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Extraction of peroxidase from various plant sources and its biodegradation studies on phenolic compounds

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ABSTRACT

The enzymatic approach for bio remediation process has gained attraction because of its significant action on pollutants and high catalytic action compared to that of the chemical catalyst. This study focuses on the action of peroxidase enzyme on phenolic compounds present in industrial wastes. Crude Peroxidase enzyme was extracted from vegetable sources-radish, tomato, turnip and cabbage. The enzyme was found to be stable at an optimum temperature range of 55-65° C and at an optimum pH of 6-6.5. Using Ammonium sulphate precipitation, the crude extract was purified and maximum yield was obtained when the salt concentration was 80%. Aqueous Two phase extraction was also carried out and peroxidise was separated from top phase. Gel permeation chromatography was also performed and it showed up to 9.43 fold purification with maximum specific activity of 10.94 U/mg. The effect of inhibitor, Sodium azide, on the enzyme was studied and it clearly indicated that the activity of the enzyme decreased with increase in concentration of the inhibitor. Phenol degradation by the enzyme peroxidase showed that increase in the degradation of phenol was observed at high concentration of enzyme. Maximum degradation was obtained by the enzyme from turnip. Thus peroxidase can be used for phenol degradation in near future.

KEYWORDS

Peroxidise;Turnip;
Aqueous two phase extraction;
Phenolic degradation.

INTRODUCTION

The isolation and purification of a biotechnological product to a suitable form for its use is popularly known as downstream processing[1]. The main aim is to minimize the number of required steps and increase its yield to maximum possible value[2]. Downstream processing implies manufacturing purified product fit for specific use, generally sufficient enough for marketing[3].

Peroxidases [E.C.1.11.1.7] a oxidoreductase produced by a number of plants and micro organisms generally has iron porphyrin ring catalyzing oxidation of many organic substrate of aromatic compound[4]. They mostly have a molecular mass that ranges from 30,000 to150,000 Da.(14) It is considered as one of the most heat stable enzyme[5]. Peroxidase a widely distributed

compound in nature catalyzes the reduction of hydrogen peroxide to water, making it harmless[6]. This enzyme is found to exist in both membrane-bound and involved in regulation of plant hormone, protective mechanisms and lignin biosynthesis[7]. Because of its several roles, the enzyme is frequently observed as several iso enzymes in plants.

Phenol occurs mostly in the waste water of industries such as petroleum refining, coal conversion, plastic, resin, textiles, iron, steel, pulp and paper manufacturing. Phenol, a carcinogen can be of major health concern, even in low concentrations. Many technologies have been determined for the removal and degradation of phenolic compounds in wastewaters. Current trend of methods for phenol removal from waste water include microbial degradation, use of oxidizing agent such as ozone and UV, solvent extraction, adsorption etc. The primary aim of the downstream processing is to develop high selective protocol for the target enzyme through economic way[8]. Using of affinity ligands and Aqueous two phase extraction methods are the emerging bioseparation technique[9]. Aqueous two phase extraction is one of the promising tool with high space of optimization and scale up mode for biologically sensitive enzymes[2,10,11]. Recovery of these enzymes naturally with high activity is the major task in bioseparation industries. The novel applications of peroxidase comprise waste water treatment of dye contaminated water. The present study is about to identify the most suitable biocompatible separation protocol for peroxidise enzyme from its natural source. The various sources of peroxidise rich natural sources were taken for this study and evaluated for the high recovery with appreciable activity.

MATERIALS AND METHODS

Materials

Radish, tomato, cabbage, turnip were procured from local market. 3,3',5,5' tetra methyl benzidine was bought from sigma, H_2O_2, phenol, $FeCl_3$, SDS-PAGE kit were of analytical grade.

Crude peroxidase extraction

Vegetable sources were washed properly and was cut, blended using chisel and mixed with the help of 200gms of pH 7 phosphate buffer to prepare the crude extract. The enzyme was filtered using chisel cloth and the enzyme along with buffer was centrifuged at 8000rpm for 10 minutes to remove other small impurities. Supernatant was taken and filtered with whatmann filter paper and was stored as crude extract in a beaker. To remove impurities like catalase, it was heated at 65°C for 3 minutes. The crude enzyme was then stored in a refrigerator and was brought to room temperature before every experiment.

