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Article *in* Preparative Biochemistry & Biotechnology · May 2020

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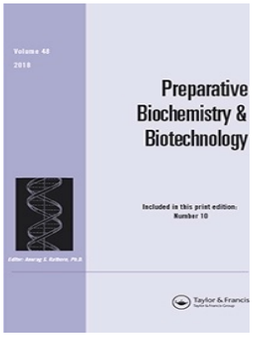

Preparative Biochemistry & Biotechnology

ISSN: 1082-6068 (Print) 1532-2297 (Online) Journal homepage: https://www.tandfonline.com/loi/lpbb20

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To cite this article: Ajinkya Dabhade, Sivaraman Jayaraman & Balasubramanian Paramasivan (2020): Colorimetric paper bioassay by horseradish peroxidase for the detection of catechol and resorcinol in aqueous samples, Preparative Biochemistry & Biotechnology, DOI: 10.1080/10826068.2020.1760883

To link to this article: https://doi.org/10.1080/10826068.2020.1760883

PREPARATIVE BIOCHEMISTRY & BIOTECHNOLOGY
https://doi.org/10.1080/10826068.2020.1760883

Colorimetric paper bioassay by horseradish peroxidase for the detection of catechol and resorcinol in aqueous samples

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ABSTRACT

Phenolic compounds such as catechol and resorcinol are toxic and persistent pollutants in the aqueous environment. Detection procedures such as chromatographic and spectrophotometric methods are time-consuming and require sophisticated instruments with skilled manpower. Development of a simple, cost effective, portable and disposable paper based biosensor could be a better alternative to the conventional methods. The present study attempted to develop a paper based biosensor by immobilizing horseradish peroxidase enzyme to detect catechol and resorcinol in aqueous samples. Horseradish peroxidase catalyzes the oxidation of phenolic compounds to semiquinones, which on reaction with a chromogen, 3-methyl 2-benzothiazolinone hydrazine (MBTH) gives faint pink to red color depending on the compound and its concentration in the sample is the basis for biosensing application. Different methods of enzyme immobilization on filter paper like physical adsorption, covalent coupling, and polysaccharide entrapment were executed. The performance of the various enzyme immobilization methods was evaluated by analyzing the developed color intensity using ImageJ software. Entrapment technique is the most effective method of immobilizing enzyme on the filter paper that produces the highest color intensity with better stability. The visible limit of detection (LoD) was observed as 0.45 mM (50 mg/L) for catechol and 0.09 mM (10 mg/L) for resorcinol in aqueous samples.

KEYWORDS

Catechol; enzyme immobilization techniques; horseradish peroxidase; paper based biosensor; phenolic compounds; resorcinol

Introduction

Catechol is an ortho-isomer of benzenediol also called pyrocatechol and has many applications as a polymerization inhibitor, photographic developer, lubricating oil and in pharmaceuticals.[1] It is a toxic and persistent water pollutant in the environment.[2,3] Catechol is classified as a possible carcinogen to humans (group 2 B) by the International Agency for Research on Cancer (IARC).[4] Phenolic compounds such as catechol have been listed as priority pollutants by EPA.[5–7] It is toxic for most aquatic animals including fish at lower concentrations.[8] Resorcinol is a meta isomer of benzenediol widely used in the manufacturing of phenolic resins and pharmaceuticals.[9] The rubber industry highly consumes resorcinol (50%) with a 0.1% loss during tire production. About 25% resorcinol usage is for high-quality wood bonding applications and other uses are in the manufacturing of pharmaceuticals, dyes, hair dye formulations and pesticides .[10] Resorcinol is an endocrine-disrupting chemical, which can cause a serious impact on wildlife on release to the environment.[11] Coal carbonization and gasification industries are a major source of catechol and resorcinol in wastewater where its concentration ranges from a few mg/L to a maximum of 2000 mg/L.[9,12]

Development of a rapid and easy detection system for phenolic compounds in wastewater is essential for environmental monitoring as it possesses serious environmental hazards mostly for aquatic flora and fauna as well as health risks to humans.[13,14] The conventional methods used for detection like spectrophotometry and chromatography are sophisticated, time-consuming, and require qualified staff.[15] Moreover, many enzymatic electrochemical biosensors integrating nanomaterials were developed for the detection of phenolic compounds such as HRP based amperometric biosensor,[16] laccase based amperometric biosensor,[17] and tyrosinase based voltametric biosensor.[18] Paper-based biosensors are the biosensors that integrate paper as a reaction platform because of its property to wick the liquid by capillary action.[19] The paper is a chemically inert, biocompatible and biodegradable material that shows its advantages to be used as a biosensing platform. Paper based biosensors are inexpensive, sensitive, user-friendly, rapid, do not require power source, readable with a naked eye, and can be easily delivered.[20] Whatman #1 filter paper has a large pore

