

PARTIAL OPTIMIZATION AND STUDY OF ANTIMICROBIAL ACTIVITY OF POLYPHENOL OXIDASE (PPO) AND PEROXIDASE (POD) EXTRACTED FROM CHILLY PEPPER PERICARP.

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ABSTRACT: Polyphenol oxidase(PPO) (E.C. number 1.10.3.1) has ubiquitous distribution in almost all living organism. Whereas, peroxidase(POD) (E.C. number 1.11.1) act as hormone regulation and defense mechanism in plants. Keeping in pace with their present-day industrial application, efforts have been made to evaluate the activity of these two enzymes (PPO and POD) using pepper pericarp (*Capsicum annuum L.*) as an experimental material using catechol and guaiacol as a substrate, respectively. The effects of enzyme extract, substrate, hydrogen peroxide concentration (only for POD), pH and temperature and antimicrobial activity against different bacterial strains were investigated.

Keyword: Polyphenol oxidase, Peroxidase, antimicrobial activity.

INTRODUCTION:

Polyphenol oxidase (PPO) is a copper-containing enzyme which is probably present in all plants. It is widely distributed enzyme involved in the biosynthesis of melanins in animals and in the browning of plants. The enzyme catalyzes the oxidation of phenolic compounds to form corresponding quinone intermediates which polymerize to form undesirable pigment. It catalyzes two types of the oxidative reaction involving molecular oxygen: the hydroxylation of monophenols to o-diphenols and the oxidation of o-diphenols to o-quinones, which lead to the formation of black or brown pigments (Lee et al., 2007). Peroxidase (POD) is an oxidoreductase that is directly involved in many plant functions such as hormone regulation, defence mechanisms, indolacetic degradation and lignin biosynthesis(Serrano et al., 2008)It catalyzes a reaction in which hydrogen peroxide acts as the acceptor and another compound acts as the donor of hydrogen atom. POD involved in enzymatic browning since diphenols may function as reducing substrate in this reaction (Chisari et al., 2007). The involvement of POD in browning is limited by the availability of electron acceptor compounds such as superoxide radicals, hydrogen peroxide and lipid peroxidase. Enzymatic browning is a significant problem in a number of fruits and vegetables such as strawberry (Chisari et al., 2007), grape (Muñoz et al., 2004), potato (Lee et al., 2007), and lettuce (Gawlik et al., 2007). The discoloration in fruits and vegetables by enzymatic browning, resulting from conversion of phenolic compounds to o- quinones which subsequently polymerize to be a brown or dark pigment. The enzymes involved these processes are PPO and POD (Jiang et al., 2004). Because PPO and POD are the main enzymes involved the phenolic oxidation of many fruits and vegetables, their activities have attracted much attention. The relationship between the degree of browning and PPO activity were studied in processing apple varieties to provide reference for raw material selection (Ye et al., 2007). The kinetic characteristic and thermal inactivation property of POD extracted from red sweet pepper (*Capsicum annuum L.*) was studied. The enzyme was partially purified and its activity was evaluated using ABTS as substrate (Martínez et al., 2008). Since residual enzyme activities were assumed to cause capsaicinoid losses, POD activity in hot chili peppers (*Capsicum frutescens L.*) were investigated a long with the changes in capsaicinoid contents (Schweiggert et al., 2006).

