on protein partitioning process and maximum purification is obtained using them (Alici and Arabaci 2016). Ammonium sulfate is involved in salting out of proteins from solution and shows high solubility and high ionic strength compared with other salts such as potassium sulfate, sodium chloride, magnesium sulfate, and sodium sulfate (Gagaoua et al. 2017). t-Butanol as a co-solvent offers greater dominion over other solvents owing to the larger size and branched chain structure so as not to disrupt or denature the completely folded protein. Furthermore, t-butanol exercises the kosmotropic as well as crowding effect at a temperature of 20–30 °C which elevates TPP purification (Niphadkar and Rathod 2015). The TPP procedure was rapid and completed within 1 h of constant stirring at room temperature. Three phases were formed in TPP which were separated carefully. Lower aqueous as well as intermediate (precipitate) phase was collected and analyzed for PPO activity. The protein content was estimated to be 0.275 mg/ml and 2.9 mg/ml for interfacial and bottom phase respectively. It was found that maximum recovery and fold purification were observed in the intermediate (precipitate) phase; hence, further experiments were carried out with intermediate phase only.

Various parameters of TPP govern the bio-separation system. Among these, t-butanol and ammonium sulfate concentrations are the most critical one. These factors influence the partition coefficient of enzyme and amount of enzyme in aqueous and intermediate phases. Hence, optimizations of

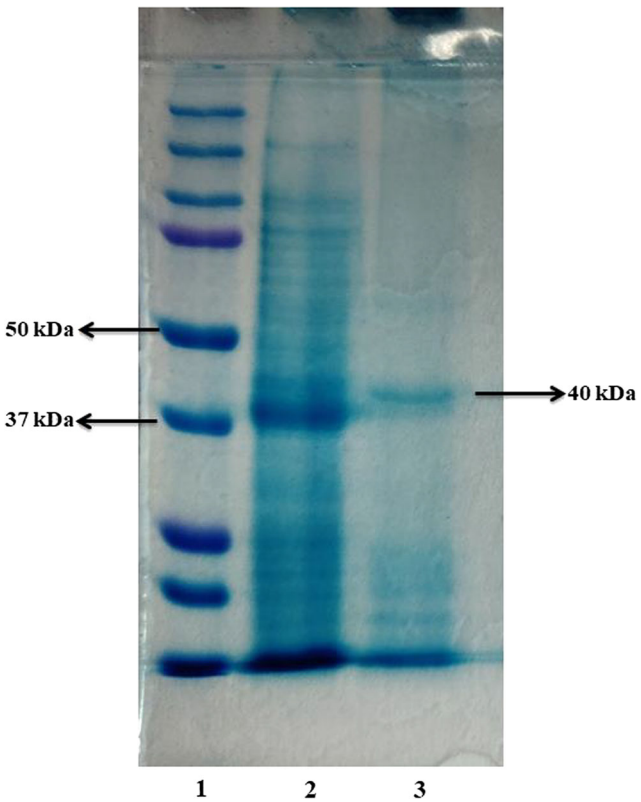

Fig. 2 SDS-PAGE analysis of crude extract and purified sample. **Lane 1**: Protein marker (250 kDa). **Lane 2**: Crude enzyme extract. **Lane 3**: TPP purified PPO

