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A Potential Peroxidase Obtained from the Juice of *Beta vulgaris* (Beet)

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Abstract In this communication, authors have reported the peroxidase enzyme in the juice of *Beta vulgaris* (beet) and studied its different enzymatic properties. The steady state enzyme kinetics of this peroxidase was studied using two substrates i.e. guaiacol and hydrogen peroxide. The K_m values of this peroxidase for the substrates guaiacol and hydrogen peroxide were 900 and 30 μM, respectively. The pH and temperature optima were 6.0 and 60°C, respectively. The enzyme has maximum stability at pH 6.0. The enzyme was most stable at 45 °C and the activation energy for thermal denaturation of the enzyme was 38.6 kJ/mole/K. Activity of this peroxidase is inhibited completely by sodium azide at the concentration of 16 mM.

Keywords: *Beta vulgaris, peroxidase, H_2O_2, guaiacol, sodium azide*

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1. Introduction

Peroxidase [E.C. 1.11.1.7] is a heme-containing enzyme, which catalyses the oxidation of different organic and inorganic substrates using hydrogen peroxide as the electron acceptor [1,2]. Peroxidases are widely distributed in living organisms such as 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 [10,11,12]. 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]. In enantioselective reduction of hydroperoxides [14], hydroxylation of arenes [15], halogenation [16], N-oxidation [17], and sulfoxidation [18] and epoxidation of olefins [19], peroxidases play promising roles.

In this manuscript, authors have identified the juice of *B. vulgaris* (beet) as a good source of peroxidase enzyme and studied the different enzymatic properties as steady state enzyme kinetics, temperature optimum, thermal stability, pH optimum, pH stability, activation energy for thermal denaturation of enzyme and inhibition of peroxidase. Since peroxidase enzyme is very active biocatalyst in several types of organic syntheses and this source of peroxidase is very easily available, thus, authors have decided to do the several possible organic biotransformations by this enzyme obtained from this source in future. In order to know the efficiency of the enzyme K_m has been determined for two different substrates along with its different properties.

2. Materials and Methods

2.1. 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.

2.2. Isolation of the peroxidase

The enzyme was isolated by cutting the *Beta vulgaris* (beet) 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 3K-30 refrigerated centrifuge at 14000 rpm for 30 min at 4°C to remove the cloudiness of the juice. The clear juice was stored at 4°C. The enzyme stored in this way has reasonable activity even after 2-3 months.

2.3. Peroxidase Activity Assay

Peroxidase activity of the enzyme was measured in 1 mL reaction solution containing 50 mM sodium phosphate buffer pH 7.0 at 30°C using 5 mM guaiacol, 0.6 mM 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 during the enzymatic reaction [20,21]. The reaction solution was allowed for thermal equilibration for 10 minutes, 20 μL of the enzyme was added and activity measurement was started immediately and was continued for 180 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.

2.4. Steady State Enzyme Kinetics

The steady state kinetics of the enzyme was studied for the peroxidase obtained from the juice of *B. vulgaris* 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%.

2.5. 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 *B. vulgaris* was 6.0 that means it is most active at pH 6.0.

pH stability of the enzyme was tested by incubating an enzyme aliquot at a particular pH for every 10 minutes time intervals upto one hour, assaying its activity and plotting the activity against time intervals for that particular pH.

2.6. Temperature Optimum and Temperature Stability

The temperature optimum was determined by measuring the relative activity of the peroxidase in the temperature range 10-80°C at fixed pH 7.0 using the reaction solution of the composition mentioned in peroxidase activity assay. Temperature optimum was 60°C.

For thermal denaturation of the enzyme, rate constant at different temperatures were determined by keeping the enzyme aliquots at different fixed temperatures and assaying the enzyme activity at regular time intervals and plotting activity against time intervals. Rate constants were calculated from $t_{1/2}$ values using equation $k = 0.693/t_{1/2}$. Energy of activation for thermal denaturation was calculated by Arrhenius plot.

2.7. 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 0-16 mM and activity of peroxidase obtained from juice of *B. vulgaris* was completely diminished at 16 mM.

3. Results and Discussion

The rapid increase of absorbance at λ = 470 nm, ΔA_{470} is due to the conversion of guaiacol to tetraguaiacol with time in a peroxidase assay solution extracted from *B. vulgaris*. 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 *B. vulgaris* juice contained a good peroxidase activity.

