Available online at www.joac.info

ISSN: 2278-1862

Journal of Applicable Chemistry

2013, 2 (5):1123-1131

(International Peer Reviewed Journal)

Study of peroxidase obtained from *Daucus carota* (carrot) juice extract

Pankaj Kumar Chaurasia, Sunil Kumar Singh, Shashi Lata Bharati *

*Department of Chemistry, D.D.U. Gorakhpur University, Gorakhpur- 273009, Uttar Pradesh, **INDIA**

Email: shashilatachem@gmail.com

Received on 26th August and finalized on 31st August 2013.

ABSTRACT

During the last decades, peroxidases have emerged as good biocatalysts for the variety of organic biotransformation reactions like oxygenation of racemic hydroperoxide, hydroxylation of arenes, the oxyfunctionalization of phenols and aromatic amines, the epoxidation, halogenation of olefins and the oxygenation of hetero atoms. Thus, the main objective of this communication is to obtain a rich source of peroxidase and study the various properties. The enzyme was isolated by cutting the Daucus carota into the small pieces and squeezing its juice and studied its kinetic, thermal, pH and inhibition properties of peroxidase by substrates like H_2O_2 and guaiacol.The studies made have shown that Daucus carota (carrot) juice contains peroxidase activity of the order of 200 IU mL^{-1}. The K_m values of this peroxidase for the substrates guaiacol and hydrogen peroxide were 1300 and 50 μM, respectively. The pH and temperature optima were 6.5 and 50 $^{\circ}C$, respectively. pH stability of this peroxidase was pH 6.0 and it is most thermally stable at 30 $^{\circ}C$ when exposed for one hour and also retains its maximum activity at this temperature. The activation energy for thermal denaturation of the enzyme was 32 kJ mol^{-1} K^{-1}. Activity of this peroxidase is inhibited completely by sodium azide at the concentration of 30 mM. Kinetic study reveals that this peroxidise can be utilized for different synthetic and biotransformation reactions. Due to its low K_m value for hydrogen peroxide, this peroxidase may be very fruitful in this area by using hydrogen peroxide as a substrate.

Keywords: Peroxidase, *Dacus carota*, H_2O_2, guaiacol, sodium azide.

INTRODUCTION

Peroxidase (E.C. 1.11.1.7) is a heme-containing enzyme, which catalyses the oxidation of a wide variety of organic and inorganic substrates using hydrogen peroxide as the electron acceptor [1, 2]. Peroxidases are widely distributed in living organisms including microorganisms, plants, and animals [3]. It is mainly located in the cell wall [4]. It is one of the key enzymes controlling plant growth and development. Peroxidases also involved in various cellular processes including construction, rigidification and eventual lignifications of cell walls [5], suberization [6], organogenesis [7], phenol oxidation [8], crosslinking of cell wall proteins [9], and protection of tissue from damage and infection by pathogenic microorganisms [10–12]. It is also used in clinical diagnosis and microanalytical immunoassays because of its high sensitivity. Apart from these applications, peroxidases have been used for biotransformations in organic synthesis [13]. There are different types of peroxidases and recent studies have revealed that not all

peroxidases are similar in their structures and functions [14–21]. Lignin peroxidase differs from horseradish peroxidase in the sense that lignin peroxidase directly oxidizes veratryl alcohol whereas horseradish peroxidase cannot [17]. Soyabean peroxidase [17] has lignin peroxidase type activity, but it is more stable at acidic pH and at higher temperatures than the lignin peroxidase. In enantioselective reduction of hydroperoxides [23], hydroxylation of arenes [24], epoxidation of olefins [20], halogenation [25], N-oxidation [26], and sulfoxidation [27], peroxidases play promising roles.

The main purpose of this communication is to find a convenient rich source of peroxidase and studies the different enzymatic properties as kinetic studies, temperature and pH optima studies and inhibition studies of peroxidase. In this order, authors started work on the *D. carota*. The present communication also demonstrates that the juice of *D. carota* is a good source of peroxidase enzyme and this source can be used for the purpose of different synthetic and biological applications.

MATERIALS AND METHODS

Chemicals: Guaiacol was from Sigma Chemical Company, St. Louis USA. All other chemicals used in these investigations were either from Himedia Laboratory Ltd, Mumbai or from E. Merck (India) Ltd., Mumbai, and were used without further purifications.

