International Journal of Botany Studies
ISSN: 2455-541X; Impact Factor: RJIF 5.12
Received: 19-02-2021; Accepted: 09-03-2021: Published: 15-03-2021
www.botanyjournals.com
Volume 6; Issue 2; 2021; Page No. 161-167

Partial purification and characterization of star fruit leaves (*Averrhoa carambola* l.) polyphenol oxidase and its immobilization study with different matrices for removal of aqueous phenol

Shruti Laad[1], Premakshi HG[2], Chandrappa Mukappa Kamanavalli[1*]
[1] P. G. Department of Studies in Biochemistry, Karnatak University, Dharwad, Karnataka, India
[2] P. G. Department of Studies in Chemistry, Karnatak University, Dharwad, Karnataka, India

Abstract
Our study focuses on a partial purification, characterization and immobilization of enzyme for phenol removal by polyphenol oxidase (PPO) enzyme from carambola leaves. A PPO partially purified about 4.20-fold with a recovery of 2.93 % yield. The PPO enzyme is a monomeric protein with a molecular weight of~61 kDa. The Km values for free and immobilized PPO were 20 and 32 mM, respectively. The entrapment of PPO by the SA–PVA–silver nanoparticles (AgNPs) showed the best result for phenol removal. The optimal pH of free and immobilized PPO enzyme activities was 7.5 pH. The optimal temperature of free and immobilized PPO was 40 ℃ and free enzyme activity lost at 60 ℃; The AgNPs immobilized PPO removed 95 % of the phenol on the fifth cycle. The immobilized carambola leaf PPO enzyme could be efficiently used for the removal of phenol from industrial effluents.

Keywords: partial purification, *Averrhoa carambola* l., polyphenol oxidase

Introduction
Increased production of plastics, dyes, pesticides, and many other chemicals has resulted in the release of hazardous chemical wastes and environmental pollution. A few persistent pollutants, including several pesticides, are carried in air and water, affecting the wildlife and general population [1]. These pollutants are non-biodegradable and are known to have chronic toxic effects. Chlorinated organic compounds, in particular, are found to be resistant to biochemical degradation. Monochlorophenols, among other phenol compounds, serve as intermediates in the production of pesticides [2]. These are also used as antimicrobial agents in various products, such as adhesives, oils, textiles and pharmaceutical products.
Phenolic compounds are produced in waste-waters from several industries, including coal conversion, petroleum refining, plastics, dyes and other organic chemicals, textile manufacturing, mining, and paper-making. Most of the phenols are toxic and some phenolic compounds are carcinogens to humans [3]. The biodegradation process majorly focuses on the use of micro-organisms in the removal of environmental pollutants. Besides, enzymes could also be used effectively for the safe removal of toxic and hazardous phenolic compounds. Oxidoreductases (i.e., polyphenol oxidase and peroxidase) have been used to remove phenols and aromatic compounds from waste-waters [4]. For the treatment of large volumes of waste-waters, reactors containing immobilized enzymes are desirable because of the high cost of enzymes. Enzyme immobilization usually provides reuse of the enzyme and has advantages such as product separation and continuous operation [5]. There is always a search for cheaper support and enzyme to prepare immobilized enzymes for such implement applications.
Polyphenol oxidase (PPO; tyrosinase, catechol oxidase; E.C. 1.14.18.1) catalyzes the oxidation of phenols to o-quinones, which then undergo spontaneous, non-enzymatic polymerization in water, eventually forming water-insoluble polymers which can be separated by filtration [6]. Several techniques depend on the PPO treatment have been developed to remove industrial waste-waters, but the processes' cost has limited their use [7]. Accordingly, it is imperative to develop new techniques for enzyme immobilization on industrially available and cheap carriers that are ideal support materials for enzyme immobilization because of their hydrophilicity, biocompatibility, and biodegradability and anti-bacterial property [8].
In this study, the first focus was finding a cheaper and readily available plant polyphenol oxidase (PPO) enzyme source for the commercially developed ones and their immobilization. Star fruit leaves (*Averrhoa carambola L.)*) are waste and have been employed in this work as a readily available and inexpensive PPO enzyme source. PPO enzyme was partially purified from Star fruit (*Averrhoa carambola L.*) leaves and immobilized on sodium alginate (SA), sodium alginate–polyvinyl alcohol (SA–PVA) and SA–PVA–silver nanoparticles (AgNPs). The biochemical activities were determined for free and immobilized Star fruit (*Averrhoa carambola L.*) leaf PPO. The second focus was to evaluate the performance of inexpensive immobilized enzymes for the removal of phenols.

Materials and Methods
Materials
The leaf of Star fruit (*Averrhoa carambola* L.) was obtained from kemmannu region (Udupi District), Karnataka State, India. Catechol (1,2-benzenediol), Sodium dodecyl sulphate (SDS) were purchased from the Sigma Chemical Co, India. Coomassie brilliant blue R-250, acrylamide, methylene-bis-acrylamide, ammonium sulphate, sodium dihydrogen phosphate (NaH_2PO_4**)**, sodium alginate (SA), sodium alginate–polyvinyl alcohol (SA–PVA) and SA–PVA–silver nanoparticles (AgNPs). and all other chemicals used were of analytical grade, purchased from Merck, India.

