Development of a paper-type tyrosinase biosensor for detection of phenolic compounds

Özge Şenyurt[1,2]
Füsun Eyidoğan[3]
Remziye Yılmaz[4]
M. Tufan Öz[5]
V. Cengiz Özalp[6]
Yakup Arıca[7]
Hüseyin A. Öktem[1,2*]

[1]Department of Biological Sciences, Nanobiotechnology R&D Lab, Middle East Technical University, Ankara, Turkey

[2]Nanobiz Ltd, METU–Technopolis, Ankara, Turkey

[3]Faculty of Education, Başkent University, Ankara, Turkey

[4]Central Laboratory, Molecular Biology and Biotechnology R&D Center, Middle East Technical University, Ankara, Turkey

[5]Centre of the Region Haná for Biotechnological and Agricultural Research, Palacký University, Olomouc, Czech Republic

[6]School of Medicine, Istanbul Kemerburgaz University, Istanbul, Turkey

[7]Department of Biology, Gazi University, Ankara, Turkey

Abstract

A low-cost, portable, and disposable paper-type tyrosinase biosensor was developed for determination of phenolic compounds, using a paper-strip absorption method. Tyrosinase and a chromophore (3-methyl-2-benzothiazolinone hydrazone) were immobilized on paper strips to manufacture the biosensor, which was tested on a nontoxic substrate (L-dopamine). The biosensor was responsive to phenolic compounds such as 4-chlorophenol, catechol, *m*-cresol, and *p*-cresol. The sensor showed stability for 70 days. The developed biosensor can be used for remote on-site qualitative monitoring of phenolic compounds in wastewater samples. Volume 00, Number 00, Pages 1–5, 2014

Keywords: *4-chlorophenol, catechol, cresol, L-dopamine, paper strip, phenol*

1. Introduction

Phenol and its derivatives, the sources of which are both natural and industrial, are widespread contaminants in water. Although phenolic compounds play important biochemical and physiological roles in living systems, their accumulation in the environment as a result of intensive human activity may result in drastic ecological problems [1, 2]. Thus, many phenolic compounds are included in lists of dangerous substances or priority pollutants by the European Commission and the US Environmental Protection Agency. The maximum limit of phenol concentration in water for protection of public health and safety is 3.5 μg/L, and the acceptable range of phenolic compounds in surface water for drinking purposes is 1 to 10 μg/L [3]. Therefore, it is of great importance to determine the presence of phenolics for health care, environmental monitoring, and product quality assessment.

Various analytical techniques are available for detection of phenol in environmental samples. Spectrophotometry, gas and liquid chromatography, and capillary electrophoresis are the commonly used methods. However, these methods are time consuming and require complicated treatment of the sample [4]. Additionally, the equipment employed is expensive and is not generally portable. Therefore, there is an interest in developing simple, sensitive, specific, accurate, and portable systems such as biosensors for determination of phenolic compounds.

Abbreviations: *L-dopa, L-dopamine; MBTH, 3-methyl-2-benzothiazolinone hydrazone; PBS, phosphate-buffered saline; PPO, polyphenol oxidase.*

**Address for correspondence: Hüseyin A. Öktem, PhD, Department of Biological Sciences, Middle East Technical University, 06800 Ankara, Turkey. Tel.: +90 312 210 5172; Fax: +90 312 210 7976; e-mail: haoktem@metu.edu.tr.*

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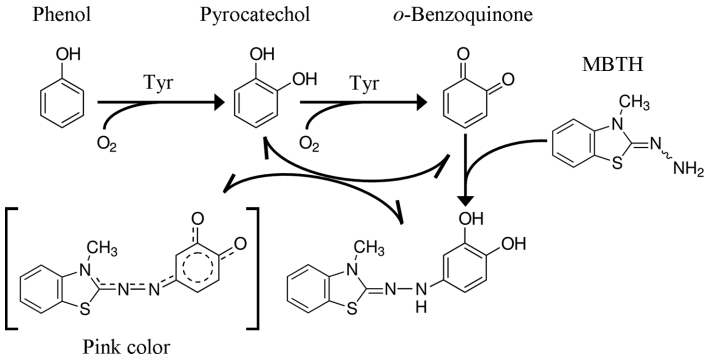