aforementioned factors were carried out to achieve the maximum possible fold purification and recovery profile. Ammonium sulfate concentration was varied from 20 to 60% (w/v) and the data was analyzed. On increasing the concentration from 20% (w/v), PPO precipitation increases due to change in protein solubility in solution which causes hydrophobic interaction with other protein molecules and ultimately its precipitation by salting out of enzyme from aqueous phase. It causes precipitation by dissipating the solvent layer (water) present around the protein (Choonia and Lele 2013). It was observed that the highest purification rate of PPO (9.2-fold) and 69% recovery was reached at 45% (w/v) ammonium sulfate, t-butanol ratio 1:1 (v/v), pH 7, and a temperature of 30 °C in the intermediate phase as represented in Fig. 1a. On further increasing the salt concentration to 60% (w/v), purification fold and recovery decrease in the intermediate phase. It may be due to reduction in selective partitioning of PPO and irreversible denaturation (Niphadkar and Rathod 2015). At low salt concentrations (0–20%), the purification fold and recovery are low in intermediate phase due to insufficiency of hydrophobic interaction between protein molecules (low precipitation) and as a result of which partition of enzyme is enhanced at bottom (aqueous) phase. However, increasing salt concentration from 20% (w/v), the enzyme gets precipitated more by salting out phenomena and forms a thick intermediate layer. In total, 45% (w/v) salt concentration was found to be sufficient at which maximum recovery and purification fold were achieved. Crude enzyme extract to t-butanol ratio was also varied at pH 7 and temperature of 30 °C as shown in Fig. 1b. The purification and recovery profile were found to be maximum at 1:1 enzyme to t-butanol ratio. Below 1:1 (v/v) ratio, the amount of t-butanol was not sufficient enough to synergize with ammonium sulfate and using a higher amount may cause protein denaturation (Dennison and Lovrien 1997). Optimum temperature and pH were also investigated to determine the TPP conditions. Temperature was optimized in the range of 0–60 °C. Temperature is an important parameter as it governs the stability as well as configuration state of enzymes (Niphadkar and Rathod 2015). As the temperature increases from 10 to 30 °C (Fig. 1c), purification as well as recovery of PPO increases in the intermediate phase and then it starts to decrease after 30 °C. Temperature of about 20–30 °C is most suited to achieve the highest yield and purification profile as t-butanol shows significant kosmotropic and crowding effect at this temperature, which stabilizes intramolecular interactions of enzyme and thus enhances partitioning process. t-Butanol

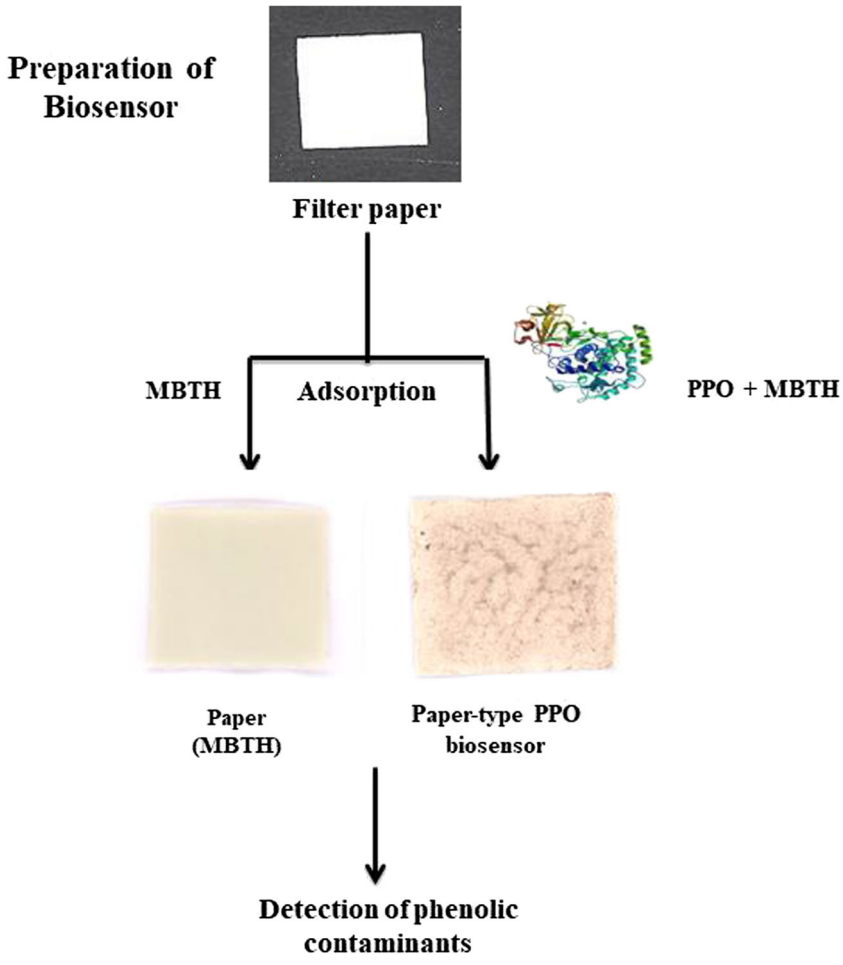

Fig. 3 The schematic representation of the methodology used in development of paper-based PPO biosensor