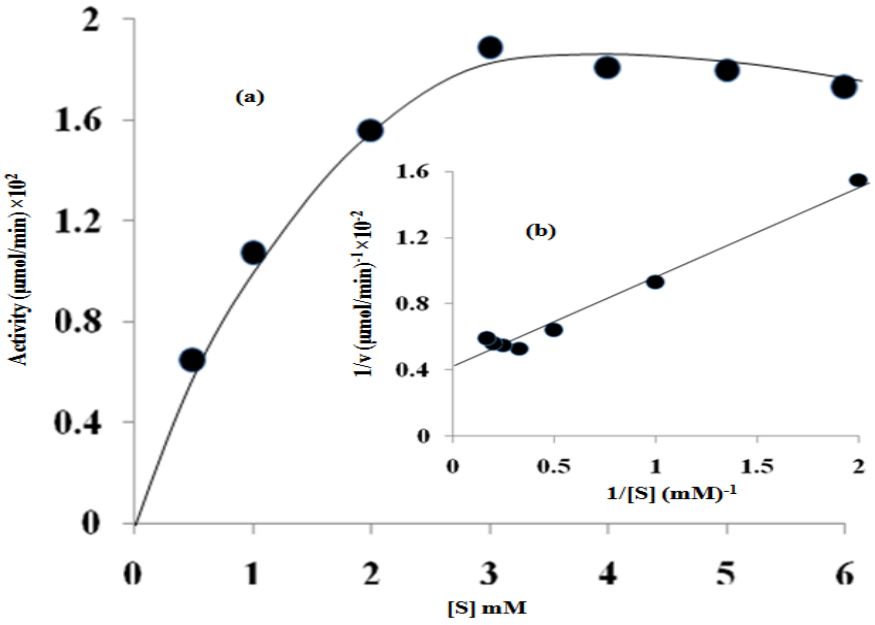

Figure 1. (a) Michaelis-Menten and (b) double-reciprocal plots for the peroxidase obtained from the *B. vulgaris* (beet) juice using guaiacol as the variable substrate

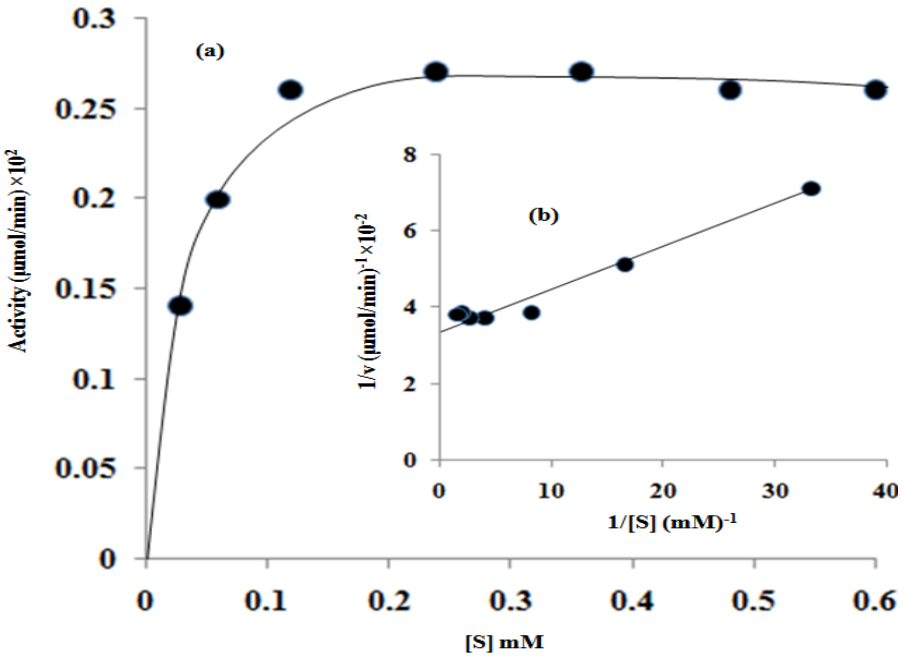

Figure 2. (a) Michaelis-Menten and (b) double-reciprocal plots for the peroxidase obtained from the juice of *B. vulgaris* (beet) using hydrogen peroxide as the variable substrate