FIG. 1 *Reaction mechanism on paper-type tyrosinase biosensor. Tyrosinase (Tyr) catalyzes oxidation of phenolic substrates to o-quinones that are rapidly converted to pink-colored complexes in the presence of MBTH.*

Tyrosinase (monophenol oxidase or *o*-diphenol:oxygen oxidoreductase, EC 1.14.18.1) also known as polyphenol oxidase (PPO), is a copper-containing monooxygenase that is widely distributed in microorganisms, animals, and plants [5]. Tyrosinase catalyzes its target molecule by involving molecular oxygen in two distinct reactions (Fig. 1), that is, hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) [6]. Different support materials and immobilization techniques have previously been used for development of tyrosinase-based biosensors [7–9]. In the present work, a tyrosinase-based paper biosensor is developed for determination of phenolic compounds, using a paper-strip absorption method. The sensor uses tyrosinase and 3-methyl-2-benzothiazolinone hydrazone (MBTH), which are immobilized on a solid support material. The working principle of the sensor depends on rapid conversion of *o*-quinones—oxidation products of tyrosinase activity—to pink-colored complexes in the presence of MBTH (Fig. 1).

2. Materials and Methods

2.1. Materials

Tyrosinase from mushroom, MBTH, catechol, 4-chlorophenol, *m*-cresol, *p*-cresol, and L-dopamine (L-dopa; L-3,4-dihydroxyphenylalanine) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Tyrosinase and L-dopa were dissolved and diluted in phosphate-buffered saline (PBS), whereas phenolic compounds were dissolved in 25% ethanol and diluted in distilled water. The solutions were stored at 4 °C. Tyrosinase solution was stored at −18 °C. Distilled water from a Millipore filter (Merck, Darmstadt, Germany) was used in all experiments, which were performed at room temperature (24 ± 2 °C).

PBS buffer (pH 7.0) was prepared with 9.95 g NaCl (Reidel-deHäen, Hannover, Germany; purity > 99.8%), 0.25 g KCl (Merck; purity > 99.5%), 1.14 g Na_2HPO_4 (Sigma; purity > 99.9%), and 0.25 g KH_2PO_4 (Sigma; purity > 99.7%), and distilled water added up to 1 L. Absolute ethanol, density 1.79 kg/L, was purchased from Pancreac Qumica (Barcelona, Spain). Filter paper No. 1 used as support material was purchased from Whatman (Chiltern, United Kingdom).

2.2. Manufacturing of paper-type tyrosinase biosensor

The filter paper was cut into 1 × 1 cm pieces that were autoclaved prior to the manufacturing of the tyrosinase biosensor. The biosensors were prepared by mixing the enzyme and MBTH solutions (1:1 ratio v/v) and transferring the mixture to 1 cm^2 pieces of filter paper, which were used as solid support. The loaded papers were dried in a vacuum desiccator (680 mmHg) for 15 Min.

Several studies were performed to determine the optimum enzyme and MBTH concentrations for paper biosensor constructions. The enzyme stock solutions were prepared by dissolving 24.0 mg/mL of tyrosinase in PBS buffer (pH 7.0) and diluted to 4, 2, 1, and 0.5 mg/mL. For optimization of enzyme concentration, the MBTH amount was kept constant at 24 mM in the enzyme–MBTH mixture. For optimization of chromophore concentration, the MBTH solution was diluted to final concentrations of 0.375, 0.75, 1.5, 3, 6, 12, and 24 mM in PBS buffer (pH 7.0). For MBTH optimization studies, enzyme concentration was kept constant at 2 mg/mL.

2.3. Determination of detection limit and sensitivity

In order to determine the detection limit of the biosensor, L-dopa was used as a substrate, and the response of the biosensor was evaluated in the range of 0 to 0.512 mM L-dopa. For the sensitivity tests of the biosensor, L-dopa as well as phenolic compounds (catechol, *m*-cresol, *p*-cresol, and 4-chlorophenol) were used in the concentration range of 0 to 0.512 mM.