Fresh peppers are good source of vitamin C and E, provitamin A and carotenoids (Chatterjee et al., 2007). Moreover, the red pepper fruit (*Capsicum annum* L.) has been used for many years as a source of pigments to add or change the color of foodstuffs, making them more attractive and acceptable for the consumer. It also shows antimicrobial activity. Plant oxylipins, the metabolic products of polyunsaturated fatty acids have been variously implicated in control of microbial pathogens. To get a better insight into the biological activities of oxylipins, in vitro growth inhibition assays were used to investigate the direct antimicrobial activities of 9 and 13- lipoxygenase (LOX) products against a set of plant pathogenic bacteria and fungi. LOX hydroperoxides derived from seeds of *Capsicum annum* showed differential antimicrobial activity. It was found that the LOX hydroperoxides, that is, 9-hydroperoxy octadecadienoic acid (9-HPODE) and 9-hydroperoxy octadecatrienoic acid (9-HPOTrE) showed antimicrobial activity against various fungal and bacterial pathogens. LOX hydroperoxides, that is, 9-hydroperoxy octadecatrienoic acid (9-HPOTrE) showed maximum antimicrobial activity than 9-hydroperoxy octadecadienoic acid (9-HPODE). The antimicrobial activity of oxylipins suggests their role in plant defense mechanism (Sucharitha et al., 2010). Thus, the aim of the present study was to evaluate PPO and POD activities in hot pepper pericarp. The optimum conditions for determination of both enzyme activities by spectrophotometric method were investigated including the amounts of enzyme extract, concentration of substrate, pH and incubation temperature.

MATERIALS AND METHODS:

Chemicals:

Polyvinylpyrrolidone was obtained from E.Merck was used. Catechol, Guaiacol, Di-sodium hydrogen phosphate, Potassium dihydrogen phosphate, Tri-sodium citrate, Citric acid and phosphoric acid were also purchased from the same.

Materials:

Pepper fruits used in this study were obtained from a local market in Burdwan. The pepper fruits were washed several times with tap water and the seeds and peduncle were removed. The pericarp was homogenized by using homogenizer for 2 min and stored at 4°C for further experiments. The codes and descriptions of the pepper samples used in this study are summarized in Table 1. The photographs of some samples are shown in Figure 1.

Enzyme Extraction:

All steps of enzyme extraction were carried out at 4°C. Five grams of the homogenized pepper pericarp of different species were extracted with 0.1 M phosphate buffer pH 7 containing 5 g of polyvinylpyrrolidone using magnetic stirrer for 15 min. The homogenate was filtered through Whatman No.41 filter paper and then centrifuged at 2,500 rpm for 20 min. The supernatant was filtered through Whatman No.42 filter paper and collected as an enzyme extract.

Enzyme Assay:

PPO activity was determined using a spectrophotometric method based on an initial rate of increase in absorbance at 410 nm (Soliva *et al.*, 2001). Phosphate buffer solution pH 7 (0.1 M, 1.95 mL), 1 mL of 0.1 M catechol as a substrate and 50 µL of the enzyme extract were pipette into a test tube and mixed thoroughly. Then the mixture was rapidly transferred to a 1-cm path length cuvette. The absorbance at 410 nm was recorded continuously at 25°C for 5 min using ultraviolet-visible spectrophotometer. POD activity was assayed spectrophotometrically at 470 nm using guaiacol as a phenolic substrate with hydrogen peroxide (Diaz *et al.*, 2001). The reaction mixture contained 0.15 mL of 4% (v/v) guaiacol, 0.15 mL of 1% (v/v) H₂O₂, 2.66 mL of 0.1 M phosphate buffer pH 7 and 40 µL of the enzyme extract. The blank sample contains the same mixture without the enzyme extract.

Effect of amounts of enzyme extract on enzyme activity:

The activity of PPO and POD as a function of amounts of enzyme extract was investigated. PPO activity was assayed at various amounts of the enzyme extract from 20 to 200 µL by mixing with 2 mL of 0.1 M catechol, and 1 mL of 0.1 M phosphate buffer pH 7. POD activity was also performed using the amounts of the enzyme extract from 25 to 100 µL. The solution of the reaction mixture contained 0.1 mL of 1% (v/v) H₂O₂, 0.1 mL of 4% (v/v) guaiacol, and 2.78 mL of 0.1 M phosphate buffer pH 7.