Preparation of carambola leaf PPO extract

The crude extract of PPO was prepared according to the method of Huang *et al*. [9]. Carambola leaf (50 g) was homogenized for 15 min in 50 ml pre-cooled 0.1 M sodium phosphate buffer (pH 7.0), with 0.3 % PVP (polyvinylpyrrolidone) to stop enzymatic browning. The homogenate of the extract was filtered through four layers of gauze. The filtrate was centrifuged at 3000 g (Remi R-4C Laboratory Centrifuge) at 5000 × *g* for 15 min at 4 ℃, and the obtained clear supernatant was used for further studies.

Extraction and purification and quantification of an enzyme

Carambola leaf polyphenol oxidase (PPO) was extracted according to the method described previously [10, 11]. The supernatant of the enzyme was precipitated with ammonium sulfate at 40-80 % saturation for 1 h with gentle stirring. The precipitate of the enzyme was collected by centrifugation at 15,000 × *g* for 30 min. The enzyme residue was dissolved in 50 mM phosphate buffer (pH 7.0) and dialyzed at 4 ℃ against 10 mM phosphate buffer (pH 7.0). Approximately 10 ml of dialyzed extract was loaded onto to Sephadex G-100 column (1.2 × 70 cm, 0.5 ml/min). The eluted fractions (4 ml) were collected and checked for PPO activity and protein. Fractions with PPO activity were pooled, dialyzed and concentrated [12, 13]. PPO activity was determined using catechol as a substrate. The reaction mixture contains 10 µl of purified enzyme sample, 1.6 ml of 50 mM of catechol solution and 0.4 ml of 0.2 M sodium phosphate buffer (pH 5.5) at 35 ℃ incubated for 15 min and the change in absorbance at 420 nm was measured spectrophotometrically. One enzyme activity unit was measured as the amount of enzyme that increased the absorbance of 0.001 per minute [14].

Denaturing SDS-PAGE

The molecular weight of the purified PPO enzyme was determined by 10 % SDS-PAGE, (biotech Model Code 05-01) and performed according to the modified method of Davis and Laemmli [15, 16]. The separating and stacking gels used were 15 % and 4 %, respectively. 0.05 M of phosphate buffer at pH 7.2 as the electrode tray buffer and bromophenol blue (0.02%) as the tracking dye were used. Electrophoresis of carambola leaf purified enzyme was carried out by applying a current of 100 V for ~5 h and was continued till the dye band reached the lower end of the gel. Standard protein markers (catalase, bovine serum albumin, ovalbumin, soybean trypsin, and lactoglobulin) were electrophoresed on the same gel slabs. SDS-PAGE gels were stained with 0.025 % Coomassie brilliant blue R-250 in methanol-acetic acid-water (4:1:5, v/v). The gels were subsequently destained with methanol-acetic acid-water (4:1:5, v/v).

Immobilization of PPO in different matrices

Sodium alginate (SA) entrapment

Carambola leaves PPO was immobilized on SA entrapment, according to Tallur *et al*. [17]. To 20 ml enzyme preparation, 400 mg of SA was added to obtain a 2 % SA solution. This resultant solution was then added dropwise into sterile, cold 0.2 M $CaCl_2$ (4 ℃) solution through a burette connected to a tapered pipette tip. Gel beads of approximately 1.5-2.0 mm diameter were obtained. The gel beads were hardened by re-suspending into a fresh calcium chloride for 2 h with gentle agitation. Finally, these beads were washed several times with sterilized distilled water and stored in 0.2 M phosphate buffer (pH 7.0) for further use.

Sodium alginate–polyvinyl alcohol (SA–PVA) gel entrapment

The SA–PVA entrapment of carambola leaf PPO was performed according to Pang *et al*. [18] with minor modification. Aqueous solutions of 6 gm of polyvinyl alcohol and 4 gm of sodium alginate were mixed well with water. In this mixture, 40 ml of enzyme preparation was dispersed and glutaraldehyde was used as a crosslinker. The gel bead spheres were prepared by dispensing the mixture dropwise manner with a 10 ml pipette into a 0.2 M $CaCl_2$ solution. The gel beads were washed with a large amount of water and stored. The beads were frozen at -20 for 24 h, kept at 4 for 12 h in 0.2 M phosphate buffer (pH 7.0), and thawed at room temperature for further use.

Entrapped in sodium alginate–polyvinyl alcohol–silver nanoparticles (SA–PVA–AgNPs) gel

Polyvinyl alcohol (PVA)/ sodium alginate (SA) nanocomposite beads containing silver nanoparticles (AgNPs) (PVA/SA/AgNPs) were prepared as described above. Silver nitrate solution (5 mM $AgNO_3$) was added gently and stirred in an ice-water bath until the solution becomes colorless. Then 10 ml of $NaBH_4$ (10 mM) aqueous solution was added dropwise under vigorous stirring at the same temperature for 1 h. The solution became light yellow. To this, 40 ml of the enzyme was added, and hydrogels were prepared by dispensing the mixture dropwise manner with a 10 ml pipette into a 0.2 M $CaCl_2$ solution. The obtained beads were filtered, washed three times with double distilled water and stored in 0.2 M phosphate buffer (pH 7.0) [19, 20].