2.4. Color intensity analysis

Paper biosensors were constructed for further tests with the optimum enzyme and MBTH concentrations. After application of substrate onto papers, the biosensors were dried and scanned by Hewlett-Packard Photosmart C3180 with parameters of 24 bits. The quantitative data for color change formed on the paper biosensors were analyzed with OBITEK ObiColor Master Software, which measures the intensity values of three colors, red, blue, and green, on the basis of the values of 0 and 255 for pure black and pure white, respectively. In this study, color intensity values of the biosensor after application of substrate were provided by subtracting the intensity value of no substrate control.

3. Results

The developed method is based on simple paper-strip absorption, which uses immobilization of tyrosinase and a chromophore on a filter paper support material.

3.1. Manufacturing of tyrosinase-based paper biosensor

The detection limit and sensitivity of the biosensor may vary with tyrosinase and chromophore concentrations and

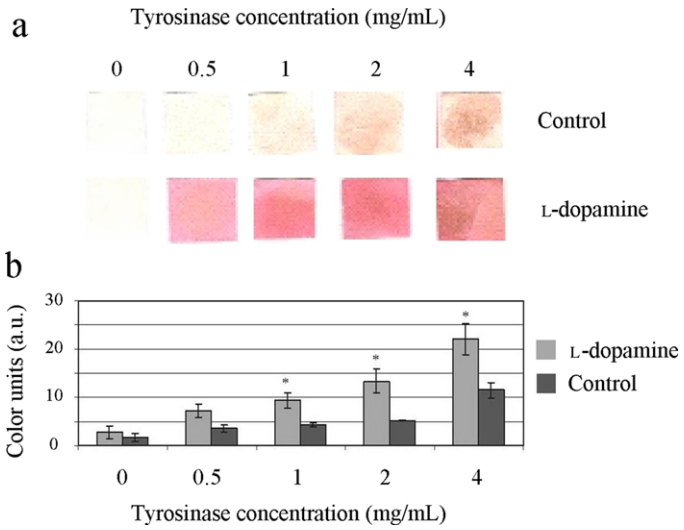

FIG. 2 *Response of biosensor to L-dopa at different tyrosinase concentrations. The biosensor paper disks after substrate (0.256 mM L-dopa) application (a) and arbitrary color units obtained with ObiColor Master Software (b) are presented. Control paper disks in the first row did not receive L-dopa application. Vertical bars and asterisks indicate SEM and significance values ($P < 0.05$) compared with control (0 mg/mL tyrosinase), respectively.*

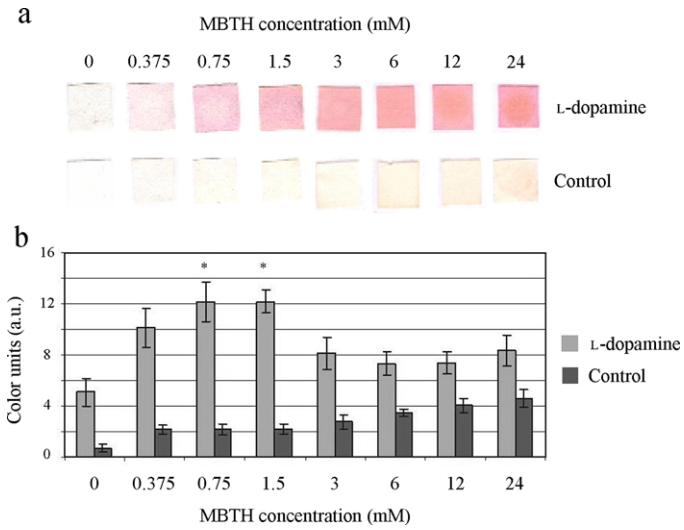

FIG. 3 *Change in biosensor response at different chromophore (MBTH) concentrations. The biosensor paper disks after substrate (0.256 mM L-dopa) application (a) and arbitrary color units obtained with ObiColor Master Software (b) are presented. Control paper disks in the second row did not receive L-dopa application. Vertical bars and asterisks indicate SEM and significance values ($P < 0.05$) compared with control (0 mM MBTH), respectively.*

pH. Analytical performance characteristics are discussed with optimization of these parameters in the following parts.