Isolation of the peroxidase: The enzyme was isolated by cutting the *D. carota* into the small pieces, crushing the pieces in mortar with pestle, and extracting the juice by keeping the pieces in four layers of cheese cloth and squeezing it. The juice was centrifuged using Sigma (Germany) model 3 K-30 refrigerated centrifuge at 14000 rpm for 30 min at 4 $^{\circ}$C to remove the cloudiness of the juice. The clear juice was stored at 4 $^{\circ}$C. The enzyme stored in this way has reasonable activity even after 2 months.

Peroxidase activity assay: Peroxidase activity of the enzyme was measured in 1mL reaction solution containing 50 mM sodium phosphate buffer pH 7.0 at 30°C using 5 mM guaiacol, 0.6mM hydrogen peroxide as the substrates and by monitoring the absorbance changes at λ = 470 nm using molar extinction coefficient value of 2.66×10^4 M^{-1} cm^{-1} for the product tetraguaiacol formed by the enzymatic reaction [28, 29]. The reaction solution was allowed for thermal equilibration for 10 minutes, 20 μL of the enzyme stock 200 IU mL^{-1} was added, and activity measurement was started immediately and was continued for 120 seconds. All spectrophotometric measurements were done with UV/Vis spectrophotometer Hitachi (Japan) model U-2000 which was fitted with electronic temperature control unit for variation of temperature in the cuvettes. The least count of the absorbance measurement was 0.001, and one enzyme unit is the amount of enzyme which produces 1 μmol/min of the product.

Steady state enzyme kinetics: The steady state kinetics of the enzyme was studied for the peroxidase obtained from the juice of *D. carota* using guaiacol and H_2O_2 as the variable substrates and monitoring the steady state formation of tetraguaiacol as mentioned in the assay section. We have studied the kinetic properties by varying the concentration of guaiacol (keeping the concentration of H_2O_2 constant at enzyme saturating value 0.6 mM) and again by varying the concentration of H_2O_2 (keeping the concentration of guaiacol constant at the enzyme saturating value 5 mM.). The K_m values were calculated in both cases (guaiacol and H_2O_2) using linear regression analysis of the data points of double-reciprocal plots. Each point of steady state velocity was an average of triplicate measurements, and the percentage standard deviation was less than 5%.

pH optimum and pH stability: The pH optimum was determined by measuring the relative activity of the enzyme in the pH range 3.0–9.0 using buffer prepared with $H_3PO_4/NaH_2PO_4/Na_2HPO_4$.The composition of the reaction solution was the same as mentioned in the peroxidase activity assay. pH optimum for the peroxidase present in the juice of *D. carota* was 6.5 that means it is most active at pH 6.5. pH stability of the enzyme was tested by incubating an enzyme aliquot at a particular pH for 60 minutes and started to measure the activity for every 10 minutes intervals. pH stability was measured for different pHs ranges from 3.0-8.0 and activity were plotted against the different time intervals. Before each measurement, the reaction mixture in the spectrophotometer cuvette was allowed for 10 min for

temperature equilibration, the reaction was initiated by the addition of 20 μL, of concentrated enzyme stock having 200 IU/mL and the activity was measured immediately.

Temperature optimum and thermal stability: The temperature optimum was determined by measuring the relative activity of the peroxidase in the temperature range 10–70 °C at fixed pH 7.0 using the reaction solution of the composition mentioned in peroxidase activity assay. Temperature optimum was 50 °C for the peroxidase present in the juice of *D. carota.* Thermal stability for the peroxidase obtained from the juice of *D. carota* has been studied for the temperature range 20-60 °C. At each temperature, activity was taken for every 10 minute intervals started from 0-60 minutes and then activity was plotted against time intervals for each temperature. Before each measurement, the reaction mixture in the spectrophotometer cuvette was allowed for 10 min for temperature equilibration, the reaction was initiated by the addition of 20 μL, of concentrated enzyme stock having 200 IU/mL and the activity was measured immediately.

Inhibition Study: The effect of sodium azide on the activity of the peroxidase was also studied for the different concentrations of sodium azide ranges from 0-30 mM and activity of peroxidase obtained from juice of *D. carota* was completely diminished at 30 mM.