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Supplemental data for this article is available online at http://dx.doi.org/10.1080/10826068.2020.1760883.

volume fraction, which can support fast lateral flow and hence it is used most commonly as a microfluidic or biosensor platform.[21] Biorecognition agents like enzymes can be immobilized on the paper by physical adsorption, covalent coupling or polysaccharide entrapment and also can be printed on the paper using a piezoelectric inkjet printer.[22,23] Because of these advantages, the paper-based biosensor can be proved as an effective tool for environmental monitoring.[2] Hossain et al. demonstrated an electrochemical biosensor for simultaneous detection of catechol, resorcinol and hydroquinones.[24] Abdullah et al. described optical biosensor for the detection of catechol using enzyme laccase and chromogen MBTH.[25] Lepore and Portaccio constructed an optical biosensor using sol-gel entrapment of laccase to detect catechol and resorcinol. Although many studies described the development of amperometric and optical biosensors for the detection of phenolic compounds using enzymes like tyrosinase and laccase having good sensitivity and limit of detection, these methods are expensive and complicated. Here we describe a simple paper-based biosensor that is easy to construct and use.[26] Arciuli et al. demonstrated paper based bioassay for phenolic compounds in wine using tyrosinase enzyme and MBTH deposition on paper. The sensor response was characterized using L-3-4-dihydroxyphenylalanine (L-DOPA). Furthermore, tyrosinase based paper biosensor was fabricated by Alkasir et al.[27] using enzyme entrapment by depositing on alternate layers of chitosan and sodium alginate. However, the specific response of the sensor to the pollutants like catechol and resorcinol in real-time water samples is not yet been widely reported. Considering the complex and time consuming biosensor fabrication methods in the above literature, there is a need to develop an easy, time saving and effective biosensor method for detecting the phenolic compounds.

In the present study, Horseradish peroxidase (HRP; donor: hydrogen peroxide; oxidoreductase; EC 1.11.1.7) was used for developing a paper-based biosensor to detect phenolic compounds that are commonly found in aqueous samples. The enzyme catalyzes the oxidation of benzenediol to benzosemiquinone that reacts with chromogen 3-methyl 2-benzothiazolinone hydrazone (MBTH) and gives color depending on the phenolic compound and its concentration. The different immobilization methods on Whatman#1 filter paper viz., physical adsorption, covalent coupling on fresh filter paper, covalent coupling on chitosan modified paper, and polysaccharide entrapment of enzyme were analyzed for the best performance of the developed biosensor. The concentration of enzyme, chromogen, hydrogen peroxide and pH were optimized for the efficient construction of biosensor to detect phenolic compounds in wastewater.

Materials and methods

Materials

Horseradish peroxidase from the root of *Arabidopsis thaliana*, (EC 1.11.1.7) optimum pH 6–6.5, 250 U/mg was purchased from Himedia, India. Pyrocatechol, resorcinol and MBTH were purchased from SRL chemicals (India). 1 mg/mL HRP solution was prepared in 0.1 M Phosphate buffer of pH 6.0 and stored at 4 °C. Catechol and resorcinol solutions in 0.1 M phosphate buffer of pH 6.0 were made fresh for each experiment. 3% (*v/v*) hydrogen peroxide (H_2O_2) was prepared in 0.1 M Phosphate buffer of pH 6.0, stored at 4 °C and the volume used according to the requirement of the concerned experiments. 0.1 M Phosphate buffer was prepared by adding 1.2 mL of 1 M NaH_2PO_4 and 8.8 mL of NaH_2PO_4 making the final volume to 100 mL. pH was set to 6.0 with 0.1 N NaOH. Chitosan (1% *w/v*) was prepared by first dissolving 0.2 g medium weight chitosan in 20 mL 0.5% acetic acid with stirring and pH adjusted to 6.0 with 0.1 N NaOH. Glutaraldehyde solutions 1% (*v/v*) and 3% (*v/v*) were prepared fresh in 0.1 M Phosphate buffer of pH 6.0. A wash buffer was prepared by dissolving 0.1% Tween-20 (*v/v*) in 0.1 M Phosphate buffer of pH 6.0.

Colorimetric assay

Whatman #1 filter paper was cut into 1 cm × 1 cm pieces, which were sterilized in UV for 20 min. The images of the immobilized enzyme assays were generated using the Cannon LIDE120 scanner at 300 dpi resolution. The images were analyzed for color intensity at grayscale in black and white format using ImageJ software. ImageJ provides color intensity in the range of 0 (for black) to 255 (for white). The image at grayscale was inverted (0 for white and 255 for black) to get increasing color intensity values with increasing darkness. Although the color generates within a minute on substrate dispensation, the images were scanned after 15 min for proper drying of the paper. All the assays were carried out in triplicates and the average color intensities with variations are displayed by the error bars.

