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Detection of Phenolic Compounds by Colorimetric Bioassay Using Crude Polyphenol Oxidase

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ABSTRACT

Monitoring of phenolic compounds in food components and industrial processes is of great importance. Routine analytical techniques used for determination of phenolics are costly and tedious. Enzymatic bioassays can be good alternate due to their inalienable specificity, simplicity and fast responsiveness. The present study was designed to develop a colorimetric bioassay for detection of phenolics using crude extract of polyphenol oxidase (PPO). Crude PPO was extracted from apple and conditions were optimized for enzyme activity. Enzyme was immobilized on solid support (Whatman paper-1 and TLC plate) by over-spotting with 3-mthyl-2-benzothiazolinone hydrazine hydrochloride hydrate (MBTH) to develop bioactive spot. Solutions of four substrates; catechol, 4-methyl catechol, L-3,4-dihydroxyphenylalanine (L-DOPA) and L-tyrosine were independently loaded on bioactive spots where the PPO catalyzed the reaction in which substrate was converted to quinones which formed pink colored adduct with MBTH. Color change was detectable by naked eye but color intensity was determined by image analysis software. The detection limit for catechol, 4- methyl catechol and L-DOPA was 64 μM, 512 μM and 4 mM respectively with 5 minutes of response time under optimized conditions (16 enzyme units, 6mM MBTH at pH 7 and 25 °C). The change in color intensity was concentration dependent for each substrate beyond its minimum detection limit. A simple, portable, disposable and low cost colorimetric bioassay was developed for detection of phenolic compounds.

1. Introduction

Phenolic compounds are ubiquitous in nature and released into environment by different industries and domestic wastes [1]. Worldwide production of phenolic compounds is about 50,000 tons per annum [2]. The industrial waste products of these phenolic compounds leach into the water reservoirs and contaminate medical, nutritional and environmental matrices [3-5]. The long and short term exposure of phenolics causes health and environmental risks [6]. Being antioxidant these polyphenolics have health benefits as risks of coronary cardiopathy and cancer are minimized by these compounds [7]. Moreover food quality is influenced by polyphenols [8]. Some phenolic compounds are precursors to neurotransmitters, for example L-3,4-dihydroxyphenylalanine (L-DOPA). The concentration of L-DOPA in body fluids remains less than few microgram per liter but the concentration of L-DOPA may exceed mg/L in patients who have been treated with exogenous L-DOPA. In pharmaceutics the level of L-DOPA may reach up to 80% by weight [9].

Different methods have been proposed for detection and quantification of phenolics present in fresh and waste waters. Commonly used methods are: gas chromatography [10], high performance liquid chromatography (HPLC) [11], electrical as well as chemical methods [12] such as Winder-Harris enzymatic assay and Folin-Ciocalteau assay. These methods involve complicated sample handling, sample pretreatment and these are costly as well [13]. Enzyme-based detection of phenolic compounds shows a considerable potential as an effective alternate [14]. PPO is considered a better candidate to be used in biosensors due to its ability to catalyze electron-transfer reactions without need of additional cofactors, oxidation of phenolic compounds in the presence of oxygen and its good stability [15]. In the recent years, approaches have been made for detection of phenolics using different types of enzymes like tyrosinase [16], laccase [17] and horseradish peroxidase (HRP) [18].

Different methods are used for immobilization of enzymes which include covalent coupling, adsorption, microencapsulation, polymer entrapment, chemical aggregation and bioaffinity [19, 20].Adsorption is the simplest method for enzyme immobilization [21].

In recent years research has been focused on development of simple, low cost and portable tools for

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analytical purposes [21, 22]. Simple and cost effective enzyme-based bioassays are valuable for remote locations where laboratories are hardly accessed or trained personnel is needed.

Keeping in view the need for development of simple and low cost phenol bioassay, current study was designed to extract and immobilize crude polyphenol oxidase from apple (golden delicious) to detect phenolic compounds. Our detection of phenolic compounds is based on PPO activity which converts phenolic substrate into quinone [23]. The enzyme was immobilized on Whatman filter paper and TLC plate. Detection of phenolic compounds was determined by pink color formation. The pink color formation is due to binding of MBTH with enzymatic oxidation product (quinone) [24]. Colorimetric detection of phenolic compounds is very promising because analyte is observed by naked eye and image can be scanned or recorded by simple camera phone and transferred to laptop or personal computer for software based analysis.