also works best as an aggregation agent at 20–30 °C (Gagaoua et al. 2017). Optimum temperature was found to be 30 °C for purification as highest purification and recovery of PPO were achieved. At high temperatures, conformational change of structure occurs due to thermal denaturation of enzyme; hence, recovery and purification fold seem to decline after 30 °C (Gagaoua et al. 2016). Moreover, the volatility of t-butanol tends to increase at higher temperatures and less solvent will be available for extraction which reduces synergistic effect with ammonium sulfate. The activity of PPO was checked in the pH range of 4–9 while keeping other parameters constant as shown in Fig. 1d. Change in pH affects the TPP system by changing the partitioning of protein due to phase-charged protein electrostatic interaction. It changes the net charge of the target enzyme. pH alters the ionizable group present in the protein (Gagaoua et al. 2015). Recovery yield and purification fold of PPO were found to be highest at pH 7 in the intermediate phase while increasing or decreasing pH resulted in low enzyme activity. When pH is acidic, PPO gets protonated and interacts less strongly with the substrate; therefore, its activity decreases. PPO enzymes show more catalytic activity in phosphate buffer at pH 6–7 and are more stable in neutral pH as reported in literatures (Fairhead and Thöny-Meyer 2012; Niphadkar et al. 2015).

The overall recovery and purification profile of PPO from waste potato peels have been summarized in the Table 1. A good yield of about 69% and purification fold (9.2) were achieved with this economical method in a single step. To determine the molecular weight, SDS-PAGE analysis of purified PPO and crude enzyme extract were carried out. In lane 2 (crude PPO), many bands of proteins were observed as shown in Fig. 2. In lane 3, one intense band of PPO with molecular weight in the range of 30–40 kDa, along with other 2–3 bands of impurities, was observed. The molecular weight of PPO from potato obtained in this study is in agreement with the previously reported literature (Niphadkar and Rathod 2015).

For development of paper biosensor, the TPP purified PPO was adsorbed on Whatman paper grade 2. It was based on colorimetric detection as a chromophore reagent MBTH was used in order to get a visible color for detection. MBTH forms an adduct or a complex with the product formed (quinones) and generally gives red/pink color which can be seen by the naked eye (Şenyurt et al. 2015). The illustration of the method used is shown in Fig. 3. FTIR analysis of the paper biosensor was carried out, and as shown in Fig. 4, it was found that an amide II peak was observed between 1550 and 1650 cm^{-1} due to adsorption of PPO on the paper. The filter paper without enzyme does not show any peak in this region.

To evaluate the analytical performance of the biosensor, certain parameters like limit of detection, sensitivity, reproducibility, selectivity, and stability were determined. The detection limit and sensitivity of PPO-based paper biosensor were investigated by using different phenolic compounds such as catechol, phenol, p-cresol, and 4-methyl catechol. The concentration of phenolic compounds was varied as low as possible over a range of 256 to 0.125 μM to check the sensitivity toward each phenolic compound. After the addition of phenolic compounds to the paper biosensor, the biosensor showed colorimetric response toward these phenolic compounds with good efficiency as depicted in Fig. 5a. A slight difference in color was produced by the biosensor depending on the phenolic compounds: reddish pink color for catechol