Activity assays of PPO

At 40 ℃, the activity of free and immobilized PPO was determined using catechol as a substrate. The assay mixture consisted of 1.6 ml of 50 mM catechol in 0.2 M potassium phosphate buffer pH 5.5 and 10 µl of an enzyme. The increase in absorbance of PPO enzyme at 420 nm was measured with the initial rate of o-quinone formation at 420 nm [21] function at a time for 1 min. One unit of PPO activity is defined as the amount of the enzyme that causes an increase in absorbance of 0.001 per min. PPO activity was assayed in triplicate measurements.

Optimal pH and temperature

The optimal pH for free and immobilized carambola leaf PPO activities was determined by Gulcin and Koksal [22, 23] with slight modification in the range of 4.0 to 9.0. Citrate buffer 0.2 M was used at a pH range of 4.0 to 6.0 and 0.2 M phosphate buffer was used at a pH range of 6.0 to 9.0 with 50 mM catechol as a substrate in both the buffers. A standard reaction mixture assayed PPO activity with a different buffer.

The PPO activity's optimum temperature was determined by adding 1.6 ml of 50 mM catechol solution, 0.2 M of phosphate buffer at pH 7.5. Before the addition of the enzyme, the mixture was pre-equilibrated for 5 min at various temperatures (20 to 60 °C), then 10 µl of enzyme solution was added and incubated the reaction mixture for 5 min residual enzyme activity was measured.

Storage stability of free and immobilized PPO

The storage stability of free and immobilized PPO of carambola leaf was systematically carried out using a partially purified enzyme. The extract was stored at 4 °C and the activity of the immobilized PPO and free PPO was monitored at regular intervals for 30 days. The residual PPO enzyme activity was measured spectrophotometrically at 420 nm.

Thermal stability measurements

The thermal stability of free and immobilized polyphenol oxidase was determined by measuring the enzyme's residual activity exposed to three different temperatures (50-70 °C) in 0.2 M, pH 7.5 for 2 h. At 10 min time intervals, a sample was removed and assayed for enzymatic activity.

Determination of the concentration of phenol

Immobilized PPO in SA, SA–PVA and SA–PVA–AgNPs matrices was separately put in artificial phenolic waste-water (20 mg phenol/ l in 0.2 M phosphate buffer, pH 7.0) to remove phenol with five cycle process. Phenol was measured using a colorimetric assay using 4-aminoantipyrine and potassium ferricyanide under alkaline conditions to form a red quinone-type dye absorbing maximally at 510 nm [24].

Results

Enzyme purification and quantification

The enzyme purification steps for carambola leaf PPO are summarized in Table 1. First, the crude extract proteins were precipitated with ammonium sulphate from 40–80 % and all the fractions were analyzed for the PPO activity. It has been observed that only 60 % fraction had PPO activity, while the other fractions did not have any detectable PPO activity. The enzyme was purified 4.20 -fold with 2.93 % recovery. Then the fraction containing partially purified enzyme was loaded onto a Sephadex G-100 column. The enzyme was eluted, and activity was determined using catechol as a substrate.

All the fractions having PPO activity were pooled, concentrated, and loaded on SDS-PAGE to check enzyme purity. In 40 % ammonium sulphate precipitation, one single protein band has appeared. And in 60 %, two protein bands appeared around 61 kD and 65 kD respectively in SDS-PAGE (Fig.1).

When the native PAGE study was performed, only one activity band was visible; and the zymographic pattern showed the protein band appeared almost at the same position as in SDS-PAGE.

The single band of the enzyme around 61 kD shows monomeric PPO enzyme in nature.

Table 1: Purification of PPO extracted from carambola (*Averrhoa carambola*) leaf

Steps	Total activity (U/mL)	Total protein (mg)	Specific activity (U/mg protein)	Fold Purification	% Yield
Crude extraction	41352	3015	13.71	1	100
$(NH_4)_2SO_4$(40-80%)	24026	1244	19.31	1.407	58.1
Gel filtration	3041	102	29.81	2.173	7.35
Ion exchange (Sephadex G-100)	1212	21	57.71	4.207	2.93