2. Methodology

2.1. Materials

Crude polyphenol oxidase was extracted from apple (golden delicious). Acetone, sodium phosphate monobasic, sodium phosphate dibasic, phenolic substrates (catechol, 4-methylcatechol, L-3,4dihydroxy phenyl-alanine and L-tyrosine) and MBTH were purchased from Sigma Aldrich. Whatman filter paper 1 and TLC plates for immobilization of enzyme were purchased from Deltalab scientific co. Rawalpindi, Pakistan. The solutions of phenolic compounds were made in sodium phosphate buffer (with varying concentration and pH). MBTH was dissolved in 25 % ethanol. All reagents were of analytical grade.

2.2 Preparation of Crude Extract of Polyphenol Oxidase (PPO) and Enzyme Activity Assay

Crude extract of polyphenol oxidase was prepared by following the method used by Waleed *et al*. (2009) [25]. MAPADA (UV-100) spectrophotometer was used for enzyme activity assay. The presence of PPO in the extract was confirmed by following the method used by Shie*et al*. (2002)[26]. Catechol was used as a substrate. One unit of enzyme activity was defined as the amount of enzyme that caused the increase in absorbance of 0.001 O.D (optical density) per minute.

There was linear increase in the absorbance of assay solution within the first 60 seconds. The absorbance change was proportional to the concentration of catechol. Thus, enzyme activity can be expressed in terms of change in absorbance (ΔAbsorbance) at 420 nm per second (instead of amount of moles converted per unit time). In this way rate of reaction and activity of enzyme equated each other for initial sixty seconds. The reaction rate ($V\ s^{-1}$) was calculated as follows:

$$V(s^{-1}) = \frac{\Delta \text{Absorbance}\ \ (420\ \text{nm})}{60\ \text{s}} \times 62.5\ \text{(dilution factor)}$$

Where : $V\ s^{-1}$ is reaction rate

ΔAbsorbance is the change in absorbance per minute

62.5 is the dilution factor

2.3 Optimization Studies in Solution Form

For optimization studies in solution form, change in absorbance of reaction mixture was measured using spectrophotometer. Polyphenol oxidase was optimized for temperature, pH and substrate concentration. During all optimization studies catechol was used as substrate of PPO. To determine the optimum temperature, enzyme activity was measured in temperature range of 15°C-30°C with 1°C increase in temperature. Substrate solution was prewarmed to the target temperature using water bath. Then 800 µL of prewarmed substrate were mixed with 200 µL of enzymatic extract and enzyme activity was measured spectrophotometrically at each temperature. Enzyme solution was assayed for pH range of 5.8-7.8 with the difference of 0.2 to determine the optimum pH. Catechol solution was prepared in 0.2M sodium phosphate buffer with desired pH. Eight hundred microliters of substrate solution (with different pH) were mixed with 200 µL of enzymatic solution at optimum temperature and change in absorbance was measured by spectrophotometer. To study the effect of substrate concentration on enzyme activity substrate solutions were prepared in 0.2M sodium phosphate buffer with varying concentrations in the range of 20 mM-240 mM with the difference of 20mM. Eight hundred microliters of substrate solution having specific concentration (mentioned above) were mixed with 200 µL of enzyme solution at optimum temperature and pH and enzyme activity was measured spectrophotometrically.

2.4 Thermal and pH Stability Studies of PPO

The enzyme solution was put into water bath for 15 minutes at different temperatures ranging from 15°C to 65°C with 5°C increase. After incubation, enzyme and catechol solution were mixed and enzyme activity was measured at 420 nm.

The pH stability was determined over a pH range of 5.8–7.8. One mL of the PPO extract was mixed with 4.6 mL of 0.2 M sodium phosphate buffer (pH 5.8, 6.2, 6.4, 6.6, 6.8, 7, 7.2, 7.4, 7.6, 7.8) for fifteen minutes. The PPO activity was measured at each pH and compared with that of optimum pH.

2.5 Enzyme Immobilization and Optimization of MBTH, Substrate and Enzyme Concentration

Enzyme immobilization was done by adsorption method of enzyme immobilization. All optimization studies were conducted by immobilizing enzyme on Whatman filter paper 1. For immobilization of PPO on

TLC plates, optimized conditions (from immobilization studies for filter paper) were used. Enzyme was immobilized by simple over-spotting of 20 µL of enzymatic extract on filter paper pieces (1x1 cm) using micropipette and allowed to air dry. After drying of enzyme (3 minutes are enough) 20 µL of MBTH was spotted over prespotted enzyme and allowed to air dry. Change in color was observed by adding 20 µL of substrate on bioactive paper platform (enzyme-MBTH mix).