Different enzyme concentrations (0.5, 1, 2, and 4 mg/mL) were investigated using 0.256 mM L-dopa as substrate. Increasing the enzyme concentration increased the intensity of the developed color, and the highest value was obtained at 4 mg/mL (Fig. 2a). The color intensity at 1, 2, and 4 mg/mL enzyme concentrations was significantly higher than that of control without tyrosinase (Fig. 2b).

Different MBTH concentrations (0.375, 0.75, 1.5, 3, 6, 12, and 24 mM) were immobilized on filter paper to determine optimum chromophore concentration (Fig. 3a). It was found that the responses of the biosensors containing 0.75 and 1.5 mM MBTH were significantly different from control without MBTH (Fig. 3b). Increase in MBTH concentration (3, 6, 12, and 24 mM) did not result in an enhanced response in color intensity. The optimum color intensity of the biosensor in response to chromophore concentration was obtained at 1.5 mM MBTH.

Biosensor response to 0.256 mM L-dopa solution was examined at different pH values, namely, 3 to 11. Although the maximum red color formation related to biosensor activity was observed at pH 7 and 9, there were no significant differences in color intensities produced in the pH range 3 to 11, which revealed that the biosensor activity was not affected in this pH range (data not shown).

3.2. Detection limit of biosensor and sensitivity to phenolic compounds

In order to determine the detection limit of the biosensor, different L-dopa concentrations, ranging from 0.001 to 0.512 mM, were used (Fig. 4a). The color units formed by adding 0.064, 0.128, 0.256, and 0.512 mM L-dopa were significantly different from that formed by control (no L-dopa).

The sensitivity of the biosensor toward different phenolic compounds has been tested using 4-chlorophenol, catechol, *m*-cresol, and *p*-cresol. Figure 4 shows the colorimetric response to these compounds in concentration ranges of 0.001 to 0.512 mM. The detection limit of the biosensor was determined as 0.032 mM for 4-chlorophenol and catechol and 0.128 mM for *m*-cresol and *p*-cresol.

3.3. Stability of biosensor

Evaluation of the stability of the biosensors was performed at room temperature (approximately 24 ± 2 °C) and in a refrigerator (4 °C) at 10-day intervals. At room temperature, there was no significant decrease in biosensor activity over 2 months (Fig. 5a). The biosensors showed good storage stability over a period of 70 days at 4 °C (Fig. 5b).

4. Discussion

We aimed to construct a biosensor in a paper format by immobilizing tyrosinase and MBTH. To test the biosensor, L-dopa was used at 0.256 mM, and the response was monitored by