RESULTS AND DISCUSSION

The increase of absorbance at λ = 470 nm, ΔA_{470}, due to the conversion of guaiacol to tetraguaiacol with time in a peroxidase assay solution extracted from *D. carota* juice, is given by the equation $\Delta A_{470} = 2.4 \times 10^{-3}\ t + 11.8 \times 10^{-3}$. There is no increase in absorbance at λ = 470 nm in assay solutions containing no enzyme or the denatured enzyme which was obtained by one hour boiling in water. These results indicated that *D. carota* juice contained a good peroxidase activity. The analysis of the steady state kinetic measurements showed the presence of 200 IU mL^{-1} of peroxidase in the juice. Thus, *D. carota* fruit juice is a rich and convenient source of peroxidase enzyme and it can be used for various biotransformation reactions.The Michaelis-Menten and double reciprocal plots for the peroxidase obtained from the juice of *D. carota* using guaiacol as the variable substrate at the saturating concentration of the other substrate, H_2O_2 (0.6mM) are shown in figures 1(a) and 1(b**),** respectively.

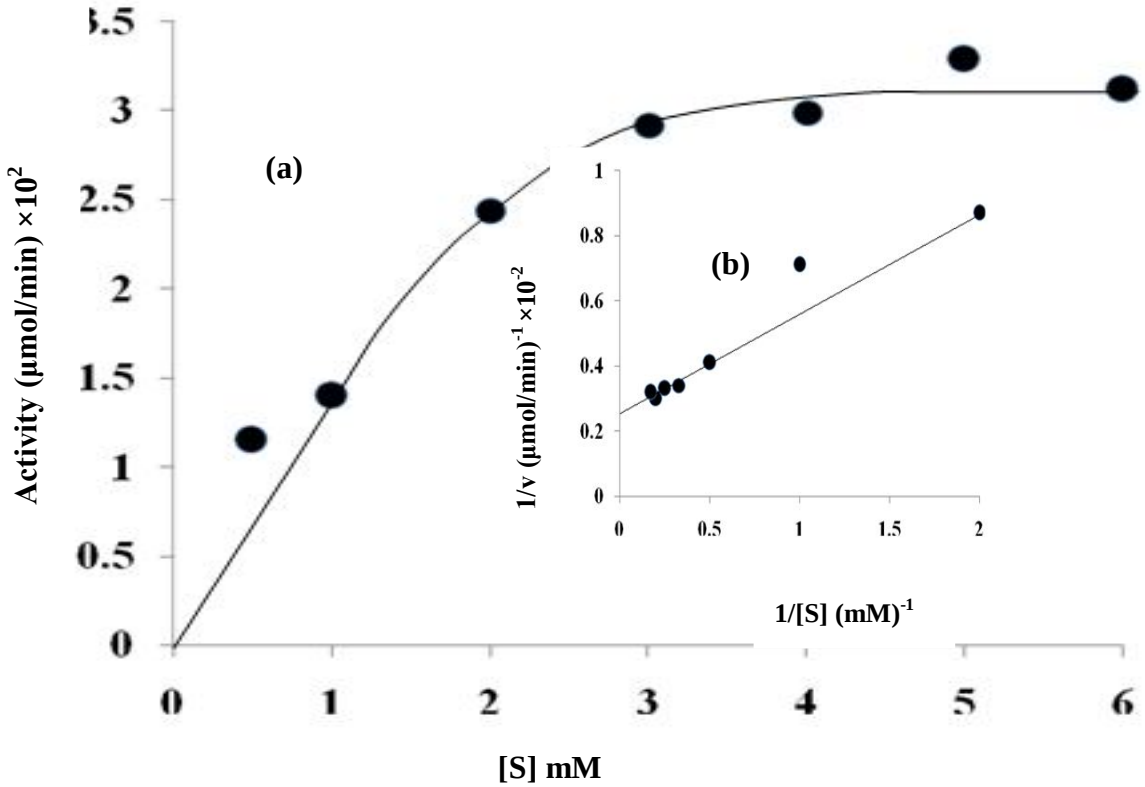

Figure 1 (a) Michaelis-Menten and (b) double-reciprocal plots for the peroxidase obtained from the *D. carota* (carrot) juice extract using guaiacol as the variable substrate.

The Michaelis-Menten and double reciprocal plots using hydrogen peroxide as the variable substrate at the fixed enzyme saturating concentration of guaiacol (5mM) are shown in figures 2(a) and 2(b), respectively.