2.5.1 Optimization of MBTH and Substrate Concentration

Whatman filter paper No. 1 was cut into 1 cm×1 cm pieces and fixed on a card in eight rows and seven columns. MBTH solutions (0.5 mM, 1 mM, 3 mM, 6 mM, 12 mM, 24 mM, 48 mM and 96 mM) were prepared in 25 % ethanol. The solutions of 4-methyl catechol (0.04 mM, 0.1 mM, 0.5 mM, 2 mM, 16 mM, 32 mM, 64 mM, 128 mM and 256 mM) were prepared in sodium phosphate buffer (0.2 M, pH 7). From enzymatic extract, 20 µL of enzyme was spotted on each filter paper and allowed to air dry for three minutes. Twenty microliters of MBTH from each dilution were spotted on the spot formed by immobilization of enzyme and again air dried for 3 minutes. Then 20 µL of substrate solution from each concentration was spotted on immobilized enzyme-MBTH mix. The change in color was observed to determine optimum MBTH and substrate concentration. Initially high concentrations of MBTH and substrate were used and later on lower concentrations were used.

2.5.2 Optimization of Enzyme Concentration

For enzyme optimization studies the concentration of MBTH solution was kept constant (6mM). Above mentioned concentrations of 4-methyl catechol were used as substrate of PPO. As our enzymatic extract was present in solution form so we determined the units of enzyme in specific volume of enzyme. From enzymatic extract, 5 µL to 40 µL of enzyme were spotted on filter paper pieces arranged into eight rows and seven columns and air dried for 3 minutes. Then 20 µL of 6 mM MBTH were spotted on each paper prespotted with enzyme solution and allowed to air dry for 3 minutes. After drying of bioactive spot, 4-methyl catechol solutions were applied on bioactive spots. Change in color was observed.

2.6 Determination of Detection Limit and Response of Bioactive Paper Platform towards other Phenolics

To determine the detection limit of bioactive paper platform, 2µM, 4µM, 8µM, 16µM, 32µM, 64µM, 128µM, 256µM, 512 µM, 1 mM and 4 mM solutions of 4-methyl catechol were prepared. Immobilization was done in the way as described for optimization studies. The sensitivity assay was conducted in the same way as detection limit was determined only number of substrates to be tested was increased (catechol, l-tyrosine and L-DOPA).

2.7 Effect of pH and Temperature on Immobilized Enzyme

Sodium phosphate buffer was made with pH 5.8- 7.8. Enzyme solution was diluted by adding 668 µL of buffer (with desired pH) into 166 µL of enzyme solution in a test tube. Twenty microliter of enzymatic solution with required pH from each test tube was picked and assayed following the same method as mentioned above for determination of detection limit. Paper was scanned after completion of reaction (5 minutes).

To determine the effect of temperature on PPO activity, enzyme-MBTH were spotted on filter paper strips and incubated in an incubator for 15 minutes at different temperatures ranging from 15°C to 65°C with ten degree increase. After temperature treatment Change in color was observed at each temperature and paper strips were scanned and merged to get single diagram.

2.8 Immobilization of PPO on Thin Layer Chromatography (TLC) Plates

PPO immobilization on TLC plate was done by following the same method used for immobilization on filter paper. Three rows of the spots were made. In one row enzyme, substrate and MBTH were mixed; in second row only enzyme and substrate were mixed. Third row contained enzyme and MBTH mixture only. Change in color was observed after 5 minutes.

2.9 Software Based Analysis of Scanned Papers

The color intensity of the spots was measured with the help of Photoshop. Color intensity was correlated with known concentrations of substrate.

3. Results and Discussion

The study was designed to extract, optimize and immobilize the polyphenol oxidase for detection of phenolic compounds. Polyphenol oxidase was extracted form golden delicious apple and enzyme was immobilized on Whatman filter paper 1 and TLC plates. Conditions for enzyme activity were optimized in solution form first and then in immobilized form. In our bioassay appearance of pink color indicated the presence of phenolic compounds.

3.1 Spectroscopic Analysis of Polyphenol Oxidase Activities

In solution form we determined the optimum temperature, optimum pH and effect of substrate concentration on PPO activity. Maximum activity was observed at 25°C. Ziyan [27] reported 20°C as the optimum temperature for apple PPO but temperature optima vary due to different factors like; type of substrate used, source of enzyme, fruit development stage and method of enzyme extraction. Ayaz [28] studied PPO

Table 1: Temperature optimization of polyphenol oxidase

No	pH	Enzyme activity (ΔAbsorbance/min)
1	5.8	0.04
2	6.0	0.042
3	6.2	0.044
4	6.4	0.045
5	6.6	0.046
6	6.8	0.048
7	7.0	0.055
8	7.2	0.048
9	7.4	0.044
10	7.6	0.038
11	7.8	0.035