Immobilization methods

Physical adsorption of HRP on Whatman paper

About 10 µL volume of HRP at a concentration of 200 µg/mL was dispensed on the filter paper using a pipette and allowed to dry for 30 min. The paper was washed three times with 100 µL wash buffer and allowed to dry for 30 min. Finally, 10 µL volume of solution containing 0.1% catechol, 24 mM MBTH and 0.6% H_2O_2, pH 6.0 was dispensed on the same paper and observed for color development. The digital colorimetric images of the assays were generated by scanning the results for every 15 min after catechol dispensation using a desktop scanner and the digital assay results were analyzed for colorimetric intensity using ImageJ software.

Hrp immobilization by covalent coupling on unmodified Whatman paper

About 20 µL of 3% glutaraldehyde in 0.1 M pH 6.0 phosphate buffer was introduced to activate the original paper for 2 hours. Then the paper was washed three times with the 100 µL of the mixed solution of the coupling buffer (1.5% (*w/v*) mannitol, 0.15% (*v/v*) glycerol and 0.01% (*v/v*) Tween 20) to the top surface of it, and then putting the bottom of

the paper in contact with a piece of cotton wool to remove the excess buffer. Subsequently, 10 µL of an enzyme solution (200 µg/mL) in the coupling buffer was spotted onto the freshly activated paper square and incubated 2 hours at 4 °C. After that, the paper was washed with 100 µL of wash buffer three times and allowed to dry for 30 min under ambient conditions.[22] Finally, 10 µL of a solution containing 0.1% catechol, 24 mM MBTH and 0.6% H_2O_2 was added onto the paper and the image was generated by scanning the paper after 15 min. The color intensity was measured using ImageJ.

HRP immobilization by covalent coupling on chitosan-coated paper

In addition to HRP coupling on unmodified paper, one more attempt was made to immobilize the HRP on chitosan-coated paper for enhancing the crosslinking behavior of the enzymes on the substratum. 20 µL of 0.1% (*w/v*) chitosan solution was dropped onto a paper square. After being dried under ambient conditions overnight, the chitosan-coated paper was ready for use. The enzyme could be covalently immobilized on glutaraldehyde activated chitosan-modified paper square.[22]

HRP entrapment in chitosan

For the preparation of 0.1% chitosan gel mixture of 1 mL volume, 600 µL of phosphate buffer pH 6.0, 100 µL of the 1% chitosan (*w/v*), 200 µL HRP enzyme (1000 µg/mL), 10 µL of 1% glutaraldehyde as a crosslinking agent were mixed in an Eppendorf tube obtaining the final concentration of the chitosan, glutaraldehyde, and enzyme as 0.1%(*w/v*), 0.01%(*w/v*), 200 µg/mL respectively. The mixture was stirred and 10 µL of the mixture was spotted on the Whatman #1 filter paper square and allowed to dry for 30 min. The paper square was washed with a wash buffer containing 0.1% Tween 20, pH 6.0. Finally, a mixture containing 0.1% catechol, 0.6% H_2O_2 and 24 mM MBTH in 0.1 M phosphate buffer was added on it and the image was generated using scanner after 15 min of incubation. The intensity of the color was measured using ImageJ. The experiments were performed for different chitosan concentrations ranging from 0.05 to 0.5% (*w/v*).

Optimization study for H_2O_2, HRP and MBTH concentrations

Paper squares were spotted with 10 µL of enzyme-chitosan gel mixture. The enzyme concentration used was 100 µg/mL. For the optimization of H_2O_2 concentrations, solutions of varying H_2O_2 concentrations from 2.5 to 60 mM with a constant 5 mM catechol and 24 mM MBTH concentration were prepared freshly. About 10 µL of each solution was spotted onto the paper squares. For the MBTH optimization study, chitosan gel mixtures with varying MBTH concentrations from 5 to 45 mM were prepared to keep a constant of 100 µg/mL enzyme concentration. About 10 µL of each mixture was spotted onto the paper squares and allowed to dry for 30 min. Each MBTH concentration was assayed with varying catechol concentrations of 4.5–45 mM keeping H_2O_2 concentration as 270 mM. For enzyme concentration optimization, chitosan gel mixtures of varying enzyme concentrations from 2.5 to 200 µg/mL were prepared with a constant 15 mM MBTH concentration. Each enzyme concentration was assayed with increasing catechol concentration. For all the assays the images were scanned after 15 min of catechol dispensation and analyzed for colorimetric intensity using ImageJ software.