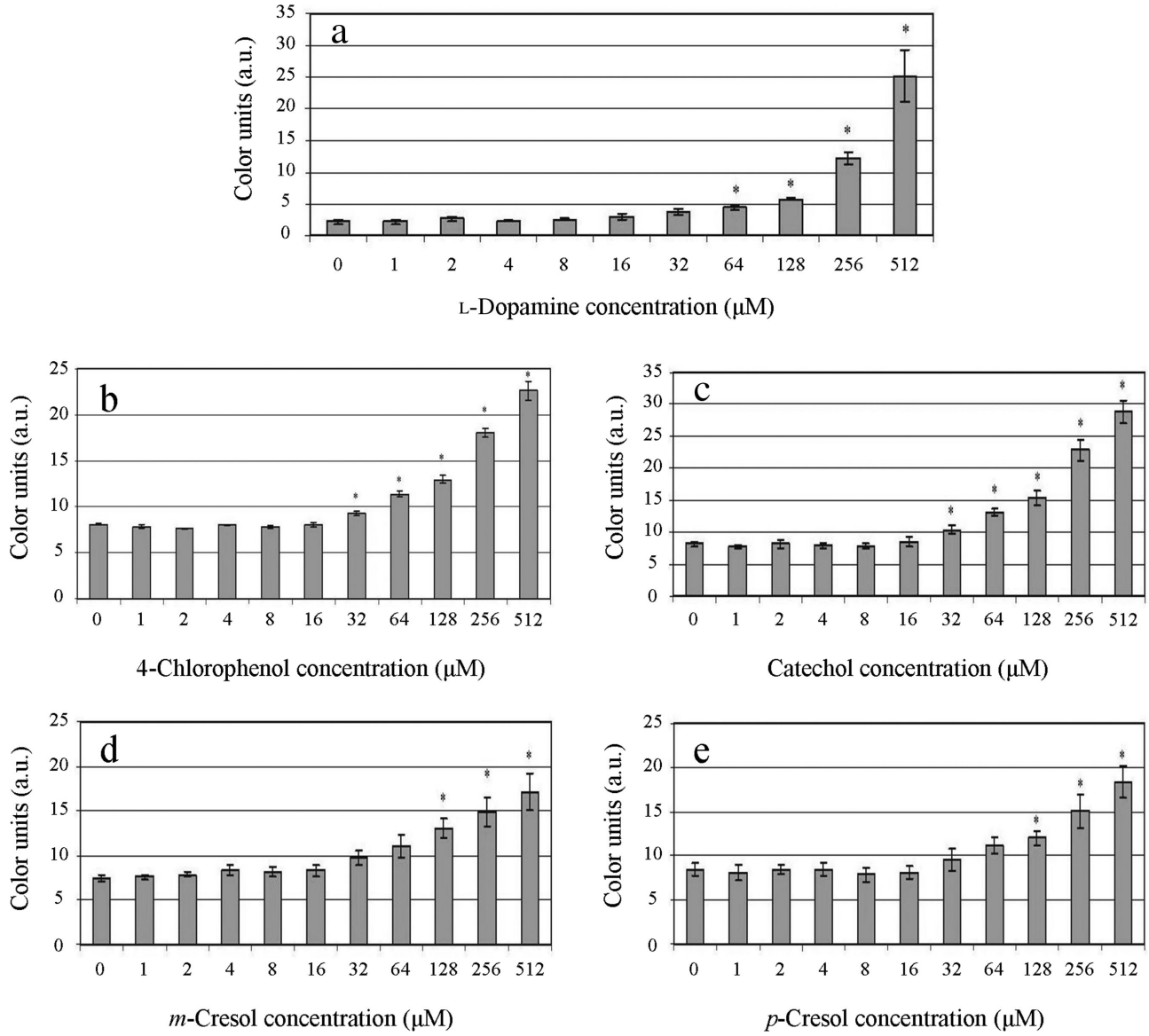

FIG. 4 *Detection limit of biosensor determined with L-dopa (a), 4-chlorophenol (b), catechol (c), m-cresol, (d) and p-cresol (e). Vertical bars and asterisks indicate SEM and significance values ($P < 0.05$) compared with the corresponding control values, respectively.*

determining the intensity of the color produced. The optimized enzyme concentration was set at 1 mg/mL, considering the cost of the biosensor and measurable response.

Sensitivity is one of the most important parameters of biosensors. In order to determine the sensitivity of biosensors toward different phenolic compounds, 4-chlorophenol, catechol, *m*-cresol, and *p*-cresol were used as substrates in concentration ranges of 0.001 to 0.512 mM. The detection limit of the biosensor was determined as 0.032 mM for 4-chlorophenol and catechol and 0.128 mM for *m*-cresol and *p*-cresol. In fact these detection limits are high compared with the detection limits of other optical or electrochemical biosensors developed earlier. These include a highly sensitive biosensor based on immobilization of tyrosinase in chitosan on a glassy carbon electrode with a detection limit of 0.05 nM [10]. Another electrochemical biosensor has been developed for remote on-site monitoring of phenolic compounds, and its detection limits were 0.2 and 0.3 μM for cresol and phenol, respectively [11]. Detection of 8.5 nM chloroguaiacol was achieved with a remote electrochemical biosensor based on mixed enzymes (laccase and tyrosinase) [12]. The detection limits of an optical biosensor, based on immobilized tyrosinase in chitosan film, were 0.9, 1.0, 1.0, and 3.0 μM for 4-chlorophenol, phenol, *m*-cresol, and *p*-cresol, respectively [13]. Another bioprobe with immobilized PPO with a detection limit of 0.05 mg/L of phenolic compounds was developed earlier [14]. Although the detection limit of a paper-based biosensor was 0.86 μg/L, the minimal sensor response detectable with the naked eye was 5 μg/L for various