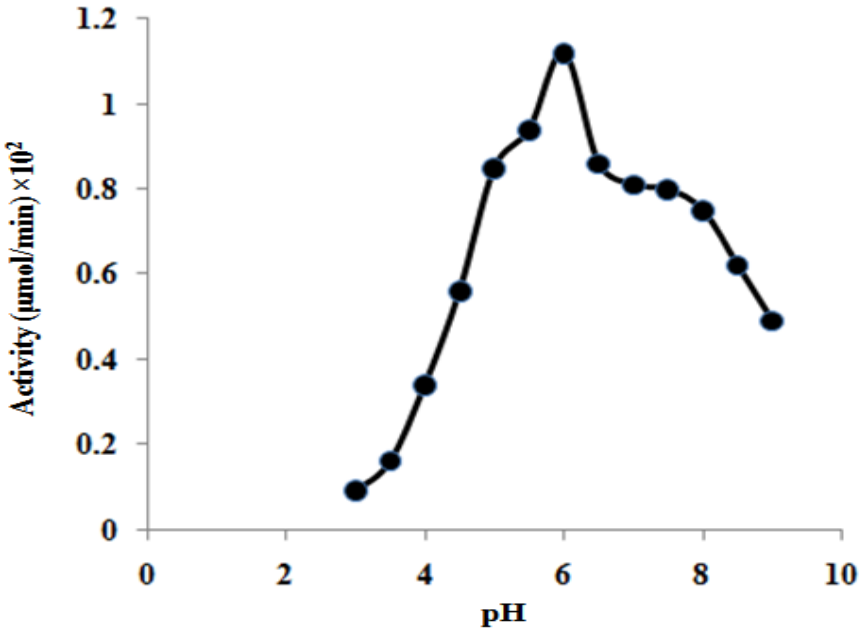

Figure 3. Dependence of the peroxidase activity on pH of the assay solution at fixed temperature 30°C

The Michaelis-Menten and double reciprocal plots for the peroxidase using guaiacol as the variable substrate at the saturating concentration of the other substrate, H_2O_2 (0.6 mM) are shown in Figure 1(a) and Figure 1(b), respectively. The Michaelis-Menten and double reciprocal plots using hydrogen peroxide as the variable substrate at the fixed enzyme saturating concentration of guaiacol (5 mM) are shown in Figure 2(a) and Figure 2(b), respectively. The calculated K_m values for guaiacol and hydrogen peroxide of peroxidase were 900 μM and 30 μM, respectively. The corresponding values of K_m for *horseradish peroxidase* [22], *Turkish black radish* [23] and *Solanum melongena* fruit juice [21] 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 peroxidise* [22]. Peroxidase obtained from the fruit juice of *B. vulgaris* 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 [24], *B. vulgaris* juice peroxidase was also analysed for this type of kinetics by measuring the steady state velocity of the enzyme-catalyzed 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 [24].

The results of the variation of the activity of the this peroxidase enzyme with the variation of pH of the reaction solution are shown in Figure 3. The determined pH optimum of the enzyme was 6.0. Plant peroxidases of *S. melongena* fruit juice [21], *Musa paradisiaca* stem juice [25] and peroxidase of fruit juice of *L. aegyptiaca* [26] 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 pH optimum near neutral pH as peroxidase obtained from the fruit juice of *B. vulgaris* i.e. pH 6.0. pH optimum for the peroxidase present in the juice of *B. vulgaris* was 6.0 that means it is most active at pH 6.0. When this enzyme was exposed for different time intervals (10 min.) upto one hour at different pH then it was found to be most stable at pH 6.0 (Figure 4).