Construction of biosensor and determination of shelf life

The paper-based biosensor was constructed using 15 µg/mL HRP, 15 mM MBTH entrapped in 0.1% chitosan using 0.01% glutaraldehyde as a crosslinking agent. A higher glutaraldehyde concentration can react with MBTH to produce intense milky white coloration and also decreases biosensor performance. The constructed biosensor paper squares were kept at room temperature and at 4°C in closed Petri dishes. Catechol and resorcinol concentrations at 50 mg/L 10 mg/L were prepared with 270 mM H_2O_2 and 10 µL of each concentration were spotted onto the paper after every 2 days and analyzed for percent decrease in colorimetric intensity.

Results and discussion

Determination of effective method for enzyme immobilization on whatman paper

All the methods of enzyme immobilization were compared by analyzing color intensities using ImageJ. The enzyme concentration, the catechol concentration, the H_2O_2 concentration, the MBTH concentration, and the time interval between enzyme dispensation and substrate-chromogen (catechol + H_2O_2+MBTH) was kept constant for all the assays. From the perspective of a colorimetric assay, the paper should not acquire background color intensity. Supplementary Figure 1a shows background color intensities of the paper squares after chitosan-enzyme gel mixture dispensation. The optimum chitosan concentration selected for enzyme entrapment was 0.1% as it gives lower background color intensity with higher colorimetric signals after catechol dispensation (Supplementary Figure 1b).

Physical adsorption on paper

From the comparative analysis of the mean colorimetric intensities, moderate signals from physical adsorption of the enzyme on fresh Whatman#1 filter paper were obtained. The enzyme was adsorbed on the paper surface by weak nonionic interactions like Van Der Waals forces, hydrophobic and hydrophilic interactions and electrostatic attraction.[28] Although the weak interactions can be disrupted in the washing step, the physical adsorption method shows higher color intensity than glutaraldehyde mediated coupling (covalent coupling) on unmodified and chitosan-coated paper (Figure 1a). However, the stability of the enzyme

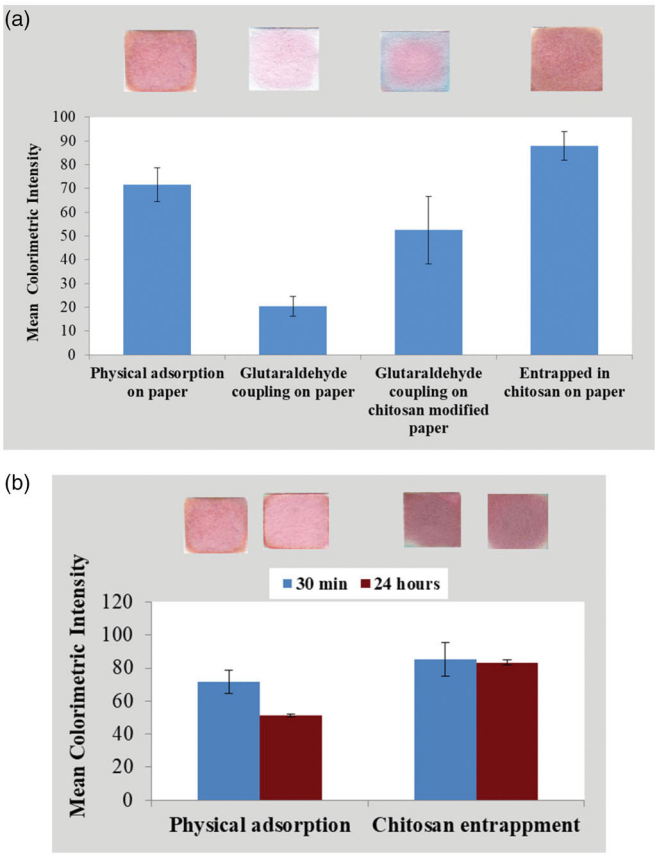

Figure 1. Development of (a) color intensity ad (b) stability by immobilizing HRP through various strategies on paper for detecting catechol.

reduced greatly compared to the chitosan immobilized enzyme.

Covalent coupling of HRP on unmodified paper and chitosan-coated paper

The covalent coupling of HRP to both chitosan modified and unmodified paper shows inefficient immobilization compared to physical adsorption of the enzyme on unmodified paper. The possible reasons could be given as (1) the incubation period of 2 hours after enzyme dispensing on paper may affect the enzyme activity even it is stored at 4°C. (2) Excess glutaraldehyde can randomly bind to the catalytic site of the enzyme resulting in inactivation of catalytic activity. (3) In the case of unmodified paper, there are no primary amine groups on paper for the covalent bonding of glutaraldehyde. The paper is washed with wash buffer containing Tween 20 which removes nonspecific bindings, the weak bonds get disrupted resulting lower signal from a covalent coupling on unmodified paper compared chitosan-coated paper.