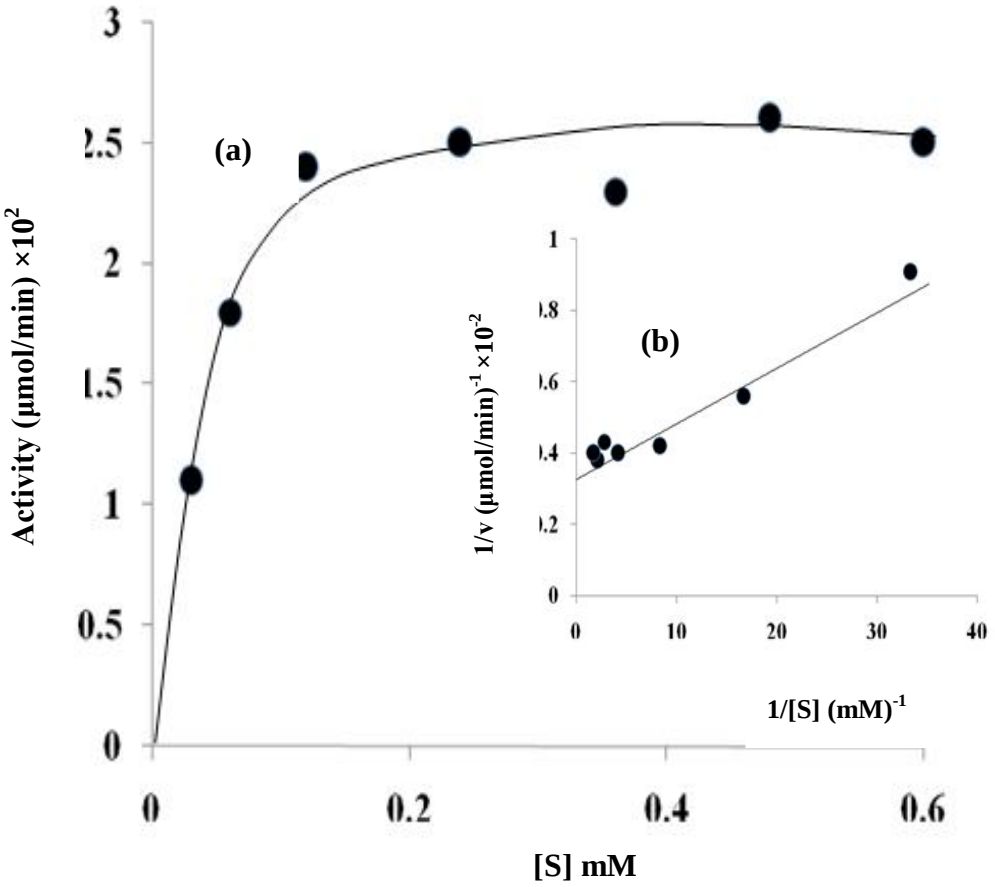

Figure 2(a) Michaelis-Menten and (b) double-reciprocal plots for the peroxidase obtained from the *D. carota* (carrot) juice extract using hydrogen peroxide as the variable substrate.

The calculated K_m values for guaiacol and hydrogen peroxide of peroxidase obtained from the juice of *D. carota* were 1300 μM and 50 μM, respectively. The corresponding values of K_m for *horseradish peroxidase* [30], *Turkish black radish* [31] and *Solanum melongena* fruit juice [29] were 800 μM and 100 μM, 36.0 μM, and 8.4 μM, and 6500 μM and 330 μM, respectively. The reported enzyme has lower affinity for both the substrates than the peroxidases of *Turkish black radish* (*Raphanus sativus*) while has lower affinity for guaiacol and higher affinity for hydrogen peroxide than *horseradish peroxidase* [30]. Peroxidase obtained from the juice of *D. carota* has higher affinity for both the substrates as compared to the peroxidases of *S. melongena* fruit juice. Since peroxidases are known to follow double displacement type kinetics [32], *D. carota* juice peroxidase was also analysed for this type of kinetics by measuring the steady state velocity of the enzyme-catalysed reaction at three different fixed concentrations of the hydrogen peroxide and varying the concentration of guaiacol at each hydrogen peroxide concentration and also at three different fixed concentrations of guaiacol and varying the concentration of hydrogen peroxide. Double reciprocal plots (not shown here) in both cases have been found to be parallel straight lines confirming that the reported peroxidase also follows double displacement type mechanism observed in case of other peroxidases [32]. The results of the variation of the activity of the peroxidase enzyme obtained from the juice of *D. carota*, with the variation of pH of the reaction solution are shown in figure 3**.**