Effect of substrate concentration on enzyme activity:

The enzyme activities were measured in a quartz cuvette of 3 mL volume. PPO activity was proceeded by mixing 50 µL of the enzyme extract, 0.1 M catechol and 0.1 M phosphate buffer pH 7 at a selected volume. POD activity was observed by using the mixture containing 40 µL of the enzyme extract, 0.1 mL of 1%(v/v) H₂O₂, 4%(v/v) and catechol 0.1 M phosphate buffer pH 7 at a selected volume. The effect of H₂O₂ concentration on POD activity was studied using the reaction system consisted of 40 µL of the enzyme extract, 0.15 mL of 4%(v/v) guaiacol, 1%(v/v) H₂O₂ and 0.1M phosphate pH 7 at a selected volume.

Effect of pH on enzyme activity:

The activity of PPO and POD were determined at pH values of 3, 4, 5, 6, 7 and 8 using 0.1 M citrate buffer (pH 3-5) and phosphate buffer (pH 6-8). The optimum pH for PPO and POD were obtained using catechol and guaiacol as substrate, respectively. The effect of pH on PPO activity was observed by using the reaction mixture containing 1 mL of 0.1 M catechol, 1.95 mL of 0.1 M buffer solution and 50 μ L of the enzyme extract. For POD, the reaction mixture contained 0.15 mL of 4% (v/v) guaiacol, 0.15 mL of 1% (v/v) H₂O₂, 2.66 mL of 0.1 M buffer solution and 40 μ L of the enzyme extract.

Effect of temperature on enzyme activity:

PPO and POD activities were determined at 20, 30, 40, 50, 60 and 70°C. The substrate and buffer solutions were incubated for 5 min at various temperatures from 20 to 70°C before adding of the enzyme extract. Spectrophotometric measurement for 5 min was carried out at 25°C. The activity of PPO under optimum temperature was determined by adding 1 mL of 0.1 M catechol, 1.95 mL of 0.1 M phosphate buffer pH 7 and 50 μ L of the enzyme extract. For POD, the reaction mixture contained 0.15 mL of 4% (v/v) guaiacol, 0.15 mL of 1% (v/v) H₂O₂, 2.66 mL of 0.1 M phosphate buffer pH 7 and 40 μ L of the enzyme extract.

Antimicrobial assay:

Antimicrobial activity of enzyme from different pepper species is assayed by Agar Cup Diffusion, using two different strain, i.e.; *E. coli* (gram negative) and *Bacillus sp.* (gram positive).

RESULT AND DISCUSSION:

PPO and POD are oxidative enzymes which catalyze the oxidation of phenolic substrates mainly due to enzymatic browning (Jiang *et al.*, 1999). They catalyze the oxidation of phenolic compounds to *o*-quinone which polymerize to form undesirable pigments (Chisari *et al.*, 2007). In this study, catechol and guaiacol were used as the substrate for PPO and POD, respectively. The enzymatic oxidation of catechol by PPO/O₂ transformed the substrate into yellow products with a maximum absorbance at 410 nm. The enzymatic oxidation of guaiacol by POD/ H₂O₂ changed the substrate into orange-pink products with a maximum absorbance at 470 nm (Figure 2).