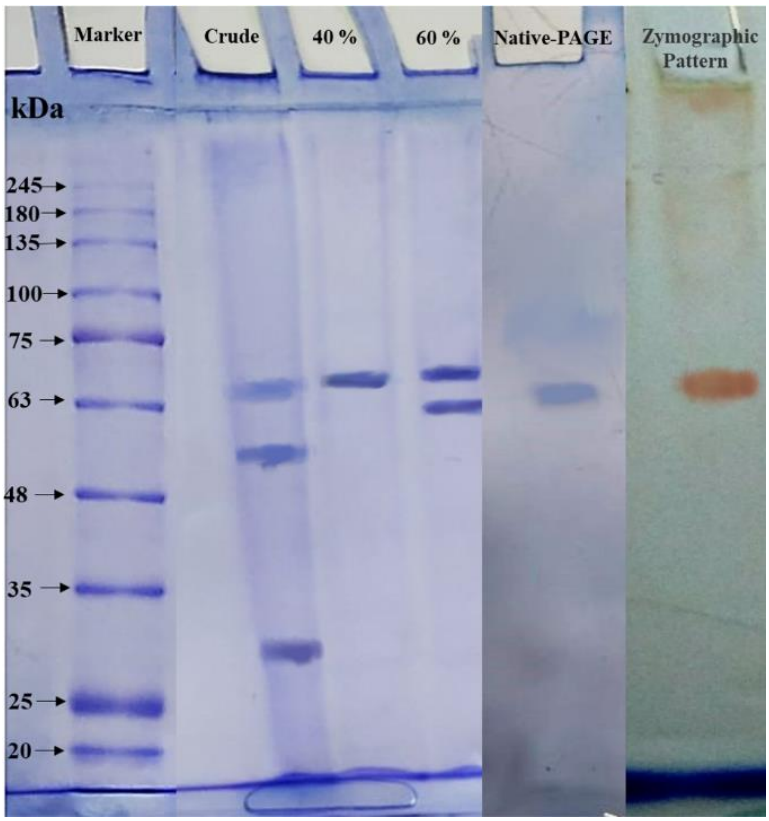

Fig 1: SDS-PAGE, activity staining and native-PAGE of *Averrhoa carambola* polyphenol oxidase (PPO) activity: Lane 1-(M) molecular mass standards; Lane 2-crude extract; Lane 3- 40 %, Lane 4- 60 % ammonium sulphate precipitation of partially purified PPO; Lane 5-native-PAGE; Lane 6-Zymography (activity staining of partially purified PPO).

Effect of pH on the activity of PPO

For the determination of pH effect on free PPO and enzyme immobilized on SA, SA–PVA and SA–PVA–AgNPs were used within the pH (0.2 M) range of 4.0-9.0. The optimum activity for free and immobilized PPO was determined at pH 7.5. Both free and immobilized carambola leaf PPO gave similar pH values (Fig. 2).

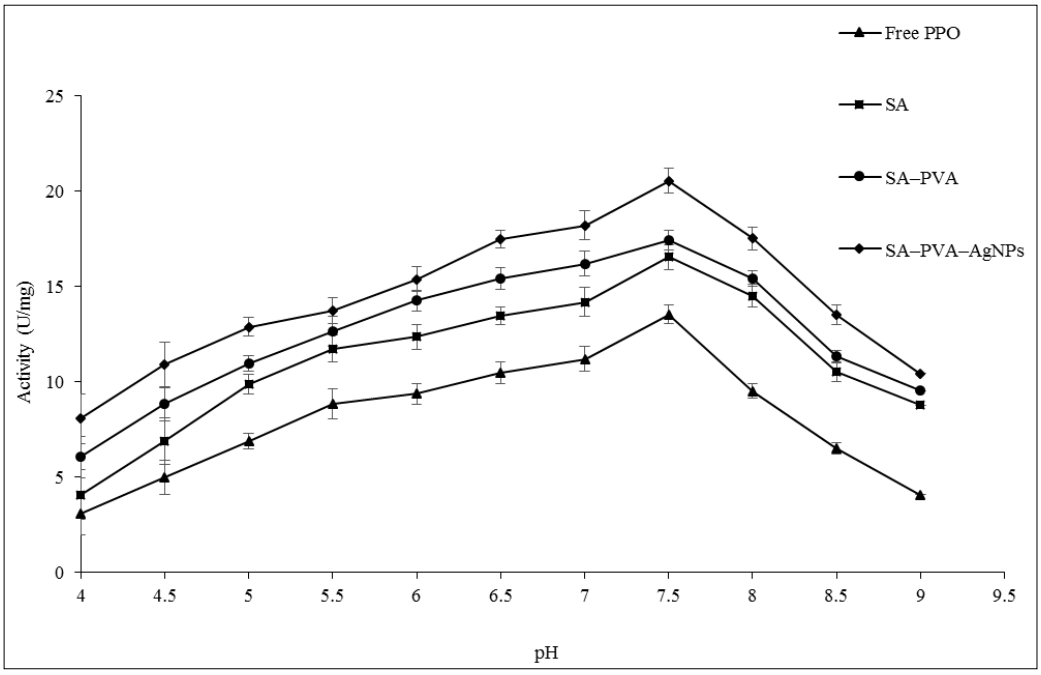

Fig 2: pH effect on free and immobilized *Averrhoa carambola* leaf polyphenol oxidase.

Effect of temperature on the activity of PPO

The activity dependence of free and immobilized PPO on temperature was shown in Fig.3. Compared to free PPO, the SA, SA–PVA and SA–PVA–AgNPs immobilized PPO has shown enhanced activity at higher temperatures at 40 ℃. In brief, the immobilized PPO in SA–PVA-AgNPs at 40 ℃ retains about half of the initial activity. Whereas the free PPO activity drastically decreased after 40 ℃ and complete loss of activity observed at 60 ℃.