The crosslinking of the enzyme to the solid support generally implies the ε-amino group of lysine residues.[29] Most proteins contain lysine residues, which are usually located on the protein surface because of the polarity of the amine group. Furthermore, lysine residues are generally not involved in the catalytic site that allows moderate cross-linking to preserve protein conformation and thus biological activity.[30] In the case of HRP, there are only six lysine residues that are probably not significant for effective crosslinking by glutaraldehyde.[31] However, the covalent coupling on chitosan modified paper gives a higher signal compared to the covalent coupling on unmodified paper as it forms a strong and stable bonding.

Enzyme entrapment in chitosan

From Figure 1a and Figure 1b, the relative high color intensity shows the efficient enzyme immobilization as well as retention of the enzyme catalytic activity compared to physical adsorption. Chitosan is an ideal polymer for enzyme entrapment due to its free amino groups which provide a site for crosslinking for a crosslinker like glutaraldehyde.[32] It is biocompatible and does not interfere in the enzymatic reaction. The chitosan fibers are crosslinked by glutaraldehyde to form a polymer matrix that entraps enzyme within it.[33,34] The enzyme is entrapped in chitosan crosslinked using glutaraldehyde shows the highest retention on paper on washing by wash buffer. The chitosan entrapped enzyme distributed over the porous paper surface exhibits a microenvironment that maintains enzyme catalytic activity over a long time. The free enzyme denatures over a period of time due to the environmental factors whereas the polymer entrapped enzyme is isolated from the external environment sustaining the confirmation and catalytic activity.[35]

Optimization study for biosensor construction and working

Determination of optimum hydrogen peroxide concentration

To analyze the proportional change in colorimetric intensty with respect to analyte concentration, firstly H_2O_2 concentration needs to be fixed. Excess amount of H_2O_2 can reduce enzyme activity by suicide inhibition mechanism and making it essential to analyze its effect on biosensor response.[36] To determine the minimum amount of H_2O_2 required to get the highest color intensity, a range of H_2O_2 concentration from 2.5–60 mM was assayed for a fixed catechol, enzyme and MBTH concentration of 5 mM, 100 µg/mL and 24 mM respectively at pH 6.0. The best results were observed at 30 mM H_2O_2 concentration which are six times higher than the catechol concentration. From Figure 2a, the color intensity is nearly the same as above of 30 mM H_2O_2 concentration, which is six times the catechol concentration. The operating H_2O_2 concentration should be more enough to oxidize the concentration of catechol present in water sample. Therefore, considering the maximum phenolic compound concentration present in wastewater as 45 mM (5000 mg/L), a six times more H_2O_2 concentration calculated as 270 mM was fixed for catechol detection. The biosensor needs to be operated at this fixed H_2O_2 concentration failing to which accurate quantitative analysis is challenging as the variable H_2O_2 concentration could contribute to color change along with phenolic compound concentration.