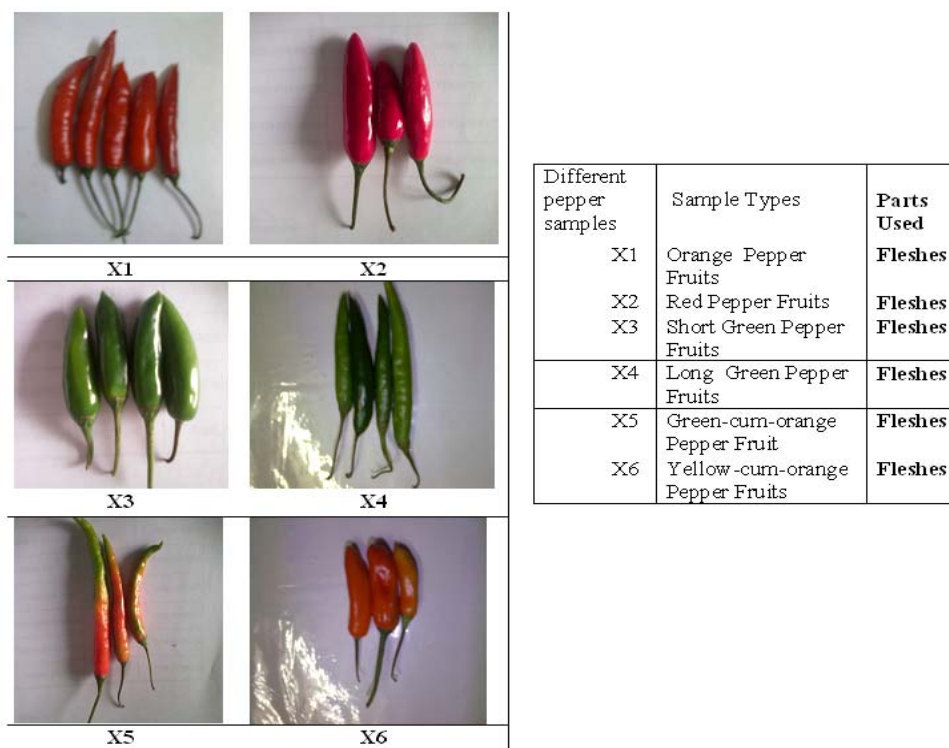


Figure 1: Different Sample variety of Pepper.

The effect of various amounts of the enzyme extract on PPO and POD activities was studied and the result was shown as the rate of substrate oxidation by the enzymes. The substrate oxidation was found to be dependent on the amounts of the enzyme extract. An increase in enzyme concentrations resulted in the corresponding linear increase in both enzyme activities (Figure 3), in the enzyme concentration range assayed (20 to 200 μL for PPO and 25 to 100 μL for POD).

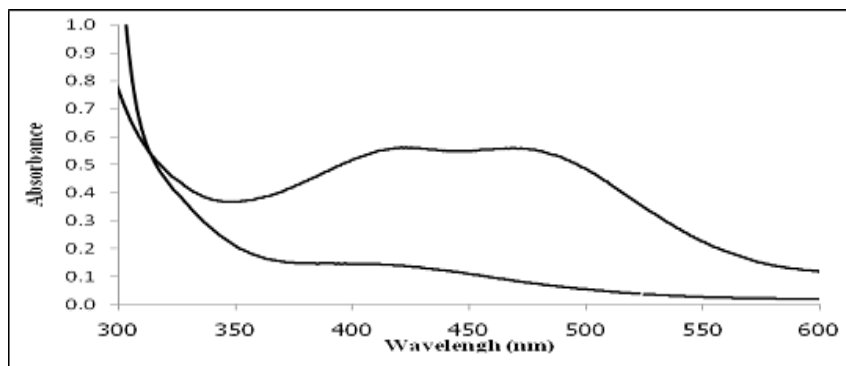


Figure 2: Absorption spectra, at 25°C, obtained for (a) the oxidation of catechol by PPO and (b) the oxidation of guaiacol by POD of chilly pepper.