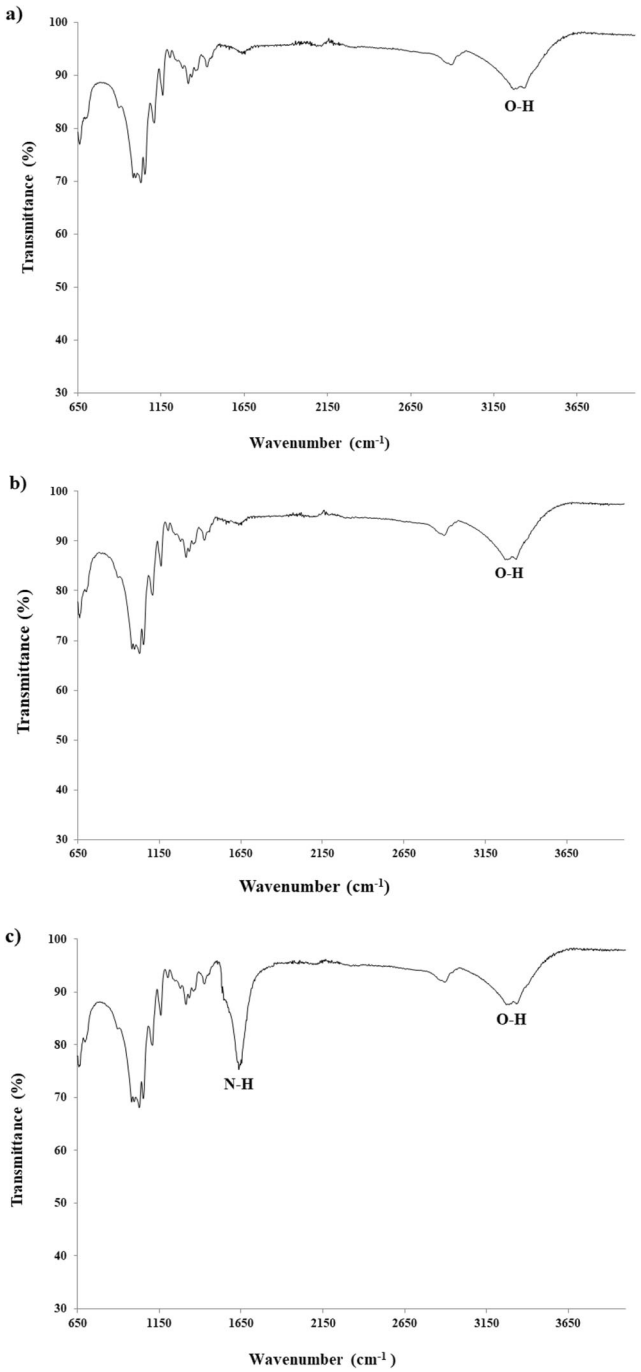

Fig. 4 FTIR graph of **a** filter paper, **b** filter paper in the presence MBTH, and **c** filter paper in the presence of PPO and MBTH

and phenol while orange-brown for p-cresol and 4-methyl catechol. The color intensity was quantified using a scanner and Adobe Photoshop, and the color unit was calculated. Figure 5b shows numerical representation of the color intensity, and the plot provided linear calibration curve between the color units and phenol concentration. The reaction required only 5–10 min depending on phenols in order to generate a visible color and color unit was analyzed after 15 min when the color change was stabilized on paper squares. It was concluded that the biosensor was sensitive toward these phenolic compounds and detection limit was determined to be almost 0.5 μM (or 0.05 mg l^{-1}) for each compound. According to IUPAC Compendium of Analytical Nomenclature, the limit of detection is the lowest concentration of an analyte that an analytical process can reliably detect (Long and Winefordner 1983). The filter paper containing only MBTH (treated as a control) was compared with the filter paper containing PPO and MBTH after substrate application. The control filter paper did not develop any color change, while the test filter paper containing PPO developed color. The color intensity was