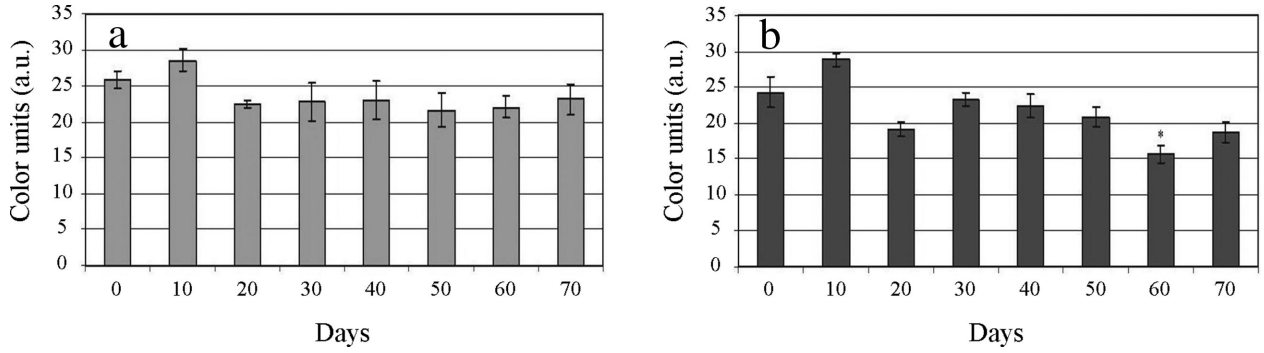

FIG. 5 *Stability of biosensor at room temperature (a) and 4 °C (b). Vertical bars and asterisks indicate SEM and significance values ($P < 0.05$) compared with day 0.*

phenolic compounds [15]. The biosensor developed in our study allows detection with a naked eye at micromolar concentrations and is practical because it requires no instrumentation. In Turkey, with the regulations published in the *Official Gazette of the Turkish Republic* (No. 25730) on February 17, 2005, the acceptable maximum concentrations of phenolic compounds in drinking water and natural water resources were set at 0.5 and 2 μg/L, respectively. Thus, the developed biosensor can be employed for qualitative evaluation of the presence of phenol in wastewater.

Operational pH range for different biosensors that employ tyrosinase varies greatly. Whereas an optical biosensor was functional at pH 6 to 7 [13], another biosensor utilizing phenol-oxidizing bacterial cells was effective in a pH range of 4 to 8.5 [16]. High activity of immobilized tyrosinase at an acidic pH of 3 was reported for a cross-linked enzyme aggregate with ammonium sulfate and glutaraldehyde. This was explained by a change in amino acid side-chain ionization in the microenvironment surrounding the active site of the enzyme [17]. In our study, it was found that the paper biosensor was operational at a wide pH range (3–11), which exhibited an important advantage for monitoring environmental samples. Furthermore, the results indicated that the immobilized tyrosinase was active at pH 3 to 11 and was able to catalyze phenolic substrates.

In the literature, various stability periods ranging from 1 or 2 months to more than 8 months have been reported [13–15]. The biosensor developed in our study was stable for 70 days and provided a suitable environment for preserving enzymatic activity for long periods of time at room temperature and 4 °C.

Biosensors cannot be compared with classical analytical methods; however, they can be used for routine analysis, screening of analytes, and determination of analyte presence. The results represented here suggest that the developed paper biosensor can be used for remote on-site monitoring of phenolic compounds present at concentrations down to 32 μM in water samples. Although the sensitivity of the biosensor is not high compared with previously developed tyrosinase biosensors, it holds substantial advantages because it requires no instrumentation or expertise and costs low to fabricate. The use of change in color for detection of phenolic compounds with a simple ready-to-use paper-type tyrosinase biosensor will facilitate evaluation and on-site qualitative monitoring of these compounds.

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