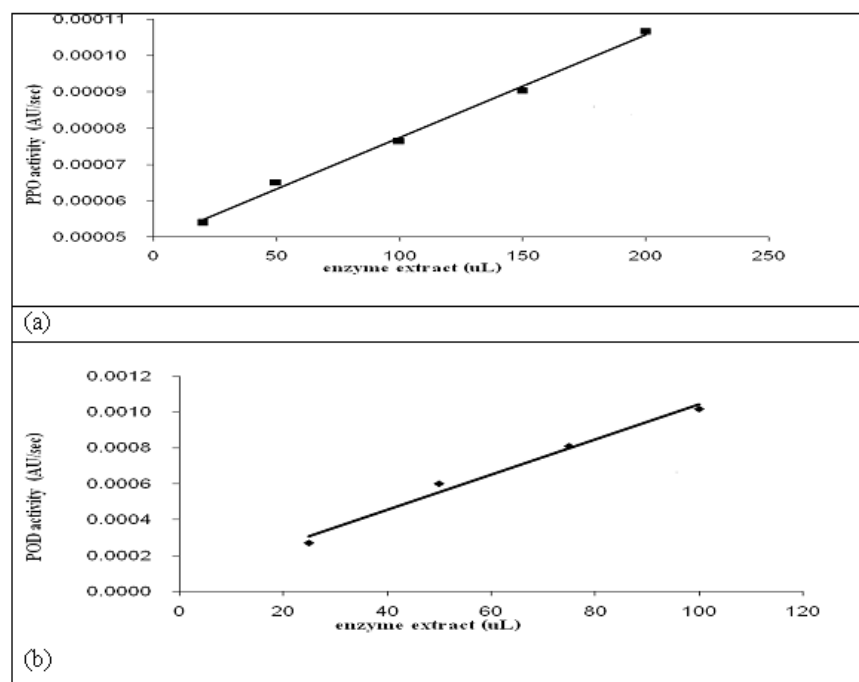


Figure 3: Effect of amounts of the enzyme extract on both PPO (a) and POD (b) activities.

As shown in Figure 2, the oxidation of catechol by hot pepper PPO generated products which had absorbance maximum at 410 nm. Thus, the optimal catechol concentration was determined by measuring the increase in absorbance at 410 nm, using different amounts of the substrate (Figure 4). As expected, an increase in the substrate concentration resulted in an increase in pigment formation. The rate of which stayed practically constant at saturating catechol concentration. Therefore, the concentration of 33.3 mM catechol was routinely chosen because at higher concentrations of the substrate did not significantly affect the formation of the *o*-quinone intermediate. When the oxidation of guaiacol by hot pepper POD was carried out in the presence of H_2O_2 , the quinone intermediate formed gave absorbance maximum at 470 nm.

The optimal guaiacol concentration was determined and the results are shown in Figure 5. The optimal concentration of guaiacol was found to be 18 mM. In addition, when the H₂O₂ concentration was increased, at a fixed saturating concentration of guaiacol, POD exhibited the highest activity at 4.9 mM of H₂O₂ (Figure 5).

The activity of PPO and POD were measured at different pH values using catechol and guaiacol as substrate, respectively. As shown in Figure 6, the optimum pH 7 of both enzymes was obtained. It is known that the optimum pH for any enzymes depends on plant materials and substrate in the activity assay. In general, most plants show maximum enzyme activity at or near neutral pH. Different optimum pH values for both enzymes obtained from various sources and substrates used have been reported. The optimum pH values are 6.8 and 5.5 for butter lettuce PPO using 4-methylcatechol and catechol as substrates, respectively (Gawlik *et al.*, 2007), pH 6.5 for longan fruit PPO using 4-methylcatechol as substrate (Jiang *et al.*, 2004), pH 6.0-8.5 for kiwifruit POD using *p*-phenylenediamine as substrate (Fang *et al.*, 2008) and pH 6.0 for spring cabbage POD using guaiacol as substrate (Belcarz *et al.*, 2008).

The optimum temperature for enzyme activity usually depends on experimental conditions. Generally, the reaction rate decreases because of thermal denaturation when the temperature is increased. This situation is similar for most enzymes. Temperature dependence in the enzyme activities is presented in Figure 7. It was found that the highest activity of PPO and POD were obtained at 30°C and 40°C, respectively. PPO showed the highest activity at 30°C, and its activity decreased slightly between 40 and 70°C. The POD activity increased when the temperature was increased from 20 to 40°C, and then decreased probably due to denaturation of the enzyme at higher temperatures. From previous studied, the temperature at which PPO showed the highest activity was in the range of 25-30°C, and then decreased at temperature above 40°C (Dogan *et al.*, 2004). In case of POD, the enzyme was highly active up to 40°C and lost its activity at higher temperatures (Fatima *et al.*, 2007). From the obtained results, the optimum temperature of the both enzymes was found between 30°C and 40°C. Thus, we determined the enzyme activities of pepper samples at ambient temperature (30 ± 3 °C).