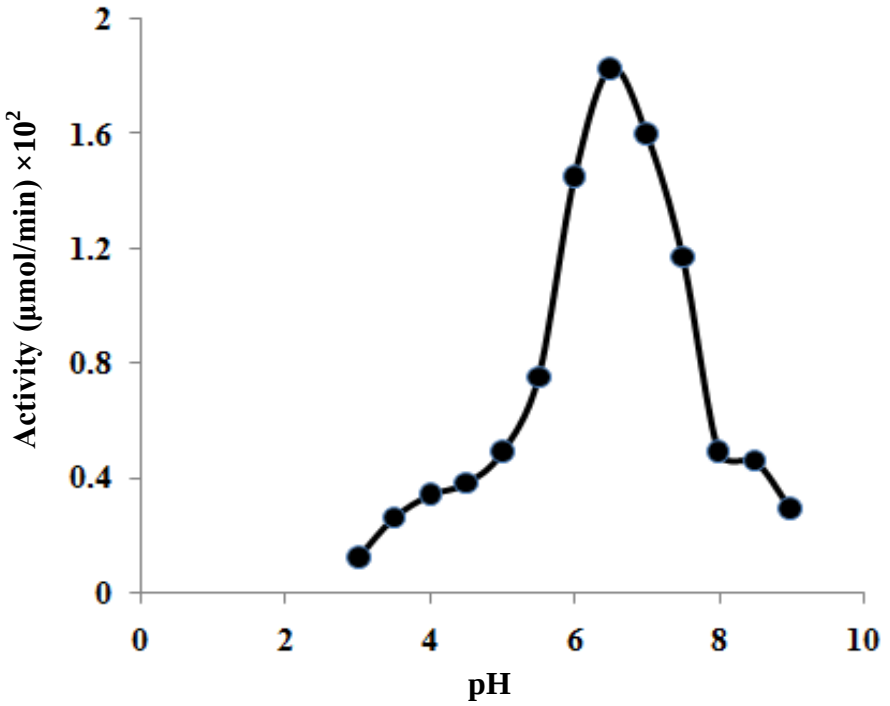

Figure 3 Dependence of the peroxidase activity on pH of the assay solution at fixed

The determined pH optimum of the enzyme was 6.5. Plant peroxidases of *S. melongena* fruit juice [29], *Musa paradisiaca* stem juice [33] and peroxidase of fruit juice of *L. aegyptiaca* [34] have been studied in our laboratory. The pH optima of the peroxidases from these sources have been found to be 5.5, 4.5 and 6.5 pHs, respectively. Thus the reported peroxidase can be used effectively near neutral pH. The first two peroxidases of three, have pH optima in more acidic regions while third one has same pH optimum as peroxidase obtained from the juice of *D. carota.*The results of the studies on the pH stability of the peroxidase are shown in figure 4 where the activities of the enzyme have been plotted against the different time intervals for each pH for which the enzyme has been exposed for 1 hr. It follows that the peroxidase obtained from the juice of *D. carota* retained its maximum activity at pH 6.0.

The results of variation of the activity of the peroxidase enzyme obtained from the juice of *D. carota*, as a result of variation of the temperature of the reaction solution of the enzyme catalysed reaction are shown in figure 5**.** The calculated temperature optimum was 50 °C. The temperature optima of the peroxidases from *S. melongena* fruit juice [29], *M. paradisiacal* stem juice [33] and fruit juice of *L. aegyptiaca* [34], peroxidases reported from our laboratory, were 84 °C, 62.5 °C and 60 °C respectively. In this way we see that the peroxidase from *D. carota* juice has the temperature optimum on the higher temperature side as *S. melongena* fruit juice, *M. paradisiacal* stem juice and *L. aegyptiaca* juice. The result of the studies on the thermal stability of the peroxidise is shown in figure 6 where the activities of enzyme have been plotted against the time intervals for which the enzyme has been exposed for 1 h. It follows that the enzyme has maximum stability at temperature 30 °C. This is the main advantage of this enzyme source because at room temperature peroxidase enzyme obtained from the *D. carota* loose its activity to a very small extent. Thus, this peroxidase can be used for the different experimental and synthetic work at room temperature. Activation energy of the thermal denaturation of the peroxidase calculated from Arrhenius plot has been found to be 32 kJ mol^{-1} K^{-1}.