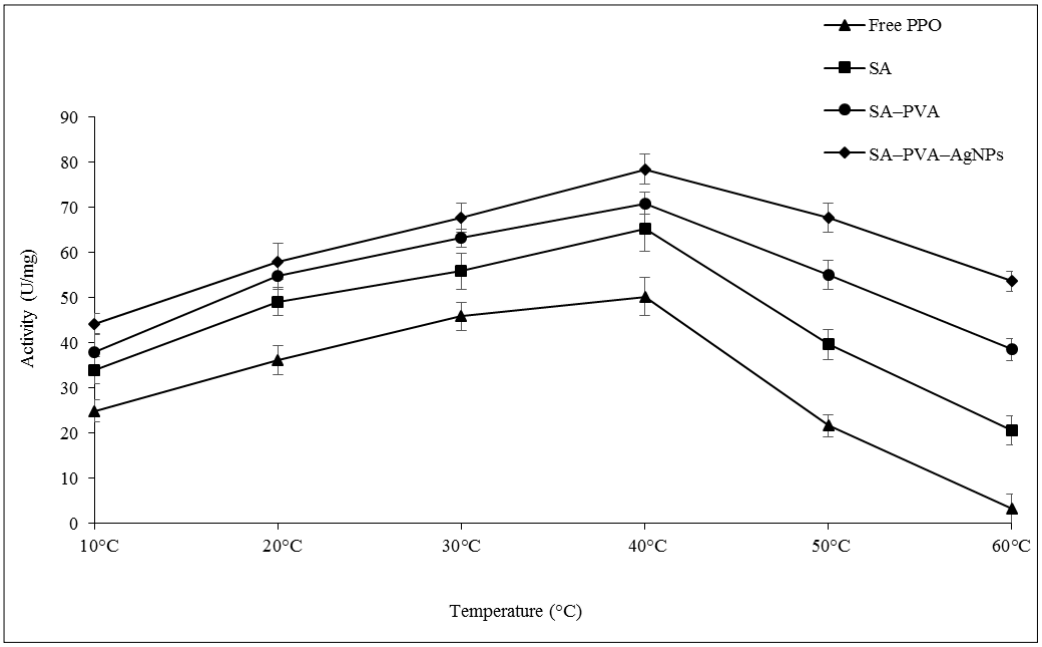

Fig 3: Optimum temperature of free and immobilized *Averrhoa carambola* leaf polyphenol oxidase.

Storage stability of immobilized PPO

The free and immobilized PPO were stored at 4 ℃. The immobilized PPO activity decreased more slowly than that of the free PPO; after 30 days, the free enzyme activity was completely lost compared to its original activity. Free PPO activity decreased significantly after 5 days and had no activity after 10 days. However, the immobilized enzyme in SA retained its activity till the 15th day. Whereas SA–PVA and SA–PVA–AgNPs retained their activity till the 25th and 28th day, respectively. The storage stability and activity of the PPO enzyme in SA–PVA– AgNPs was better compared to SA and SA–PVA encapsulated matrices (Fig. 4).

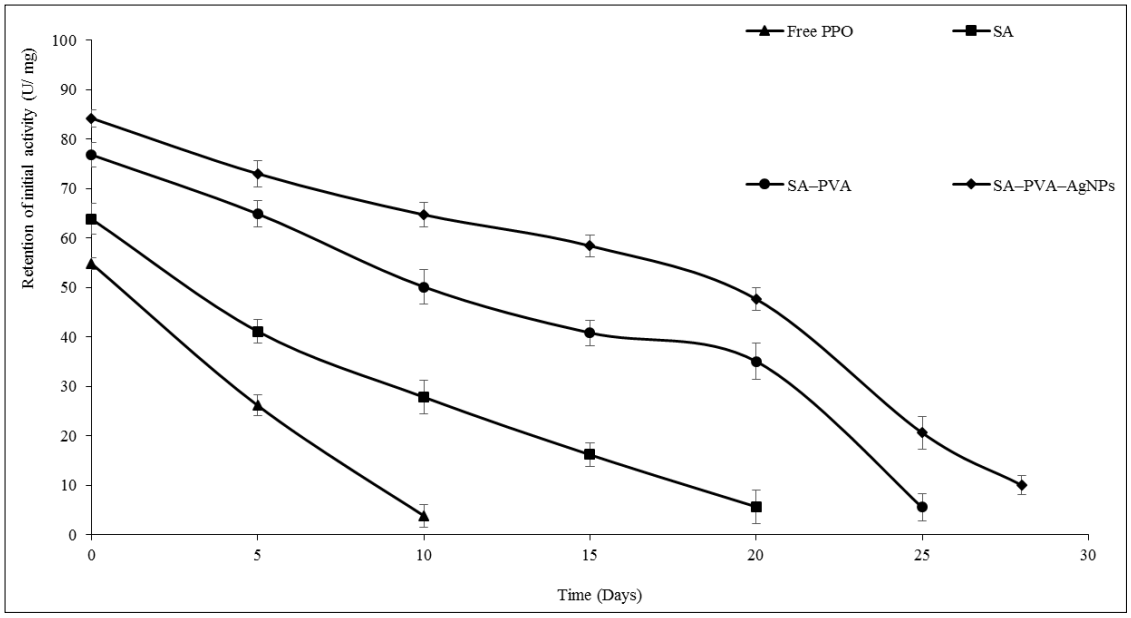

Fig 4: Storage stability of free and immobilized *Averrhoa carambola* leaf polyphenol oxidase.