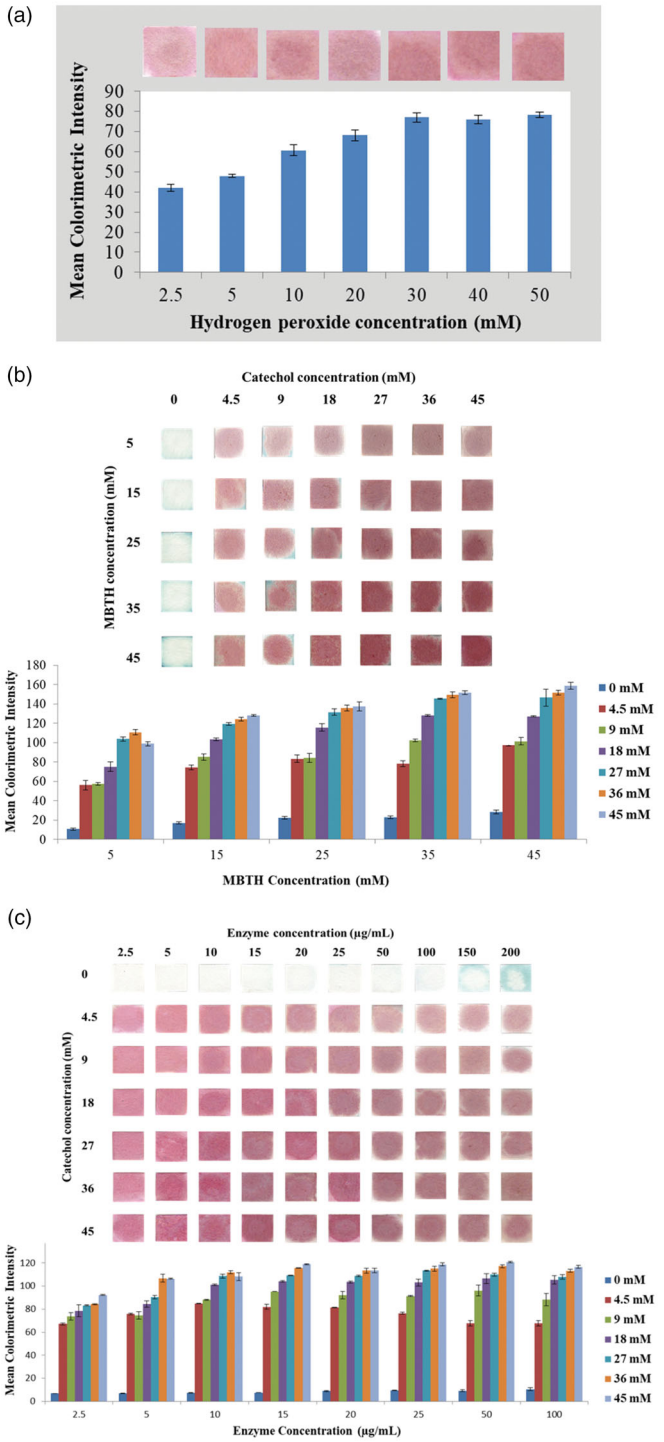

Figure 2. Optimization of (a) hydrogen peroxide (b) MBTH and (c) enzyme concentrations for the catechol detection along with its developed color intensity on paper squares.

Determination of optimum MBTH concentration

Optimization of MBTH is required for the same purpose as explained above for H_2O_2 optimization. The effect of MBTH concentration was investigated as shown in Figure 2b. The lowest background color intensity at 0 mM catechol concentration as well as stably increasing color intensity with increasing catechol concentration was obtained at 15 mM MBTH concentration. Higher MBTH concentrations give bluish color on reaction with HRP, which is not desirable from the perspective of colorimetric analysis. In addition, lower MBTH concentration does not give a stable increment in color intensities with respect to catechol concentration.[37] Oktem et al. developed laccase based biosensor for phenolic compounds detection using optimum MBTH concentration of 24 mM.[24] Abdullah et al. reported the construction of laccase based optical biosensor with 100 mM MBTH concentration.

Determination of optimum HRP concentration

For cost effective fabrication of a biosensor, it is important to find out the minimum amount of enzyme required to give desireable results without affecting the functionality of the biosensor. The enzyme concentration was optimized following the same criteria used for MBTH optimization. A range of enzyme concentration from 2.5–200 µg/mL was assayed with 4.5–45 mM catechol concentrations at 15 mM MBTH and 270 mM H_2O_2. From Figure 2c, the optimum enzyme concentration obtained was 15 µg/mL with the lowest background color intensity and stably increasing color intensity with increasing catechol concentration. The enzyme concentration obtained in optimization experiment is very low that could sufficiently reduces the fabrication cost.[25] Ariciuli et al. used 10 mg/mL enzyme to conctruct tyrosinase-MBTH based colorimetric paper biosensor for phenolic compound detection giving LOD of 5 µM.[36] Oktem et al. reported optimum enzyme (laccase) concentration as 2 mg/mL for the construction of paper-based biosensor with the limit of detection of 64 µM for catechol compared to the limit of detection (LOD) of 0.45 mM obtained in the current study. The comparative higher LOD might be due to the lower HRP concentration used but it is sufficient for catechol and resorcinol detection in effluent from coal carbonization and gasification industries as the pollutant concentration ranges from few ppm to 2000 ppm.[9]

Determination of optimum pH for biosensor

For analyzing the effect of pH on the biosensor response, the color formation was observed at pH range 3.0–9.0 using the lowest detectable concentrations of catechol and resorcinol (Figure 3). The desired pink color for 50 mg/L catechol is observed at pH 6.0–6.5.[38] Chen et al. reported optimum pH as 6.0 for HRP based amperometric biosensor for H_2O_2 detection.[27] Alkasir et al. also reported optimum color intensity at pH 6.4 in response to phenolic compounds addition for tyrosinase-chitosan based paper biosensor, which comes in the observed pH range getting desired color in response to catechol for the constructed biosensor. A purple coloration is observed along with pink color with a decrease in pH is due to the reaction of the enzyme with chromogen MBTH. HRP catalyzes the oxidation of MBTH to give purple color below pH 5.0.[39] At alkaline pH, the biosensor produces an orange color that is not desirable. For 10 mg/L of resorcinol concentration, the biosensor produces the desired pink color above pH 6.0 and the color intensity increases with an increase in pH. The pH range is required