Peroxidase and protein assay

The enzyme activity was measured using a chemical that does not occur in plants, but that changes colour, making the reaction easy to monitor. A 50ul of 5% 3, 3', 5, 5' tetra methyl benzidine was taken and mixed along with 100ul of pH6 citrate buffer and 100ul enzyme extract. The test tube was shaken and 100ul of H_2O_2 was added and then enzyme activity was monitored by measuring the OD at 620nm. The total protein estimation of each enzyme extract was determined using Lowry's et al[12].

Ammonium sulphate precipitation

Protein precipitation was carried out with ammonium sulphate method, in which different concentration of salt of ammonium sulphate was added to the sample. The concentration varied from 30% to 80%, amount of salt required was calculated and added to the sample, which was then vortexed, till the salt is solubilised. This was followed by centrifugation at 8000rpm for 10 minutes. Due to centrifugation, supernatant and pellet were separated. The reading for protein and enzyme assay from the pellet and supernatant was noted separately.

Aqueous two-phase extraction

ATPS was prepared using different concentrations of PEG, ammonium sulphate along with crude extract. After addition, sample was vortexed and then allowed to settle down, till the two phases were formed. The top and bottom phases were separately collected and the enzyme activity and protein concentration of both phases were calculated. The analysis on the partition coefficient and specific activity of the enzyme were made.

Gel filtration chromatography

1ml of purified peroxidase enzyme obtained, was

subjected to gel filtration chromatography with the help of sephadex-G-200 column. The glass column having inner diameter of 1.5 cm was packed with the column of 15 cm height. Sample was poured in the column and eluted with phosphate buffer at pH 7. Fractions of purified enzyme were collected and the enzyme activity with protein content was resolved, as mentioned in the previous section.

Phenol degradation study

The standard graph for pure phenol sample without any enzyme was plotted, where purified peroxidase from all the sources was taken and various concentration of enzyme extract was added to a particularly fixed concentration of phenol. The OD was monitored at 580nm for the activity of phenol. The peroxidase source which gives the lowest OD for phenol activity was found and was considered as the best source of peroxidase for the removal of phenol.

RESULTS AND DISCUSSION

Crude peroxidase extraction

The crude extracted from all the four sources were collected and checked for enzyme activity after which the impurities were denatured by heating. It was found that turnip gave the best result for crude among all the sources available and as shown in TABLE 1, the value was found to be 1.5U/ml, whereas cabbage gave the least enzyme activity of 0.9U/ml.

TABLE 1 : Peroxidase in its Crude sample

Source	Enzyme Activity (U/mg)	Total Protein (mg/ml)	Specific Activity (U/ml)
Radish	1.10	0.75	1.46
Turnip	1.5	0.86	1.74
Cabbage	0.90	0.82	1.09
Tomato	0.96	0.84	1.14

Ammonium sulphate precipitation

The specific activity and purification fold resulted from ammonium sulphate treatment were found to be twice that of the crude extract. In this precipitation process, no precipitate was observed from 0-20%, and peroxidase began to precipitate only when at least 30% ammonium sulphate was added, reaching the highest specific activity at 80% saturation, so it was concluded that, as the value of salt increased, enzyme activity also started increasing and maximum yield was obtained when the salt concentration was 80%, this was the case for all the vegetable sources.

Aqueous two-phase extraction

Different concentrations of PEG and salts were used and varied phase compositions make the volume of the bottom phase (which needs to be subjected to further processing) to reduce. Figure 1 shows that the system having significant effect on partition coefficient was studied for further use. It was found that PEG6000 along with ammonium sulphate gave best results from the different compositions, and the combination giving best result was chosen for each source. From Figure 2 it was found that With PEG2000, peroxidase prefers the top phase; however at low molecular weight of PEG, many proteins having high k value led to negligible extraction from contaminant protein, whereas high molecular weight PEG prevented successful separation of peroxidase in the top phase and therefore separating it from bulk precipitate.