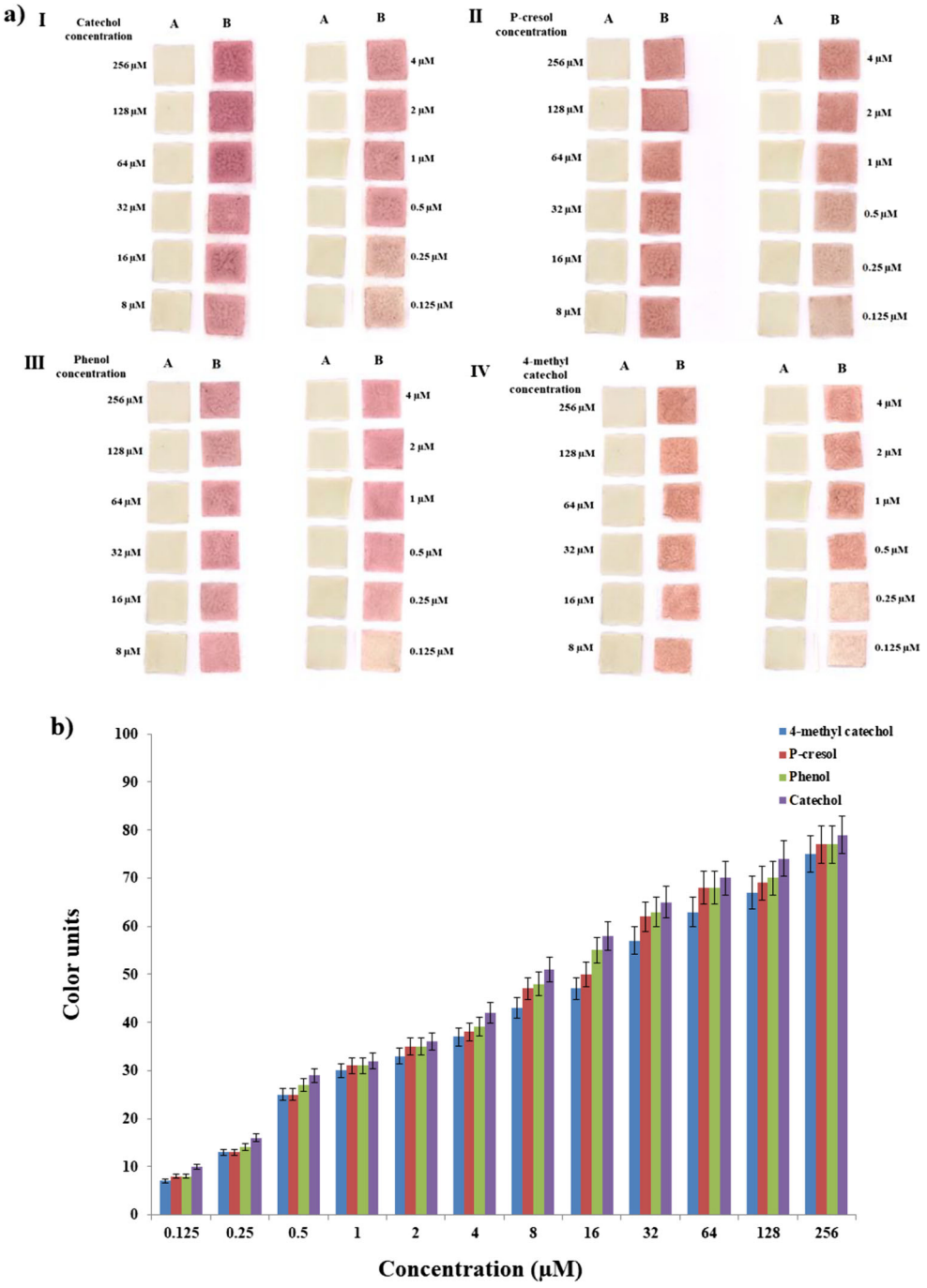

Fig. 5 **a** Scanned image of colorimetric response of biosensor to the addition of (**I**) catechol, (**II**) p-cresol, (**III**) phenol, and (**IV**) 4-methyl catechol at different concentrations. **A** represents control paper with MBTH and substrate application, while **B** represents paper with PPO + MBTH and substrate application. **b** Quantitative representation of color intensity produced by biosensor for catechol ($y = 6.1189x + 19.227$ and $r^2 = 0.9856$), p-cresol ($y = 5.9091x + 14.924$ and $r^2 = 0.9672$), phenol ($y = 5.7727x + 17.894$ and $r^2 = 0.9881$), and 4-methyl catechol ($y = 5.8741x + 2.9848$ and $r^2 = 0.9857$) in terms of color units obtained with Adobe software. All the values are means of triplicate ($n = 3$) ± SD. ANOVA significant at $P \leq 0.05$

found to be proportional to the concentration of phenolic compounds. On increasing the concentration, color intensity increases. These results were in agreement with Russell and Burton work (Russell and Burton 1999). They reported a membrane-based bioprobe of polyphenol oxidase which had a sensitivity of 0.05 mg l^{-1} for phenolic compounds and it was operational within pH range of 4–10. The biosensor developed in this work was found to be more sensitive than other paper-type biosensors reported by other investigators; this has been summarized in Table 2. Various types of phenolic compounds are present in water due to industrial discharge; therefore, in this study, the efficiency of biosensor for detecting mixture of phenolic compounds was tested. Phenolic mixture (containing catechol, phenol, p-cresol, and 4-methyl catechol) was added on filter paper, and it was found that the biosensor gave a color response toward the mixture also (Fig. 6).