Thermal stability measurements

The thermal stability may indicate the efficiency of the immobilization method and reflect the delicate balance between the acquired conformational stability and the resulting microenvironment created around the enzyme [25, 26]. Thermal stability experiments were carried out with free and immobilized enzymes. Fig.5 shows that complete inactivation of free PPO was observed at 70°C. At 50 °C enzyme immobilized on SA, SA–PVA and SA–PVA–AgNPs matrices showed good activity. At 60 and 70 °C, immobilized PPO on SA showed a drastic loss in activity. At 60 to 70 °C SA–PVA–AgNPs immobilized PPO was inactivated at a much slower rate than the native form (Fig. 5).

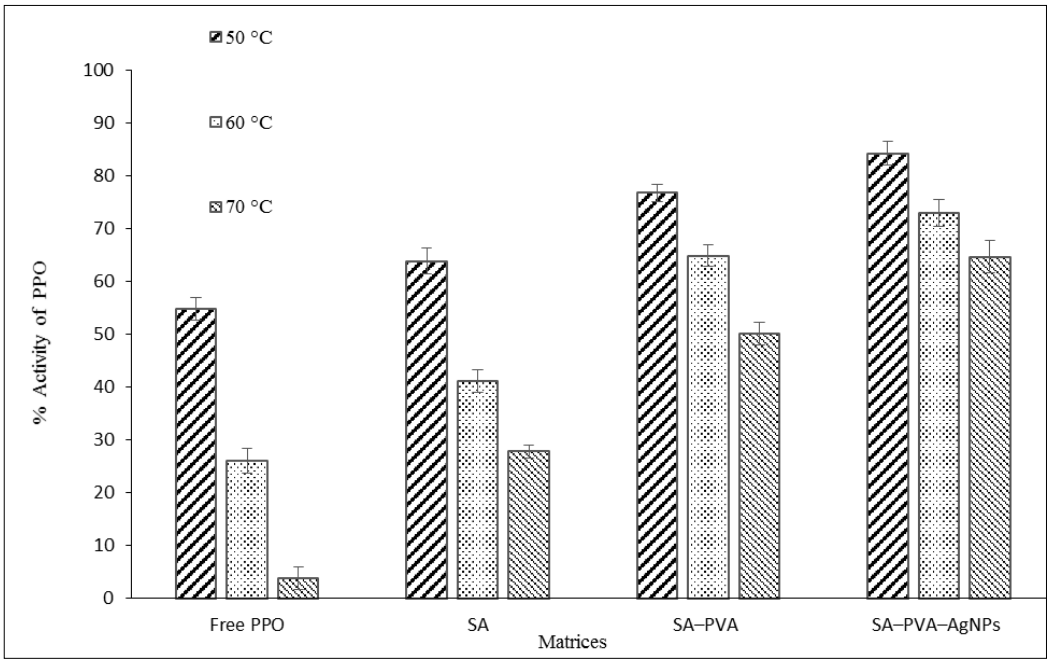

Fig 5: Thermal stability of free and immobilized *Averrhoa carambola* leaf polyphenol oxidase.

Determination of Michaelis constant

The Michaelis-Menten constants of free and immobilized carambola PPO were calculated by using Lineweaver-Burk double reciprocal models with catechol as substrate [27]. The calculated Km values for free and immobilized PPO at 40 ℃ were 20 and 32 mM at pH 7.5, respectively. The higher Km values of immobilized enzymes than those for free enzymes show an affinity change for the substrate [28].

Phenol treatment using immobilized PPO

When 5 g immobilized, PPO was mixed with 20 ml 20 mg phenol/l (as artificial phenolic wastewater) for 5 cycles at 40 ℃. The SA–PVA–AgNPs immobilized PPO removed 95% of the phenol on 5th cycle. Unlike free enzymes, the immobilized enzyme could be easily separated from the reaction solution and reused. After five repeated tests, immobilized PPO removed phenol efficiently than free PPO each over 24 h (Fig. 6).