The Inhibiting clear zones of different enzyme (such as, PPO and POD) on *E.coli* and *Bacillus sp.* ensure their antimicrobial property. And to check the result in the 3rd well an antibiotic is loaded which also give a clear inhibiting zone, which ensures the proper growth of the specific bacterial strain. And a control well is also made to make the result more perfect and easy to tally. For this experiment first spread plate technique is used on the agar plates and the on it agar cup diffusion process is carried out on it. And the ultimate result shows their antibacterial activity on a broad spectrum, as shown in figure 8.

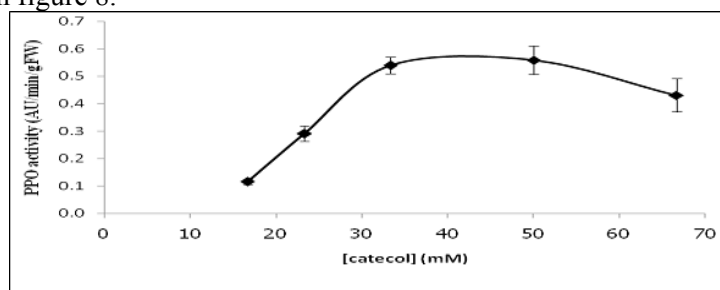


Figure 4: Effect of catechol concentration on the hot pepper PPO activity.

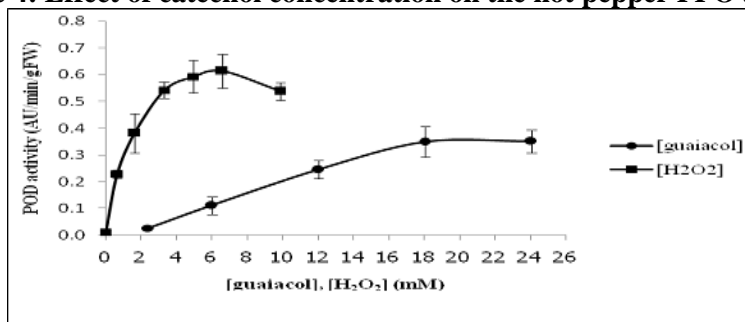


Figure 5: Effect of guaiacol and hydrogen peroxide concentrations on the hot pepper POD activity.

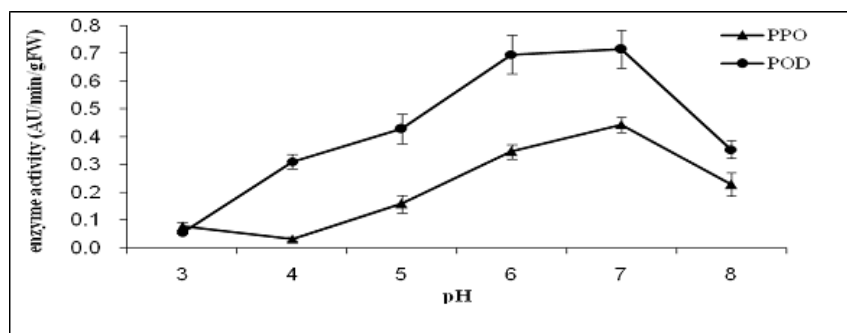


Figure 6: Effect of pH on the hot pepper PPO and POD activities.