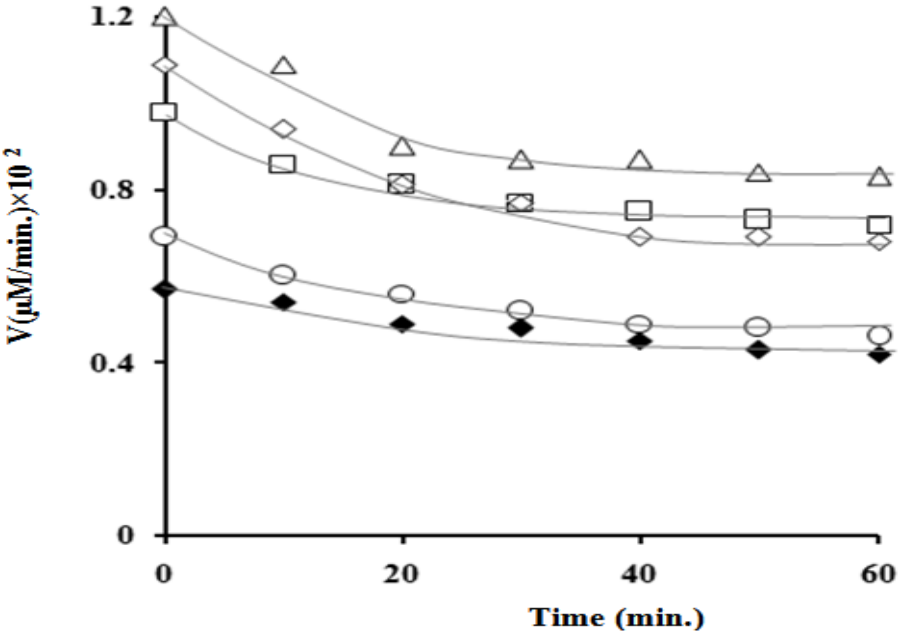

Figure 4. Stabilities of peroxidise at different pH when exposed for one hour at 10 minutes time intervals. (pH 4.0 (o),, 5.0 (◊), 6.0 (Δ), 7.0 (□), 8.0 (♦))

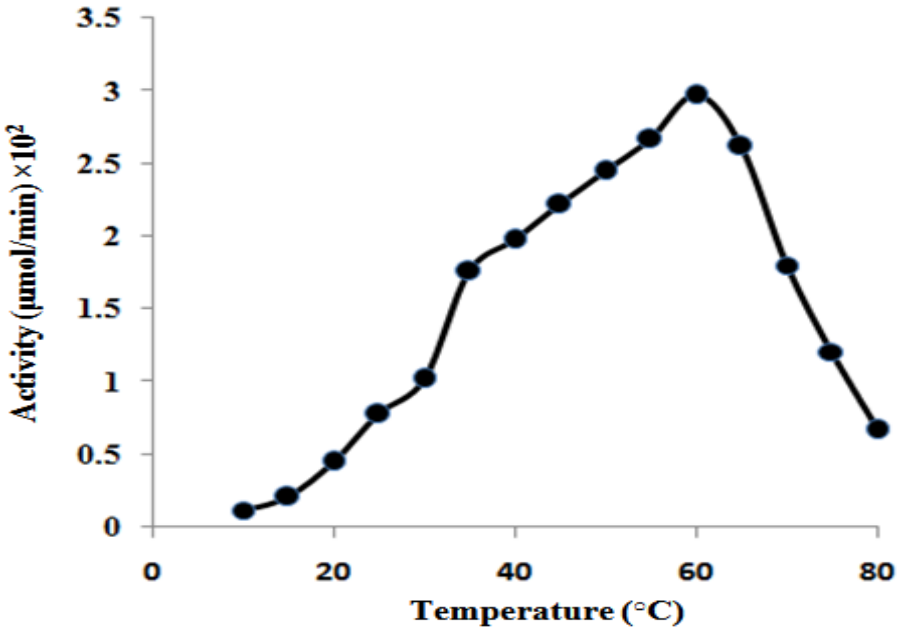

Figure 5. Dependence of the peroxidase activity on temperature of the assay solution at fixed pH 7.0

The results of variation of the activity of peroxidase enzyme obtained from the juice of *B. vulgaris*, as a result of variation of the temperature of the reaction solution of the enzyme catalyzed reaction are shown in Figure 5. The calculated temperature optimum was 60°C. The temperature optima of the peroxidases from *S. melongena* fruit juice [21], *M. paradisiacal* stem juice [25] and fruit juice of *L. aegyptiaca* [26], peroxidases reported from our laboratory, were 84°C, 62.5°C and 60°C respectively. In this way, we see that the peroxidase from *B. vulgaris* juice has the temperature optimum toward the higher temperature side as like *S. melongena* fruit juice, *M. paradisiacal* stem juice and *L. aegyptiaca* fruit juice. When this enzyme was exposed for different time

intervals (10 min.) for one hour at different temperatures then it was found to be most stable at 45°C. The result of the studies on the thermal stability of the peroxidase is shown in Figure 6. Activation energy of the thermal denaturation of the peroxidase calculated from Arrhenius plot was found to be 38.6 kJ/mole/K.