P<0.05

dynamics at three stages of fruit development and they reported 30, 20 and 30°C as the optimum temperatures for apple PPO at initial, intermediate and final fruit development stage respectively. Results from temperature optimization studies have been shown in Table 1. As shown in Table 1 enzyme activity increased with increase in temperature up to 25°C. Above 25°C there is decrease in enzyme activity. So 25 °C was considered as optimum temperature for PPO activity. All other optimization studies were conducted at 25°C.

pH changes the ionization state of amino acids present in protein, hence disturbs structure and affects the function of enzyme. To determine the optimum pH for PPO activity sodium phosphate buffers were made in pH range of 5.8 to 7.8. Maximum activity was observed at pH 7. There was sharp decrease in enzyme activity after pH 7. Least activity was observed at basic pH which indicates that basic pH affects PPO activity more as compared to acidic pH. Effect of pH on enzyme activity has been shown in Table 2.

Substrate concentration also affects enzyme activity. To determine optimum substrate concentration, catechol solutions were made with different concentrations. Initially increase in substrate concentration, activity of enzyme increased because free active sites of enzyme are occupied with increase in substrate concentration. There was slight increase in absorbance from 160 to 200 mM concentration of substrate. Maximum activity was observed at 220 mM concentration (table 3).In solution form thermal and pH stability studies were conducted as well. Polyphenol oxidase was subjected to different temperatures ranging from 10°C to 60°C with 5°C increase. Enzyme solution was preheated for 15 minutes because 10-15 minutes are quite enough to deactivate the enzyme. It was observed that low temperature has least effect on enzyme activity but there is sharp decrease in enzyme activity at high temperatures. The enzyme activity was not zero even at 60°C which indicates that

Table 2: Optimization of pH for polyphenol oxidase activity

No	Temperature (°C)	Enzyme activity (ΔAbsorbance/min)
1	15	0.044
2	16	0.046
3	17	0.048
4	18	0.049
5	19	0.051
6	20	0.052
7	21	0.055
8	22	0.058
9	23	0.059
10	24	0.062
11	25	0.07*
12	26	0.068
13	27	0.065
14	28	0.062
15	29	0.059
16	30	0.058

P<0.05

enzyme was active at high temperatures as well. The enzyme activity was not zero but there was 79 % decrease in enzyme activity when enzyme was incubated at 60°C.

Polyphenol oxidase was highly stable at neutral pH. Acidic and basic pH affects the ionization state of active site of enzyme which inhibits its activity. There was 28 % decrease in PPO activity at pH 7.8 when compared with that of optimum pH. Storage stability of PPO was also determined. Enzyme was active for 3 months when stored at -18°C.

3.2 Optimization Studies in Immobilized Form

For optimization studies in immobilized form of PPO, we used 4-mthyl catechol as a substrate because high intensity pink color was formed with 4-methyl catechol as compared to other substrates.

3.2.1 Optimization of MBTH and Substrate Concentrations

To find out the optimum concentration of MBTH and substrate solutions to be immobilized, initially MBTH solutions were made with 6,12,24,48 and 96 mM concentration and that of 4-methyl catechol were 16, 32, 64, 128 and 256 mM. Enzyme concentration was kept constant (16 units). When enzyme and MBTH were mixed no color was produced but loading the substrate on immobilized enzyme-MBTH mix (bioactive spot) gave pink color which is shown in Fig. 1a. Enzyme, substrate and MBTH solutions were separately immobilized because separate immobilization gives better color as compared to the mixture of these solutions. Fig. 1a shows that increase in substrate concentration color intensity of the spot increased. With increase in substrate concentration, quinone formation increased in

increased MBTH-quinone adduct, hence color intensity increased.

Using high concentrations of MBTH and substrate solutions, high intensity pink color was produced and there was no distinguishable difference among the colors of adjacent spots. To get better contrast and observable difference among pink color produced in each bioactive spot, the concentration of MBTH and substrate solutions was decreased. As shown in Fig. 1b, the concentration of MBTH solutions was kept 0.5, 1, 3,6,12, 24, 48 and 96 mM whereas, the concentration of 4-methyl catechol solutions was 0.04, 0.1, 0.5, 2, 16, 64 and 256 mM. When substrate was loaded over the bioactive spot, pink color was observed after 5 minutes of loading the substrate. Upto 0.1 mM concentration of substrate there was no observable pink color but at 0.5 mM concentration pink color formation started. This concentration of substrate was used to find optimum concentration of MBTH for bioassay. At 0.5 mM concentration of substrate there was clearly observable difference in color with change in concentration of MBTH solution. Oktem [29] used laccase from *Trametes versicolor* in their bioassay and described 24 mM concentration of MBTH as an optimum concentration. In our bioassay stable pink color was observed at 6mM concentration of MBTH. These conditions are different from each other because the source and purity level of enzyme are defferent in both cases. We used apple as a source of crude PPO but Oktem *et al* (2012) used purified microbial laccase.