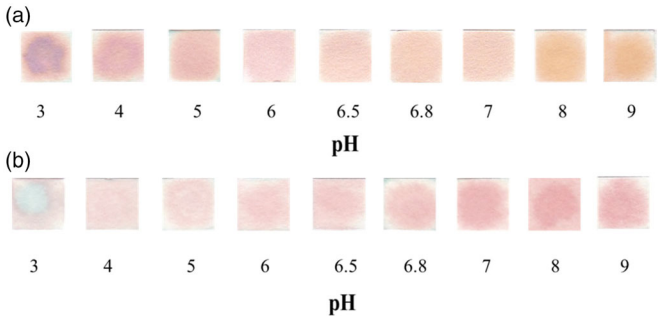

Figure 3. Effect of pH on working of the biosensor for (a) catechol and (b) resorcinol detection.

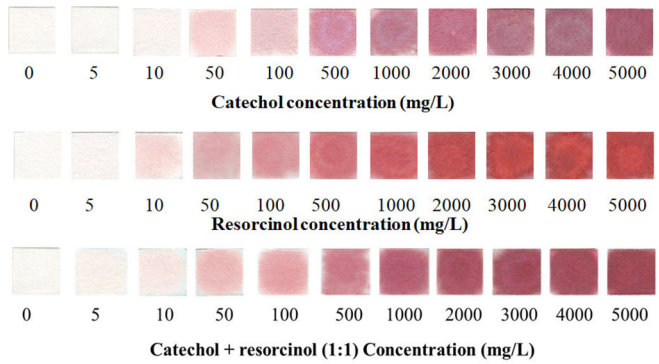

Figure 4. Limit of detection (LOD) of the HRP based paper biosensor for catechol and resorcinol detection in aquoeus samples.

to be fixed for analysis as acidic and basic pH gives undesirable colors as the color change should be proportional to the analyte concentration and not due to pH change.

Determination of the limit of detection (LOD) of the biosensor

The biosensor fabricated with all the optimized components and pH has to check for visually detectable lowest concentration. As illustrated in Figure 4, the biosensor shows a stable increase in color intensity with increasing catechol concentration as well as resorcinol concentration at pH 6. However, it is more sensitive for resorcinol showing faint pink to dark orange color with visible LOD of 10 mg/L (0.09 mM). The biosensor gives faint pink to red color for catechol with the visible LOD of 50 mg/L (0.45 mM). The color intensities after 2000 mg/L are not prominently distinguishable by the naked eye showing nearly the same color intensities.[40] Sánchez-Obrero et al. proposed a tyrosinase based electrochemical biosensor with the detection limit of 0.64 mM for catechol. An optical biosensor is demonstrated by Lepore and Portaccio,[24] which shows LOD of 4.5 µM for resorcinol and 0.6 µM for catechol. However, paper-based biosensor provides several advantages like no electronic device involved during analysis, powerless, portable and easy to use without having any background information. Moreover, HRP is quite stable at a wide range of temperatures than tyrosinase.[41] Abdullah et al.[24] described the fabrication of an optical biosensor by immobilization of laccase in chitosan and MBTH in hybrid nanofion/sol-gel

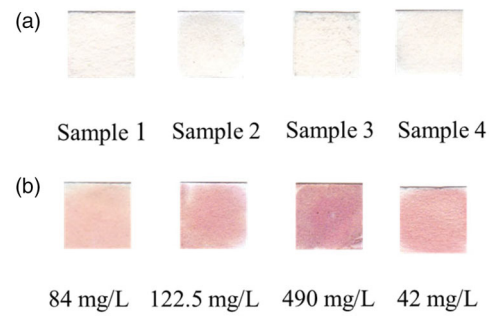

Figure 5. Validation of paper based biosensor on real-time water samples for the detection of catechol and resorcinol. (a) real-time samples collected (b) different concentrations of phenolic compounds added in real-time samples. [Visually compare the developed color intensity for real-time samples with the precaliberated color chart presented in Figure 4 for semi-quantitative measurement of phenolic compounds].

silicate film. Laccase oxidizes phenolic compounds to quinones and its reaction with chromogen MBTH is detected spectrophotometrically. The biosensor showed LOD for catechol as 0.33 mM with a linear range of 0.5–8 mM. Although the detection limit of the biosensor developed in the current study is higher (0.45 mM for catechol and 0.09 mM for resorcinol), it is a cheap, portable and disposable biosensor, which can easily be used by non-experts for instant detection. Enzyme immobilization in paper greatly reduces the fabrication cost and retains the catalytic activity of the enzyme due to polymer entrapment.