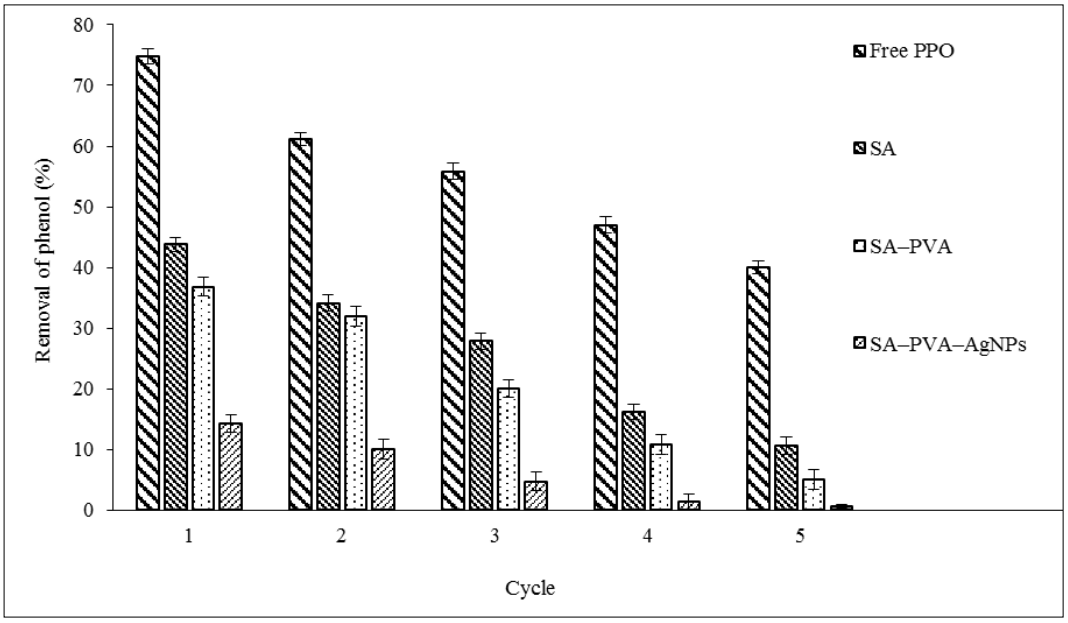

Fig 6: Removal of phenol by immobilized PPO with matrices

Discussion

It was evident from the results that, *Averrhoa carambola* leaf PPO enzyme oxidizes catechol in phosphate buffer at pH 7.5 containing PVP as a stabilizer to form respective quinones. These compounds are unstable products spontaneously polymerized to form a brown precipitate [29]. Similar results were described in mushrooms using tyrosine as a substrate [30]. Under fully denaturing conditions, we determined that the molecular mass of PPO was 61 kDa, which is monomeric, whereas a similar result of the monomeric form of protein reported for Indian pineapple fruit PPO (25 kDa) [31] and also fruit of olive (27.7 kDa) [32].

From the above immobilization of PPO, it is confirmed that immobilized PPO on SA–PVA–AgNPs removes phenol at a higher rate than SA, SA–PVA and free enzyme. Therefore, the addition of AgNPs to SA–PVA matrix significantly enhanced the degradation of phenol. The immobilization process provides a kind of membrane protection that might stabilize enzyme activity and better degradation rates in the immobilized enzyme [17]. PPO enzyme is readily available as extracts with high activity from inexpensive sources and does not require extensive purification. The enzyme act as a biocatalyst for applications involving biotransformation of phenols or bioremediation of phenol-polluted water. Thus, the application of polyphenol oxidase immobilized in different matrices like SA, SA–PVA and SA–PVA–AgNPs highly effective high flux rate membrane bioreactors was found to facilitate the highest conversion of phenols to quinones.

Acknowledgements

Authors are thankful to the DST, New Delhi, for providing financial assistance (Grant no. PURSE-Phase-2/3 (G), SR). Authors are thankful to Dr. Avila D'souza, Euchem Biologicals, Santhekatte Udupi, for providing laboratory facilities.

Conflict of Interest

The authors declare no conflict of interest in this work.

References

1. Hakulinen R, Woods S, Ferguson J, Benjamin M. The role of facultative anaerobic micro-organisms in anaerobic biodegradation of chlorophenols. Water Science and Technology. 1985; 17(1):289-301.
2. Zuzana S, Katarina D, Livia T. Biodegradation and ecotoxicity of soil contaminated by pentachlorophenol applying bioaugmentation and addition of sorbents. World Journal of Microbiology and Biotechnology. 2008; 25(2):243-252.
3. Atlow SC, Bonadonna-Aparo L, Klibanov AM. Dephenolization of industrial wastewaters catalyzed by polyphenol oxidase. Biotechnology and Bioengineering. 1984; 26(6):599-603.
4. Arica MY. Immobilization of polyphenol oxidase on carboxymethylcellulose hydrogel beads: preparation and characterization. Polymer international. 2000; 49(7):775-781.
5. Tatsumi K, Wada S, Ichikawa H. Removal of chlorophenols from wastewater by immobilized horseradish peroxidase. Biotechnology and Bioengineering. 1996; 51(1):126-130.
6. Edwards W, Bownes R, Leukes WD, Jacobs EP, Sanderson R, Rose PD *et al*. A capillary membrane bioreactor using immobilized polyphenol oxidase for the removal of phenols from industrial effluents. Enzyme and microbial technology. 1999; 24(3-4):209-217.
7. Khan AA, Akhtar S, Husain Q. Direct immobilization of polyphenol oxidases on celite 545 from ammonium sulphate fractionated proteins of potato (*Solanum tuberosum*). Journal of Molecular Catalysis B: Enzymatic. 2006; 40(1-2):58-63.
8. Yang YM, Wang JW, Tan RX. Immobilization of glucose oxidase on chitosan–SiO_2 gel. Enzyme and Microbial Technology. 2004; 34(2):126-131.
9. Huang H, Zhu Q, Zhang Z, Yang B, Duan X, Jiang Y. Effect of oxalic acid on antibrowning of banana (*Musa* spp., AAA group, cv.'Brazil') fruit during storage. Scientia Horticulturae. 2013; 160:208-212.