3.2.2 Effect of MBTH on PPO Activity

When enzyme units and concentration of 4-methyl catechol were kept constant, by changing the concentration of MBTH, appearance of yellowish color was observed at higher concentration of MBTH. Appearance of yellowish color indicates that high concentration of MBTH inhibits enzyme activity. With decrease in enzyme activity formation of *o*-quinone decreased which lowered the intensity of pink color. As shown in Fig. 2 the units of yellow color are highest at 96 mM concentration of MBTH. Low concentration of MBTH increased the units of pink color. At 6 mM concentration of MBTH, units of pink color are higher than yellow color. Highest intensity of pink color was observed at 6 mM concentration of MBTH so, this amount of MBTH was considered as optimum concentration for further studies.

3.2.3 Optimization of Enzyme Concentration

We didn't get enzyme in powdered form; enzyme was present in solution. Optimum concentration of enzyme was determined by changing the volume of enzymatic solution in the range of 5 µL to 40 µL and amount of enzyme was determined in terms of units. Units of enzyme were determined by correlating the change in absorbance with 0.001 OD value that defines one unit of

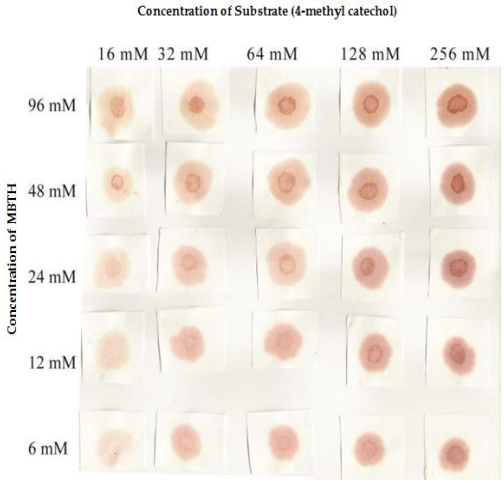

Fig. 1a. Results of MBTH-substrate optimization studies using high concentration of substrate (4-methyl catechol)