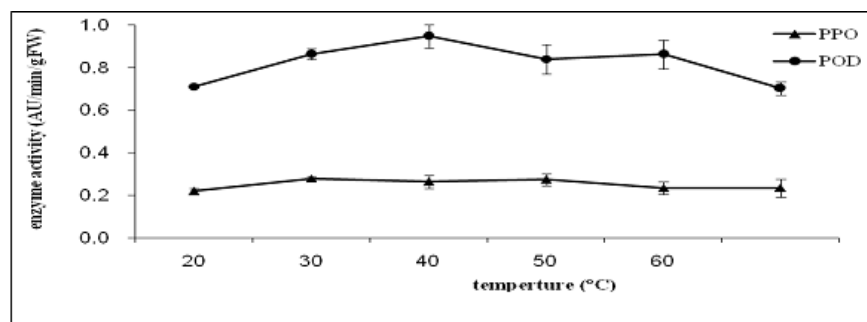


Figure 7: Effect of temperature on the hot pepper PPO and POD activities.

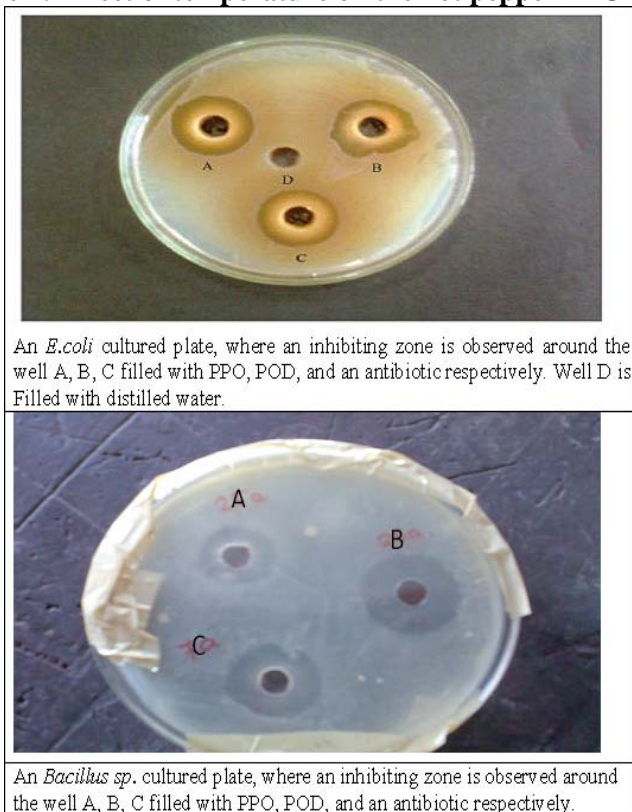


Figure 8: The inhibiting zone of different enzyme extract,like, PPO and POD on *E.coli* and *Bacillus sp.*

Note: All the source of enzymes gives more or less same result. So, the repetition of the experiment is not done again and again.

From the above set of experiments, we came to a conclusion it shows spectrophotometric method for the measurement and optimization of enzymatic activity of hot pepper PPO and POD was developed along with its antimicrobial activity, which shows a broad spectrum activity.

Abbreviation: POD –Peroxidase, PPO- Polyphenol Oxidase, LOX-Lipoxygenase, 9-HPODE - Hydroperoxy octadecadienoic acid, 9-HPOTrE - 9-hydroperoxy octadecatrienoic acid