Shelf life of paper based biosensor

The percent decrease in colorimetric intensity was found as 5.33% for catechol and 22.28% for resorcinol after 2 days of biosensor storage at room temperature (Supplementary Figure 2a). The biosensor was found more stable at 4◦C where the colorimetric intensity decreased by 0.83 and 2.16% for catechol and resorcinol respectively after a week. Even though 10.35% and 31.29% decrease in measured colorimetric intensity for catechol and resorcinol was observed after 3 weeks at 4◦C, the analyte can be detected visually for qualitative and semiquantitative determinations (Supplementary Figure 2b).

Validation of paper based biosensor

For inspecting the sensitivity and the validation of the fabricated biosensor, the real-time water samples collected around the industrial area were subjected to analysis for the detection of catechol and resorcinol. However, no color developments were observed might be due to the lesser or nil concentrations of catechol and resorcinol in the collected real-time water samples. Therefore, those samples were spiked with the known concentration of catechol and resorcinol at pH 6 and further subjected to analysis with the developed paper based biosensor. The color developed (Figure 5) was matched with the existing color chart (Figure 4) for semiquantitative detection. The calibration curve (Supplementary Figure 3) for catechol and resorcinol were

Table 1. Effluent water sample quantitative analysis of catechol/resorcinol.

	Concentration of phenolic compunds added to real-time water samples (mg/L)				
Real-time water samples	Catechol	Resorcinol	Mean colorimetric intensity	Calculated concentraion (mg/L)	Variation (%)
Sample 1	84.00	–	30.78	80.65	+3.97
Sample 2	122.50	–	45.64	128.58	−4.96
Sample 3	490.00		68.63	486.60	+0.69
Sample 4	–	42.00	44.40	41.63	+0.87

earlier prepared by plotting the concentration from 5 to 5000 mg/L versus measured colorimetric intensity.

The sample volume used was 700 µL and 300 µL of 3% H_2O_2 was added to it making final volume 1 mL before spotting onto the biosensor paper squares. The addition of H_2O_2 causes dilution of the original concentration, which can be calculated as follows:

$$C = \times \frac{1000}{700} \quad (1)$$

For catechol at a colorimetric intensity less than 42

$$x = \frac{y}{0.3817} \quad (2)$$

For catechol at a colorimetric intensity more than 42

$$x = e^{\frac{y+38.267}{17.277}} \quad (3)$$

For Resorcinol at colorimetric intensity less than 63

$$x = \frac{y}{1.1759} \quad (4)$$

For resorcinol at colorimetric intensity more than 63

$$x = e^{\frac{y+23.978}{19.252}} \quad (5)$$

where ***C*** is the concentration of catechol/resorcinol present in the water sample, ***x*** is the calculated concentration using the calibration curve, ***y*** is the mean colorimetric intensity obtained using ImageJ software at grayscale. The colorimetric intensity is directly proportional to the natural log of the concentration of catechol and resorcinol. The linearity was obtained up to 100 mg/L of catechol and 50 mg/L of resorcinol (Supplementary Figure 3). The variation in the quantitative analysis of catechol and resorcinol in real-time water samples is around 5% as given in Table 1.

Conclusion

In the present study, a paper-based biosensor was developed using horesradish peroxidase for the detection of phenolic compounds such as catechol and resorcinol in aqueous samples. The immobilization of enzyme on paper was acheived by an easy and efficient method of chitosan entrapment with glutaraldehyde crosslinking. A very less volume of 10 µL and a concentration of 15 µg/mL of enzyme were used for the biosensor construction.The mixing of all the components in chitosan and deposition on the paper instead of layer by layer deposition is easy and time saving, one step method for biosensor fabrication. The necessity of hydrogen peroxide to carry out the reaction might prove a limitation. From the experimental observations, the working pH for the biosensor is determined as 6.0–6.5. The hydrogen peroxide concentration and pH range have to be maintained for accuracy in the quantitative determination of phenolic compounds. However, pH maintainance may not be required often as the pH of most of the conventional water resources will be in the range of 6.0–6.5. The concentration of catechol and resorcinols in the aqueous sample could be estimated by matching the developed color with the preexisting color charts. The use of paper as an immobilization matrix with polymer entrapment allows the construction of cost effective, portable, disposable and stable biosensors for the implementation in real-time scenarios.

Acknowledgments

The authors thank the Department of Biotechnology and Medical Engineering of National Institute of Technology Rourkela (India) for providing the necessary research facilities.

Funding

The authors thank the Ministry of Human Resources and Development (MHRD) of Government of India (GoI) for sponsoring the Master's program of the first author.

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