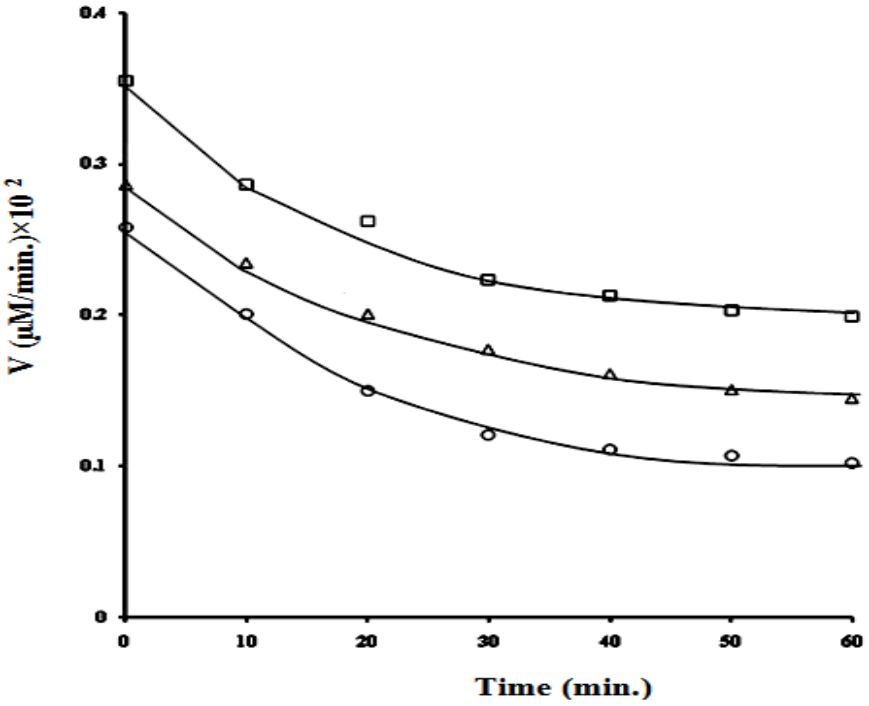

Figure 6. Thermal denaturation of the enzyme at different temperatures. (45 ^{0}C (□), 50 ^{0}C (Δ) and 55 ^{0}C (o))

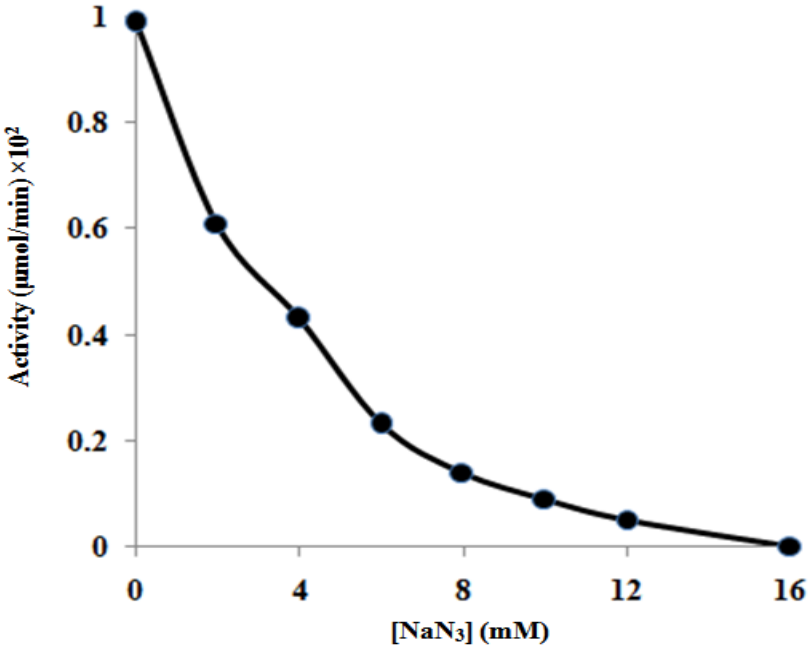

Figure 7. Inhibition of the activity of the peroxidase obtained from the fruit juice of *B. vulgaris* (beet) by sodium azide

Studies on inhibition of the peroxidase has also been performed for various concentrations of sodium azide started from 0.0 mM to 16 mM. Activity of this peroxidase has been found to be completely diminished at 16 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. Due to the significant scope of peroxidases, the peroxidase enzyme obtained from the juice of *B. vulgaris* may be effective because peroxidase obtained from this source is very active for hydrogen peroxide, especially, and does not lose activity at room temperature for several hours.

4. Conclusions

This communication demonstrates mainly the kinetic, thermal and pH properties. Activation energy for this peroxidase has also been calculated. It is clear from the K_m

values determined for different substrates as guaiacol and hydrogen peroxide, clearly explain the fact that this peroxidase can be used as potential biocatalyst for various biotransformations and organic synthetic reactions using hydrogen peroxide as substrate in comparison to guaiacol.

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