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REFERENCES

- A. Sucharitha , P. Uma. Maheswari Devi, (2010), Antimicrobial properties of chilly Lipoxygenase products. African Journal of Microbiology Research Vol. 4(9), pp- 748 – 752.
- Belcarz, A., Ginalska, G., Kowalewska, B. and Kulesza, P. (2008). Spring cabbage peroxidase – Potential tool in biocatalysis and bioelectrocatalysis. *Phytochemistry* 69: 627-636.
- Chatterjee, S., Niaz, Z., Gautan, S., Adhikari, S., Pasad, S.V. and Sharma, A. (2007). Antioxidant activity of some phenolic constituents from green pepper (*Piper nigrum* L.) and fresh nutmeg mace (*Myristica fragrans*). *Food Chemistry* 102: 515-523.
- Chisari, M., Barbagallo, R.N. and Spagna, G. (2007). Characterization of polyphenol oxidase and peroxidase and influence on browning of cold stored strawberry. *Journal of Agriculture and Food Chemistry* 55: 3469-3479.
- Deepa, N, Kaur C., George, B., Singh, B. and Kapoor, H.C. (2007). Antioxidant constituents in some sweet pepper (*Capsicum annuum* L.). *LWT-Food Science and Technology* 40: 212-219.
- Díaz, J., Bernal, A., Pomar, F. and Marino, F. (2001). Induction of shikimate dehydrogenase and peroxidase in pepper (*Capsicum annuum* L.) seedlings in response to copper stress and its relation to lignification. *Plant Sciences* 161: 179- 188.
- Doğan, S. and Doğan, M. (2004). Determination of kinetic properties of polyphenol oxidase from Thymus (*Thymus logicaulis* subsp. *chaubardii* var. *chaubardii*). *Food Chemistry* 88: 69-77.
- Fang, L., Jiang, B. and Zhang, T. (2008). Effect of combined high pressure and thermal treatment on kiwifruit peroxidase. *Food Chemistry* 109: 802-807.
- Fatima, A. and Husain, Q. (2007). A role of glycosyl moieties in the stabilization of bitter melon (*Momordica charantia*) peroxidase. *International Journal of Biological Macromolecules* 41: 56-63.
- Gawlik-Dziki, U., Złotek, U. and Świeca, M. (2007). Characterization of polyphenol oxidase from butter lettuce (*Lactuca sativa* var. *capitata* L.). *Food Chemistry* 107: 129-135.
- Jiang, Y., Duan, X., Joyce, D., Zang, Z. and Li, J. (2004). Advance in understanding of enzymatic browning in harvested litchi fruit. *Journal of Food Chemistry* 88(3): 443-446.
- Jiang, Y-M. (1999). Purification and some properties of polyphenol oxidase of longan fruit. *Food Chemistry* 66: 75-79.
- Lee, M-K. and Park, I. (2007). Studies on inhibition of enzymatic browning in some foods by Du-Zhong (*Eucommia ulmoides* Oliver) leaf extract. *Food Chemistry* 114: 154-163.
- Lee, M.Y., Lee, M.K. and Park, I. (2007). Inhibition of onion extract on polyphenol oxidase and enzymatic browning of taro (*Colocasia antiquorum* var. *esculenta*). *Food Chemistry* 105: 528-532.
- Muñoz, O., Sepúlveda, M. and Schwaerz, M. (2004). Effects of enzymatic treatment on anthocyanin pigments from grapes skin from Chilean wine. *Food Chemistry* 87: 487-490.

- Saraiva, J.A., Nunes, C.S. and Coimbra, M.A. (2007). Purification and characterization of olive (*Olea europaea* L.) peroxidase – Evidence for the occurrence of pectin binding peroxidase. *Food Chemistry* 101: 1571-1579.
- Schweiggert, U., Schieber, A. and Carle, R. (2006). Effects of blanching and storage on capsaicinoid stability and peroxidase activity of hot chilli pepper (*Capsicum frutescens* L.). *Innovative Food Science and Emerging Technologies* 7: 217-224.
- Serrano-Martínez, A., Fortea, F.M., Del Amor, F.M. and Núñez-Delgado, E. (2008). Kinetic characterization and thermal inactivation study of partially purified red pepper (*Capsicum annuum* L.) peroxidase. *Food Chemistry* 107: 193-199.
- Soliva, R.C., Elez, P., Sebastián, M and Martín, O. (2001). Evaluation of browning effect on avocado purée preserved by combined methods. *Innovative Food Science and Emerging Technologies* 1: 261-268.
- Ye, S., Yo-Xin, Y., Heng, Z., Yuan-Peng, D., Feng, C. and Shu-Wei, W. (2007). Polyphenolic compound and the degree of browning in processing apple varieties. *Agricultural Sciences in China* 6: 607-612.