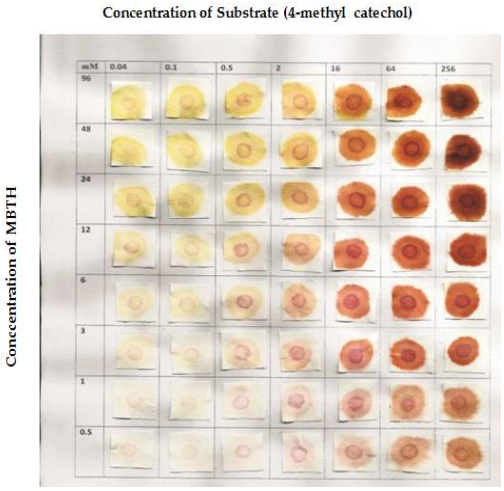

Fig. 1b. Results of MBTH-substrate optimization studies using dilute solutions

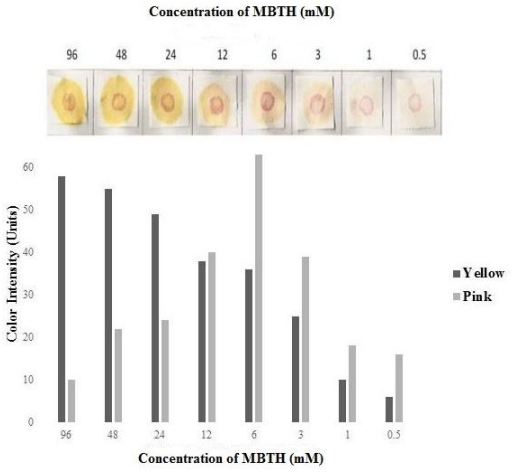

Fig. 2. Effect of MBTH on PPO activity

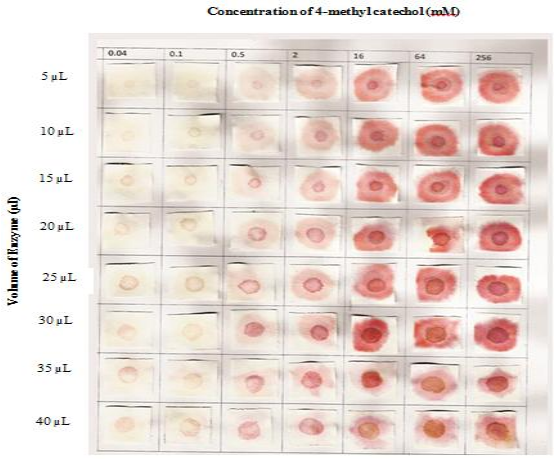

Fig. 3: Determination of optimum enzyme concentration

enzyme. Concentration of MBTH was kept constant (6 mM) and volume of enzyme and concentration of 4-methyl catechol was changed. As shown in Fig. 3 no color formation was observed upto 0.1 mM substrate concentrations but at 0.5 mM concentration of 4-methyl catechol, color formation started. There was linear increase in color intensity from 0.5 mM to 256 mM concentration of substrate. With increase in units of enzyme the intensity of pink color also increased. High intensity pink color was observed when 20 µL of enzymatic solution were used at 0.5 mM substrate concentration. High volume of enzyme also produced high intensity color but we used 20 µL of enzyme solution to avoid over wetting of paper. At higher concentrations of substrate, intensity of pink color increased but we used 0.5 mM concentration of substrate to get optimumvalues because at lower concentration of substrate there was obvious difference in color intensity of adjacent spots. At high concentrations intensity of color was very high but it was not easy to differentiate the color intensity among adjacent spots visually but use of image analysis software makes it easy to find intensity levels at higher concentrations as well.

3.3 Detection Limit and Response of Bioactive Paper Platform Towards Other Phenolics

After optimization of enzyme, MBTH and substrate concentrations, detection limit of bioactive paper platform was determined for 4-methyl catechol. Substrate solutions were made in the range of 2 µM-4 mM concentrations and assayed. Fig. 4 shows that visible detection limit of the bioassay for 4-methyl catechol was 256 µM. By increasing the contrast of the scanned image, pink color was observed up to 64 µM.

Response of bioactive paper platform was tested for catechol, L-DOPA and L-tyrosine. Bioactive platform was responsive to L-DOPA, catechol and 4-methyl catechol. No response was observed for tyrosine. Even at

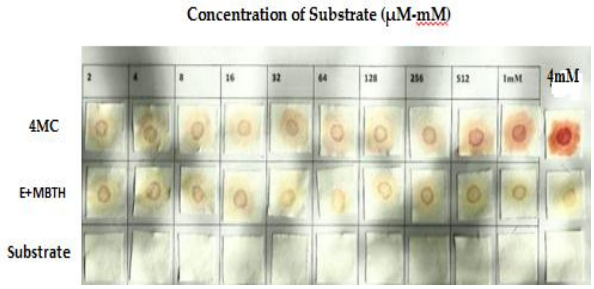

Fig. 4: Detection limit of bioactive paper platform

50 mM concentration of tyrosine solution, no pink color was observed. The bioactive paper platform was more responsive to catechol as compared to other phenolics. For catechol the detection was observed at 32 µM, for L-DOPA bioactive paper was least responsive. The L-DOPA was detectable at 4 mM concentration; below 4 mM concentration of L-DOPA no color formation was observed. As shown in Fig. 5, light pink color was observed when substrate and MBTH (S+MBTH) were mixed without enzyme. It indicates that MBTH can bind with phenolic compounds in addition to quinones formed by the catalytic action of PPO but color intensity was low even at higher concentrations of substrate. No color formation was observed in enzyme-MBTH mix. Substrate alone didn't give color. No response for L-tyrosine indicates that PPO extracted from apple shows catecholase or diphenolase activity.

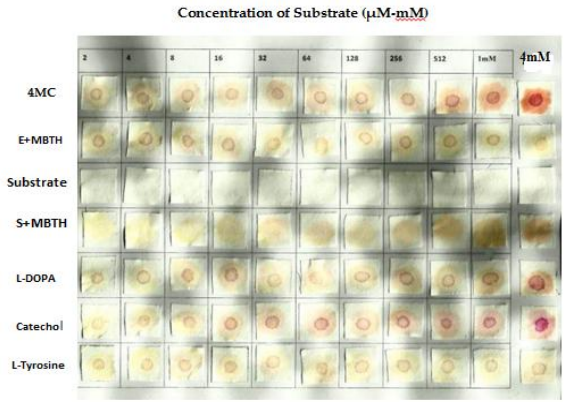

Fig. 5: Determination of senstivity of bioactive platform towards other phenolics

The detection limit and response time of our bioassay for catechol was higher than the paper based biosensor developed by Oktem [29] but for L-DOPA our bioactive platform was responsive only at higher concentration of L-DOPA (4 mM). Our results are in accordance to the results of other paper based colorimetric bioassys developed by Ramiz [30] and Arciuli [31]. In above described paper based bioassays purified enzymes (tyrosinase and laccase) obtained from microbial sources have been used which are costly. We have used crude extract of PPO from apple that is more economic , source

is readily available and extraction is easy.The market price of twenty five kilo units of mushroom tyrosinase at Sigma-Aldrich is EUR 119.00(http://www.sigmaaldrich.com /catalog/product/sigma/t3824?lang=en&region=PK&gclid=CJHF-sm6_t ACF dWIa AodLcYCRA) and 1g of laccase costs EUR 83.00 (http://www.sigmaaldrich.com/catalog/ product/ sigma/ 38429?lang=en&region=PK&gclid= CPX18ZG5_tACFR UXa Aod1QMIcA).