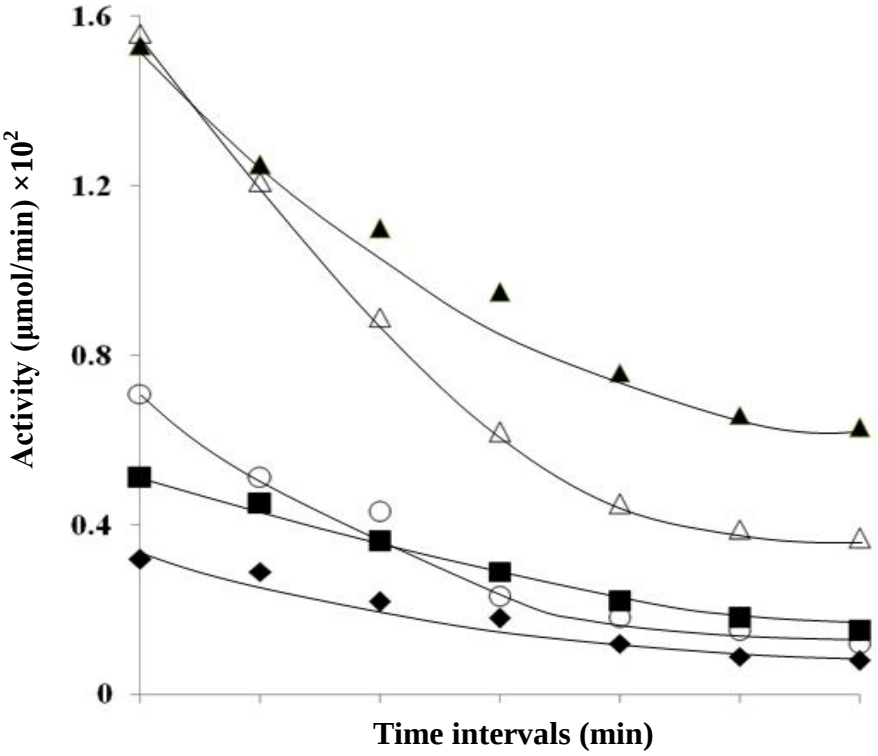

Figure 4 pH stability curves for the peroxidase at different pH ranges from pH 3.0-8.0 at fixed temperature 30°C. pH 4.0 (♦), 5.0 (■), 6.0 (▲), 7.0 (Δ), 8.0 (○)

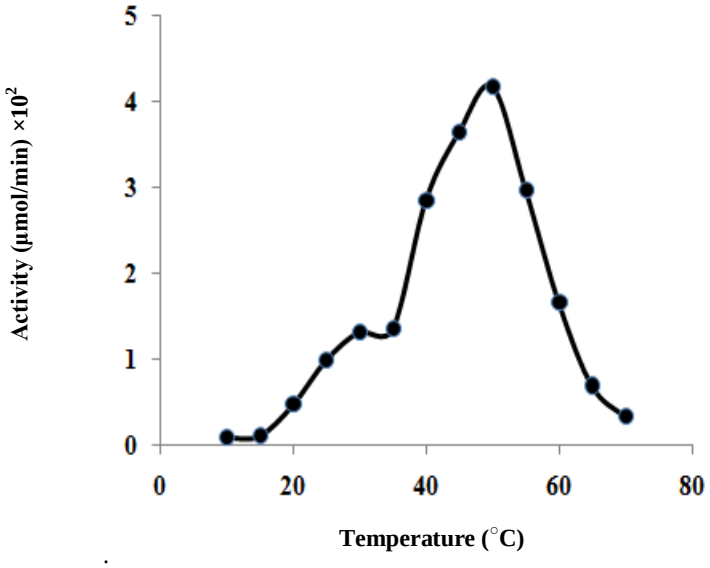

Figure 5: Dependence of the peroxidase activity on temperature of the assay solution at fixed pH 7.0.

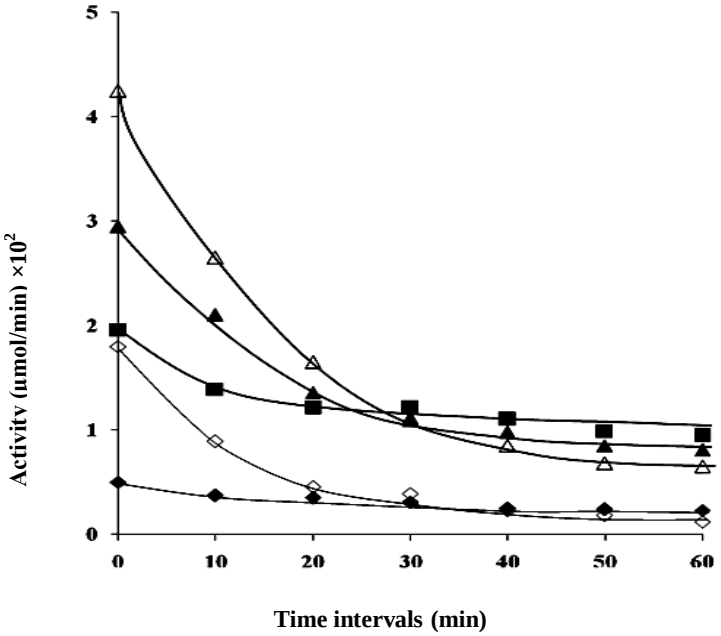

Figure 6: Thermal stability curves for the peroxidase at different temperatures ranges from 30-60°C at fixed pH 7.0. 20°C (♦), 30°C (■), 40°C (▲), **50°C (Δ),** 60°C (◊)