PPO-based paper biosensor was applied to the water sample collected from Yamuna River from three different locations near industrial areas in order to check the presence or absence of phenolic substrates within the detection limit of biosensor. The color produced in water samples was compared with the color response shown individually by catechol, phenol, p-cresol, and 4-methyl catechol. The visual detection showed that out of three locations, a slight color change was observed in one of the samples collected (Yamuna Ghat). The other two samples (collected from Okhla barrage and Yamuna Canal) did not show any color change; i.e., it denotes that either phenol was not present or the concentrations of phenolic compounds in water were lower than the detection limit of this biosensor (Fig. 7). This proved the potential of paper biosensor to be used for on-site monitoring of phenolic compounds in environmental water sample or it could be used to check the presence of level or concentration of phenols in industrial effluents (Mazhari et al. 2017). Furthermore, the colorimetric response of biosensor to urine samples spiked with catechol in the range of 0.125 to 4 μM is shown in Fig. 8a. The color intensity obtained as shown in Fig. 8b was almost the same as the color response shown by the catechol solution despite the considerable background activity in urine samples. The urine matrix showed no significant effect on the sensitivity of biosensor. This proves that elevated concentration of catecholamine in urine samples taken from patients can be detected from the paper biosensor. Tillyer and Gobin (1991) described a preparation of polyphenol oxidase enzyme electrode for detection of catecholamines in urine of patients (Tillyer and Gobin 1991).

For evaluation of reproducibility, 3 identical paper biosensors were fabricated independently following the above protocol. After the addition of 256 μM phenol, the average color intensity obtained was 77.3 (± 1.2 for $n = 3$) and calculated RSD (relative standard deviation) was found to be 1.6%.

Table 2 Comparison of paper-based PPO biosensor

S. No.	Enzyme and its source	Mode of immobilization	Detection limit	Compounds detected	pH range	Reference
1.	Polyphenol oxidase (*Solanum tuberosum* peels)	Physical adsorption on paper	0.5 μM	Phenol, p-cresol, 4-methyl catechol and catechol	3–11	This study
2.	Tyrosinase (*Agaricus bisporus*)	Physical adsorption on paper	0.032 mM 0.128 mM	4-Chlorophenol and catechol m-Cresol and p-cresol	–	Şenyurt et al. (2015)
3.	Laccase (*Trametes versicolor*)	Physical adsorption on paper	64 μM	L-DOPA and catechol	–	Oktem et al. (2012)
4.	Polyphenol oxidase (brown *Agaricus bisporus*)	Physical adsorption on nylon membrane	0.53 μM	Phenol, catechol, 4-methyl catechol and p-cresol	4–10	Russell and Burton (1999)
5.	Tyrosinase (*Agaricus bisporus*)	Physical adsorption on paper using chitosan and alginate polyelectrolytes	9.13 nM	Phenol, bisphenol A, catechol, p- and m-cresol	5.5–8.5	Alkasir et al. (2012)
6.	Tyrosinase (*Streptomyces tuirus*)	Physical adsorption on paper using AuNPs -tyrosinase bioconjugate	0.991 mM 0.487 mM 0.78 mM	Phenol Catechol Dopamine	–	Mazhari et al. (2017)

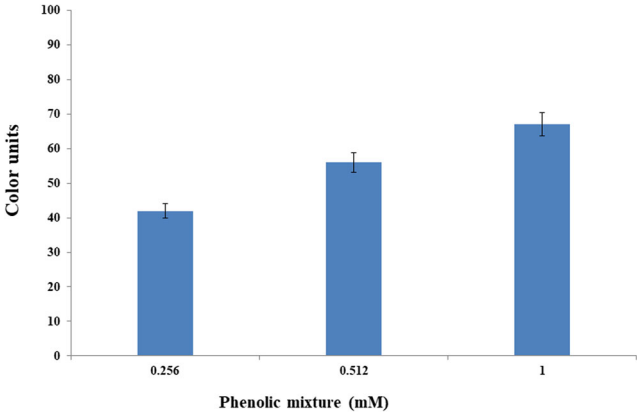

Fig. 6 Numerical representation of color intensity produced by biosensor for mixture of phenolic substrates at different concentrations in terms of Adobe color units. All the values are means of triplicate ($n = 3$) ± SD. ANOVA significant at $P \leq 0.05$

Low RSD corresponds to less deviation and better sensing performance (Alkasir et al. 2012). A pH range of 2–13 was used to check the effectiveness of the biosensor response toward the phenols (Fig. 9). It was found that the optimum pH appears to be 7–8 according to the numerical value of the colors produced. The activity of PPO in solution toward phenol at different pH was also performed to check the optimum pH for free enzyme. It was found to be at around pH 7. The colorimetric response of PPO biosensor towards phenol was achieved at pH 7–8, which corresponds to optimum pH of free enzyme. The biosensor worked in the wide pH of 3–11 and pH value which are below or above this range; the color change was not significant. The biosensor was, thus, operational within this range of pH (Russell and Burton 1999; Yang and Wu 2006).