3.4 Effect of pH on Bioactive Paper Platform

The effect of pH on immobilized enzyme is shown in Fig. 6. As shown in Fig. 6, intensity of pink color was highest at pH 7. Intensity of pink color decreased in basic pH. It indicates that enzyme showed good activity on neutral and slightly acidic pH values. Change in pH changes the ionization state of amino acids present in active site which leads to structural changes in enzyme and change in structure affects the function of enzyme (Yahsi*et al.*, 2005) [32]. In Fig. 6, C is control used to compare the activities of immobilized enzyme on different pH values with that of pH 7.

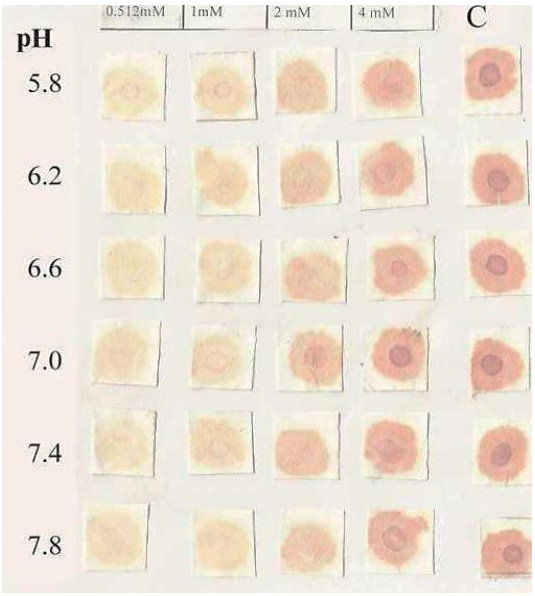

Fig. 6: Effect of pH on enzyme activity

3.5 Effect of Temperature on Response of Bioactive Paper Platform

Fig. 7 shows that increase in temperature enzyme activity decreased. Enzyme was stable upto 65°C but its limit of detection decreased with increase in temperature. At 25°C intensity of pink color was maximum. Above 25°C the intensity of pink color decreased which indicates that high temperature affects enzyme activity in immobilized form as well. Results from immobiliztion of enzyme showed that immobiliztion increases enzyme stability as compared to solution form. In solution form enzyme was less stable at higher temperatures as compared to immobilized form. increase in temperature, sensitivity of bioassay decreased. When temperature was increased the intensityof pink color at lower concentrations of substrate (0.5 mM, 1 mM and 2 mM) decreased because production of enzymatic product decreased with increase in temperature.

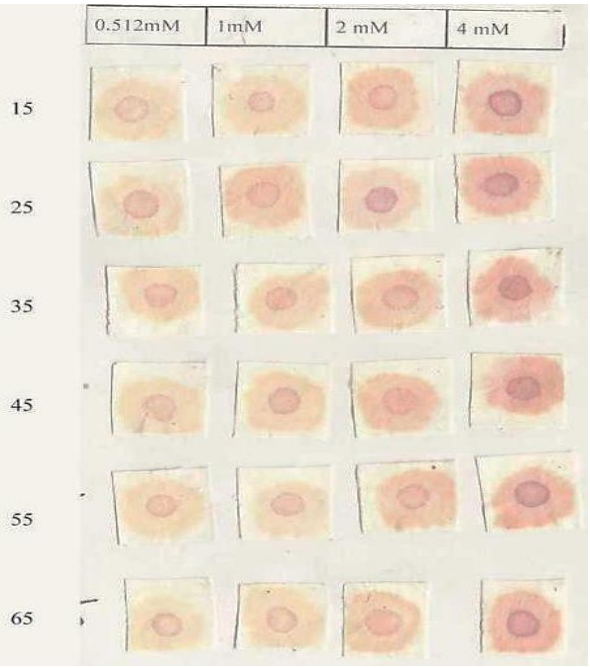

Fig. 7: Effect of temperature on bioactive paper platform 15-65 is temperature in °C and from 0.512-4mM is substrate concentration

3.6 Effect of Substrate Concentration on Response of Bioactive Paper Plateform

Increase in substrate concentration intensity of pink color increased. The response of bioactive paper for increasing concentration of catechol has been shown in Fig. 8. As shown in Fig. 8 with increase in substrate concentration quinone formation increased. Binding of MBTH with high concentration of quinones produced high intensity color.