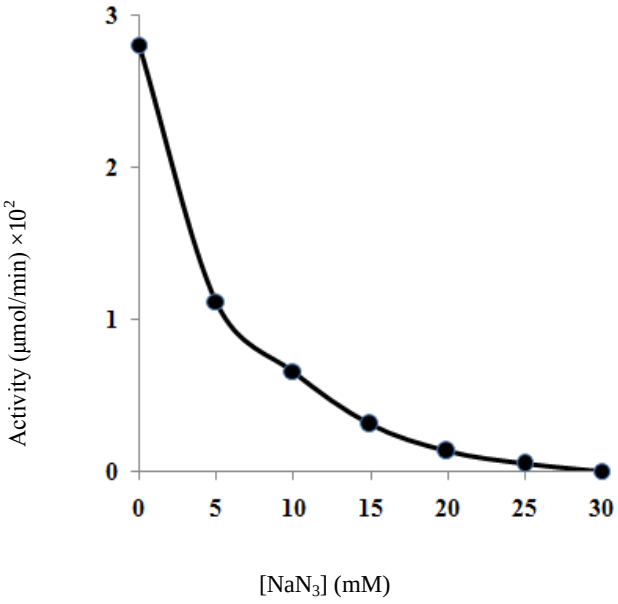

Figure 7: Inhibition of the activity of the peroxidase by

Studies on inhibition of the peroxidase of *D. carota* have also been performed for various concentrations of sodium azide strated from 0.0 mM to 30 mM. Activity of this peroxidase has been found to be completely diminished at 30 mM. It is shown in figure 7. Peroxidases are the active biocatalysts which catalyses the several types of organic conversions like epoxidation, halogenation of oleifins, oxygenation of heteroatoms, several enantioselective organic reactions and oxygenation of racemic hydroperoxide, etc.

They have also a significant role in removal of recalcitrant organic pollutants, polymerisation, decolourization etc. Due to the significant scope of peroxidases, the peroxidase enzyme obtained from the *D. carota* may be very fruitful because peroxidase obtained from this source is very active and does not lose activity at room temperature for several hours. The present communication demonstrates clearly the kinetic, thermal and pH properties. Its K_m values for different substrates explain the fact that it can be utilised as potential biocatalyst for various biotransformations and organic synthetic reactions.

APPLICATIONS

The studies made have shown that Daucus carota (carrot) juice contains peroxidase activity of the order of 200 IU mL^{-1}. The K_m values of this peroxidase for the substrates guaiacol and hydrogen peroxide were 1300 and 50 μM, respectively.

ACKNOWLEDGMENTS

Pankaj Kumar Chaurasia acknowledges the financial support to CSIR-HRDG, New Delhi, in the form of CSIR-SRF (NET), award no. 09/057(0201)/2010/EMR-I, under which this research work has been done. S.K. Singh is also thankful to CSIR New Delhi for the award of RA, award no. 09/057(0206)-2012/EMR-I.