Alkasir et al. developed a colorimetric paper biosensor of tyrosinase for detection of phenolic contaminants. This sensor was fabricated by a layer-by-layer assembly approach, formed by alternatively depositing layers of chitosan and alginate

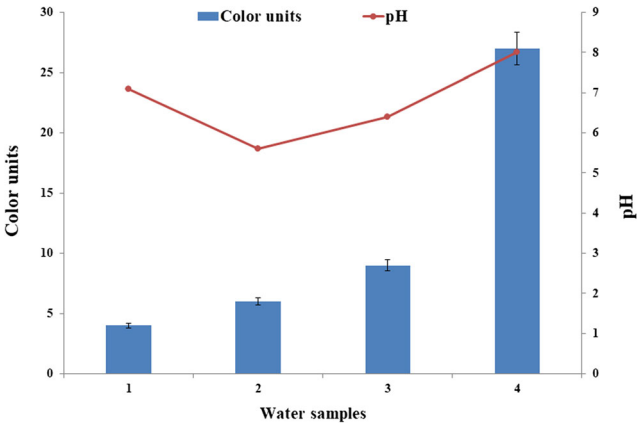

Fig. 7 Quantitative analysis of colorimetric response showed by water sample collected from different locations, (1) tap water, (2) Okhla barrage, (3) Yamuna River canal, and (4) Yamuna River ghat. All the values are means of triplicate ($n = 3$) ± SD. ANOVA significant at $P \leq 0.05$

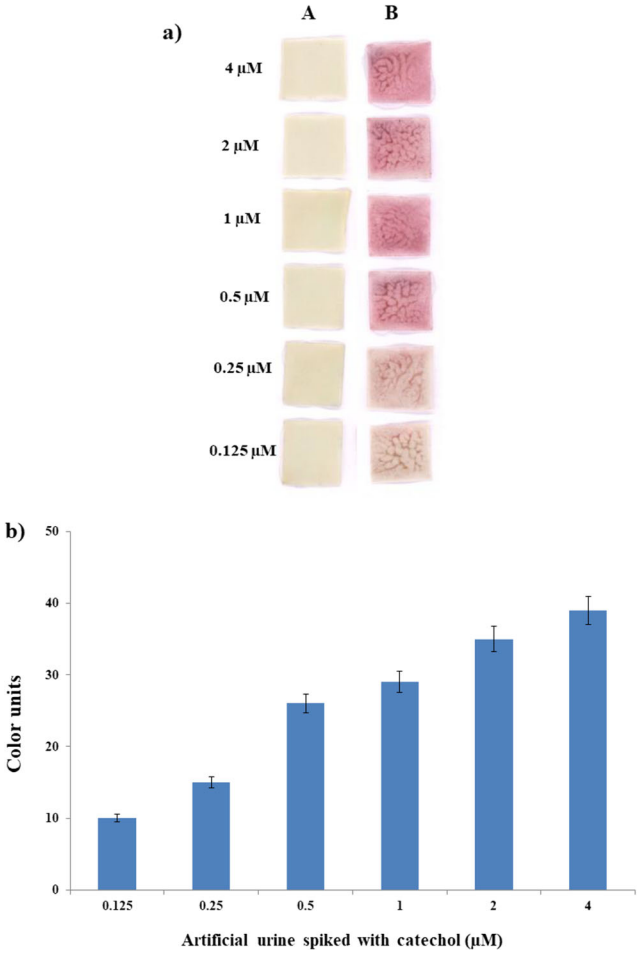

Fig. 8 **a** Scanned image of colorimetric response of biosensor (**A** represents control paper with MBTH and urine sample application while **B** represents paper with PPO + MBTH and urine sample application) and **b** quantitative estimation of color intensity of biosensor to the addition of urine sample spiked with catechol at different concentration