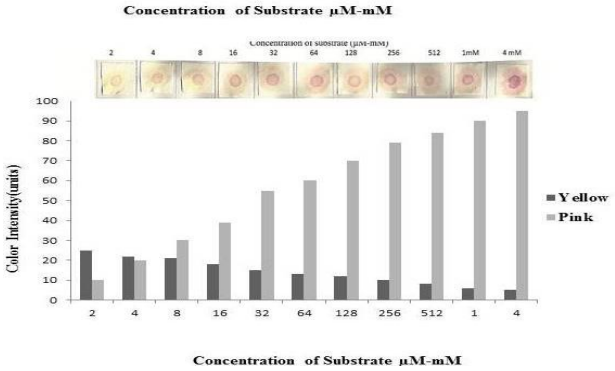

Fig. 8: Effect of substrate concentration on response of bioactive paper plateform

3.7 Immobilization of PPO on Silica (TLC) Plates

For immobilization of PPO on TLC plates, optimized conditions (from immobilization on paper) were used. Immobilization of PPO on TLC plate has been shown in Fig. 9. Enzyme was immobilized on TLC plates in the same way as described for immobilization on paper. Same results were observed as with paper but intensity of pink color formation on TLC plate was lower than that of paper based immobilization. This decrease in color intensity indicates that support material has impact on enzyme activity. Silica is slightly acidic so enzyme activity may be decreased due to slight acidity of silica. Paper based bioassay is better as compared to TLC plate because paper is least reactive for PPO and it is cost effective as well. No one has reported the use of silica plate as support material for immobilization of PPO. So we introduced a new support material for immobilization of PPO for colorimetric detection of phenolic compounds.

Table.3: Effect of substrate concentration on PPO activity in activity of PPO at 60 °C

Sr. No	Substrate Concentration (mM)	Enzyme activity (ΔAbsorbance/min)
1	20	0.046
2	40	0.050
3	60	0.073
4	80	0.075
5	100	0.077
6	120	0.079
7	140	0.081
8	160	0.084
9	180	0.085
10	200	0.087
11	220	0.090*

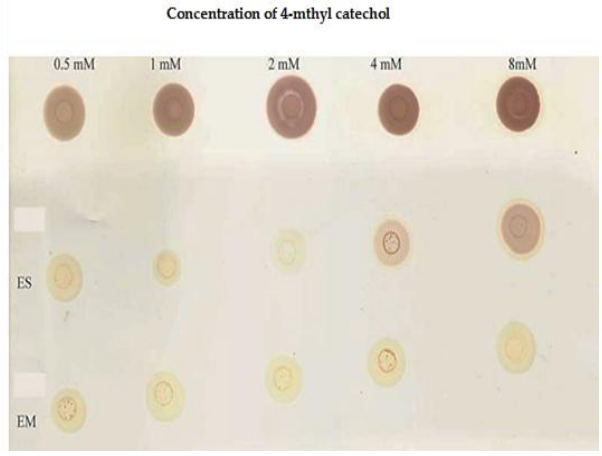

Fig. 9. Immobilization of PPO on TLC plate for detection of phenolic compounds. Top row contains enzyme, substrate and MBTH, middle row contains enzyme and substrate (ES) only and bottom row has enzyme and MBTH (EM) only

4. Conclusion

The present study demonstrated that crude extract of apple polyphenol oxidase can be used for detection of phenolic compounds. Our bioassay gives almost equal limit of detection as given by the bioassays developed using purified form of enzyme. In literature bioassays have been developed using pure enzymes extracted from microbial sources but we used apple as a source of PPO which is readily available and cost effective as well. Extraction of PPO from apple is more convenient than microbial sources. Our bioassay is simple and portable which can be used in field as well. Polyphenol oxidase is immobilized by simple over-spotting of enzyme and there is visual detection of phenolics. Simple Whatman filter paper 1 and TLC plate can be used as a support material. Image can be quantified by image analysis software. There is no requirement for trained personnel to conduct assay because it is very simple. This research suggests potential, feasibility and benefits of crude extracts for their fabrication in biosensors and bioassays. This study can pave the way for further studies on different sources of PPO and their application for detection of phenolics. Combination of crude PPOs from different sources (fruits, vegetables and microbes) can be immobilized for designing of hybrid bioassays.

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