REFERENCES

[1] L. Banci, *J. Biotechnol.*, **1997,** 53(2-3), 253–263.
[2] A.Yemenicioglu, M. O¨zkan, B. Cemeroglu; *J. Agricult. Food Chem.*, **1998,** 46(10), 4158–4163.
[3] J.N. Rodriguez-Lopez, A.T. Smith, R.N.F. Thorneley, *J. Biol. Inorg. Chem.*, **1996,** 1(2), 136–142.
[4] E.L. Chen, Y.A. Chen, L.M. Chen, Z.H. Liu, *Plant Physiol. Biochem.*, **2002**, 40(5), 439–444.
[5] M. Quiroga, C. Guerrero, M.A. Botella et al., *Plant Physiol.*, **2000,** 122(4), 1119–1127.
[6] M.A. Bernards, W.D. Fleming, D.B. Llewellyn et al., *Plant Physiol.*, **1999,** 121(1), 135–145.
[7] D.J. Lee, S.S. Kim, S.S. Kim, *Plant Sci.*, **2002**, 162(3), 345–353.
[8] L.M. Lagrimini, *Plant Physiol.*, **1991**, 96(2), 577–583.
[9] L.S. Schnabelrauch, M. Kieliszewski, B.L. Upham, H. Alizedeh, D.T.A. Lamport, *Plant Journal*, **1996**, 9(4), 477–489.
[10] I.Y. Sakharov, J.L. Castillo, J.C. Areza, I.Y. Galaev, *Bioseparation*, **2000**, 9(3), 125–132
[11] I.G¨ulc¸in, A.Yildirim, *Asian J. Chem.*, **2005**, 17(4), 2175–2183.
[12] I.G.S¸at, *Afr. J. Biotechnol.*, **2008**, **7**(13), 2248–2253.
[13] W. Adam, M. Lazarus, C.R. Saha-M¨oller et al., *Adv. Biochem. Engin. Biotechnol.*, **1999,** 63, 73–108.
[14] I.G. Gazarian, L.M. Lagrimini, S.J. George, R.N.F. Thorneley, *Biochem. J.*, **1996**, 320(2), 369–372.
[15] M. Kvaratskhelia, C. Winkel, R.N.F. Thorneley, *Plant Physiol.*, **1997,** 114(4), 1237–1245.
[16] I.G. Gazaryan, L.M. Lagrimini, *Phytochem.*, **1996**, 41(4), 1029–1034.
[17] J.P. McEldoon, A.R. Pokora, J.S. Dordick, *Enz. Microb. Technol.*, **1995**, 17(4), 359–365.
[18] B.C. Finzel, T.L. Poulos, J. Kraut; *J. Biol. Chem.*, **1984,** 259(21), 13027–13036.
[19] T.L. Poulos, S.L. Edwards, H. Wariishi, M.H. Gold, *J. Biol. Chem.*, **1993,** 268(6), 4429–4440.
[20] M. Sundaramoorthy, K. Kishi, M.H. Gold, T.L. Poulos, *J. Biol. Chem.*, **1994**, 269(52), 32759–32767.
[21] W.R. Patterson, T.L. Poulos, *Biochem.*, **1995,** 34(13), 4331–4341.
[22] R. Noyori, *Asymmetric Catalysis in Organic Synthesis*, Willy Inter Science, New York, NY, USA, 1994.

[23] R. Akasaka, T. Mashino, M. Hirobe, *Bioorg. Med. Chem. Lett.*, **1995**, 5, 1861–1864.
[24] E.N. Jacobsen, **1993**, "Asymmetric catalytic epoxidation of unfunctionalized olefins," in *Catalytic Asymmetric Synthesis*, I. Ojima, Ed., p. 159, VCH,Weinheim, Germany.
[25] M. Hofrichter, R. Ullrich, *Appl. Microbiol. Biotechnol.*, **2006**, 71(3), 276–288.
[26] P. Yadav, J.K. Sharma, V.K. Singh, K.D.S. Yadav, *Biocat. Biotrans.*, 28(3), 222–226 **(2010)**.
[27] S.Colonna, N.Gaggereo, G.Carrea, P.Pasta, *Tetrahedron Lett.*, 35(48), 9103– 9104 **(1994)**.
[28] J.R.Whitaker; *Principles of Enzymology for Food Sciences*, Marcel Dekker, New York, NY, USA **(1972)**.
[29] S.K.Vernwal, R.S.S.Yadav, K.D.S. Yadav; *Indian J. Exp. Biol.*, **2000**, 38(10), 1036–1040.
[30] J.N. Rodriguez-Lopez, A.T. Smith, R.N.F. Thorneley, **1996**, *J. Biol. Chem.*, 271(8), 4023–4030.
[31] M. Sisecioglu, I. G¨ulcin, M. Cankaya et al., *J. Med. Plants Res.*, **2010**, 4, 1187–1196.
[32] M. Tien, T.K. Kirk; *Proceedings of the National Academy of Sciences of the United States of America*, **1984**, 81(8), 2280–2284.
[33] S.K. Vernwal, R.S.S. Yadav, K.D.S. Yadav, *Indian J. Exp. Biol.*, **2000**, 38(10), 1036–1040.
[34] R.S.S. Yadav, K.S. Yadav, H.S. Yadav, **2011**, *Enz. Res.* ID 319105 doi:10.4061/2011/319105.