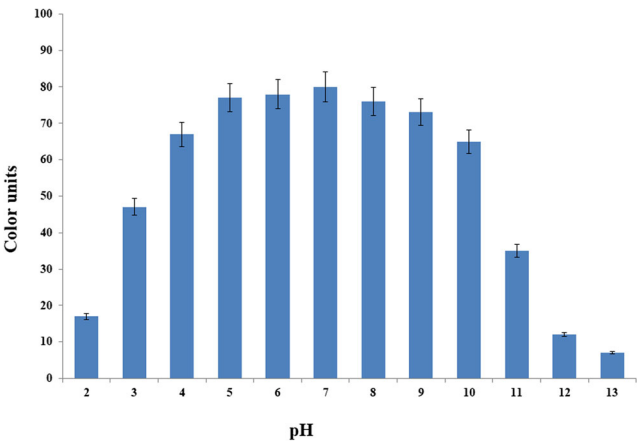

Fig. 9 Quantitative analysis of color produced by biosensor in response to 256 µM phenol solution at various pH value given in Adobe color units. All the values are means of triplicate ($n = 3$) ± SD. ANOVA significant at $P \leq 0.05$

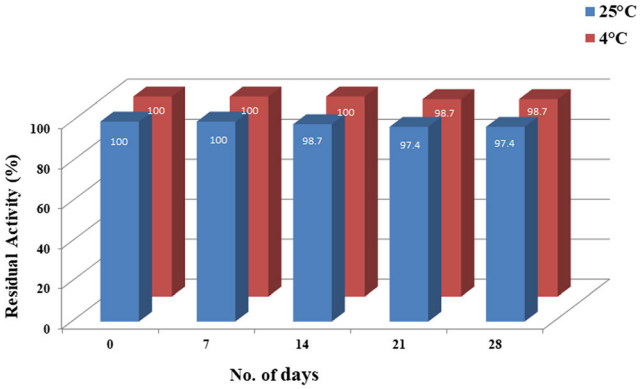

Fig. 10 Stability of biosensor in terms of residual activity (%) at 4 °C and room temperature for over a month. All the values are means of triplicate ($n = 3$) ± SD. ANOVA significant at $P \leq 0.05$

polyelectrolytes onto filter paper, and tyrosinase was physically entrapped between these layers (Alkasir et al. 2012). However, a paper bioassay developed by Alkasir et al. showed color response until pH 9 but no data regarding color change above pH 9 is given (Alkasir et al. 2012), and our study reported a biosensor which developed color toward phenolic substrates at highly basic pH (11.0) also. Thus, the biosensor developed in in this study can be used at alkaline pH. Moreover, low RSD of the biosensor, and cost-effectiveness and simplicity of the method used in the present study were observed compared with the biosensor developed in the bioassay of Alkasir et al. (2012). The stability of PPO biosensor was checked over a period of 28 days by evaluating the colorimetric response of biosensor stored in dried state at 4 °C and room temperature. Up to 98.7% and 97.4% of residual activity were retained by biosensor in our study after 1-month storage in refrigerated condition (4 °C) and room temperature respectively (Fig. 10). Free PPO enzyme in solution was also tested for storage stability. The free enzyme lost 40% of its initial activity at 4 °C and 90% of its activity when stored at room temperature after 1 month. The stability of the biosensor was carried out to determine its feasibility for commercialization. These results showed excellent storage capability. It can also be evaluated that the enzyme activity could be preserved for a longer duration, i.e., more than a month, efficiently.

Conclusions

Polyphenol oxidase is an important enzyme which has a wide stretch of application in different areas. Among all chromatographic methods used for protein purification, three-phase partitioning was found to be simpler, cost-effective, novel, and manageable which results in great purity and recovery of proteins/enzymes. In this research, PPO was purified using this technique and was obtained with high yield and fold purification. The purified free enzyme was characterized and utilized for application such as the development of paper biosensor. The biosensor showed analytical characteristics like excellent performance, sensitivity to phenols, reproducibility, pH dependency, and stability. It showed good performance over a period of 1 month when stored at 4 °C or room temperature, and was effective when environmental river water sample was applied to analyze the presence of phenolic compounds. It can also be applicable for on-site monitoring of hazardous phenolic components present in drinking water and industrial effluents. It was also used to detect the elevated catecholamine level in urine samples for early diagnosis of neural disorders. It is appropriate for field use and can be handled by non-experts. This shows the reliability of biosensor in real samples.

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Compliance of ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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