10. Bevilaqua JV, Cammarota MC, Freire DMG, Anna Jr S. Phenol removal through combined biological and enzymatic treatments. Brazilian Journal of Chemical Engineering. 2002; 19(2):151-158.
11. Edalli V, Kamanavalli C. Removal of phenolic compounds by mushroom polyphenol oxidase from *Pleurotus* species. The Bioscan. 2010; 4:89-92.
12. Flurkey A, Cooksey J, Reddy A, Spoonmore K, Rescigno A, Inlow J, Flurkey WH. Enzyme, protein, carbohydrate, and phenolic contaminants in commercial tyrosinase preparations: potential problems affecting tyrosinase activity and inhibition studies. Journal of agricultural and food chemistry. 2008; 56(12):4760-4768.
13. El-Shora HM, Metwally M. Use of tyrosinase enzyme from *Bacillus thuringiensis* for the decontamination of water polluted with phenols. Biotechnology. 2008; 7(2):305-10.
14. Hou MF, Tang XY, Zhang WD, Liao L, Wan HF. Degradation of pentachlorophenol by potato polyphenol oxidase. Journal of agricultural and food chemistry. 2011; 59(21):11456-11460.
15. Davis BJ. Disc electrophoresis- II method and application to human serum proteins. Annals of the New York Academy of Sciences. 1964; 121(2):404-427.
16. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227(5259):680-685.
17. Tallur PN, Mulla SI, Megadi VB, Talwar MP, Ninnekar HZ. Biodegradation of cypermethrin by immobilized cells of *Micrococcus* sp. strain CPN 1. Brazilian Journal of Microbiology. 2015; 46(3):667-672.
18. Pang Y, Zeng GM, Tang L, Zhang Y, Liu YY, Lei XX *et al*. Cr (VI) reduction by *Pseudomonas aeruginosa* immobilized in a polyvinyl alcohol/sodium alginate matrix containing multi-walled carbon nanotubes. Bioresource Technology. 2011; 102(22):10733-10736.
19. Mbhele ZH, Salemane MG, Van Sittert CGCE, Nedeljkovic JM, Djokovic V, Luyt AS. Fabrication and characterization of silver- polyvinyl alcohol nanocomposites. Chemistry of Materials. 2003; 15(26):5019-5024.
20. Shameli K, Ahmad MB, Yunus WMZW, Ibrahim NA, Jokar M, Darroudi M. Synthesis and characterization of silver/polylactide nanocomposites. World Academy of Science Engineering and Technology. 2010; 64:28-32.
21. Aydemir T. Partial purification and characterization of polyphenol oxidase from artichoke (*Cynara scolymus* L.) heads. Food chemistry. 2004; 87(1):59-67.
22. Gulcin I, Yildirim A. Purification and characterization of peroxidase from *Brassica oleracea* var. Acephala. Asian Journal of Chemistry. 2005; 17(4):2175-2183.
23. Koksal E, Gulcin I. Purification and characterization of peroxidase from cauliflower (*Brassica Oleracea L*. var. botrytis) buds. Protein and Peptide Letters. 2008; 15(4):320-326.
24. Edwards W, Bownes R, Leukes WD, Jacobs EP, Sanderson R, Rose PD *et al*. A capillary membrane bioreactor using immobilized polyphenol oxidase for the removal of phenols from industrial effluents. Enzyme and Microbial Technology. 1999; 24:209-217.
25. Arica MY, Senel S, Alaeddinoglu NG, Patir S, Denizli A. Invertase immobilized on spacer-arm attached poly (hydroxyethyl methacrylate) membrane: preparation and properties. Journal of Applied Polymer Science. 2000; 75(14):1685-1692.
26. Goncalves AM, Schacht E, Matthijs G, Barros MA, Cabral JMS, Gil MH *et al*. Stability studies of a recombinant cutinase immobilized to dextran and derivatized silica supports. Enzyme and microbial technology. 1999; 24(1-2):60-66.
27. Lineweaver H, Burk D. The determination of enzyme dissociation constants. Journal of the American Chemical Society. 1934; 56(3):658-666.
28. Shao J, Huang LL, Yang YM. Immobilization of polyphenol oxidase on alginate-SiO_2 hybrid gel: stability and preliminary applications in the removal of aqueous phenol. Journal of Chemical Technology and Biotechnology. 2009; 84(4):633-635.
29. Saiedian S, Keyhani E, Keyhani J. Polyphenol oxidase activity in dormant saffron (*Crocus sativus* L.) corm. Acta Physiologiae Plantarum. 2007; 29(5):463-471.
30. Espin JC, Varon R, Fenoll LG, Gilabert MA, Garcia-Ruiz PA, Tudela J *et al*. Kinetic characterization of the substrate specificity and mechanism of mushroom tyrosinase. European Journal of Biochemistry. 2000; 267(5):1270-1279.
31. Das JR, Bhat SG, Gowda LR. Purification and characterization of a polyphenol oxidase from the kew cultivar of Indian pineapple fruit. The Journal of Agricultural and Food Chemistry. 1997; 45:2031-2035.
32. Ortega-Garcia F, Blanco S, Peinado MA, Peragon J. Polyphenol oxidase and its relationship with oleuropein concentration in fruits and leaves of olive (*Olea europaea*) cv. 'Picual'trees during fruit ripening. Tree physiology. 2008; 28(1):45-54.