Gel filtration chromatography

Gel permeation chromatography was performed by sephadex G-100. Fractions collected, were tested for enzyme activity and total protein value as can be seen in TABLE 2, which proved very efficient as fraction showed up to 8.0 fold purification with maximum specific activity of 12.43 *U/mg* for turnip with highly reduced protein contents and it is shown in Figure 5(a,b,c and d) separately for the sources. This technique provided higher degree of purification for all the vegetable sources thus we can appreciate and encouraging its further use for purification purposes.

Effect of various parameters

Various parameters were monitored to check the effect of these parameters on the activity of peroxidase and then all the monitored parameters were optimized. Enzyme extract was incubated at various temperatures ranging from 40 to 90 °C for 10 minutes in a water bath, and then they were checked for OD and enzyme activity was determined for each temperature.A 5ml of enzyme extract was taken in each test tube and adjusted

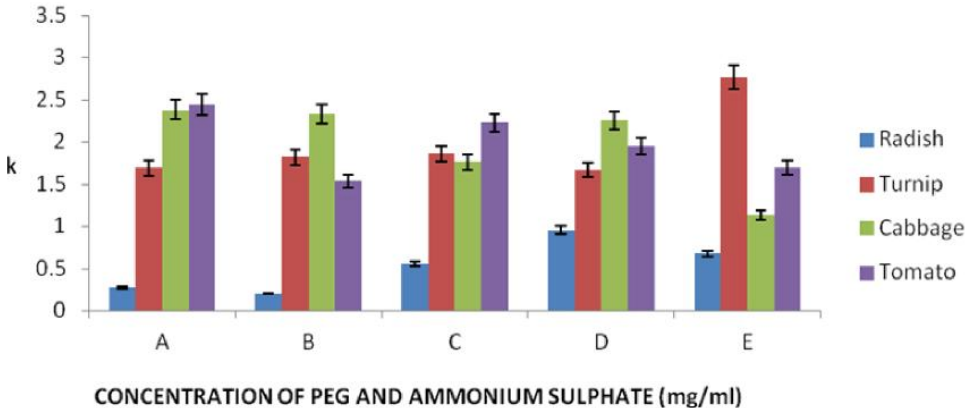

Figure 1 : Effect of ATP Phase composition on partition coefficient of peroxidase

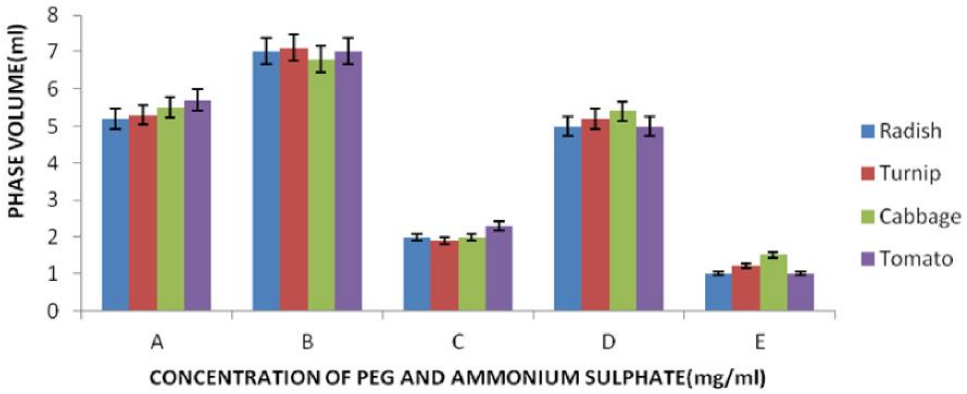

Figure 2 : Effect of ATP composition on Phase volume

for pH ranging between 4 to 9 by the addition of 1M HCl or 1M NaOH. Each sample was then kept at that particular pH for 30 minutes at room temperature, after which enzyme activity is monitored by finding the OD.

Effect of temperature

Peroxidase, being one of the most heat stable enzyme, can tolerate very high range of temperature without losing its activity. But it was found from the experiment that the optimum temperature, where the enzyme showed maximum activity was around 60-65°C as can be seen in Figure 3b, after which the enzyme is active, its activity starts decreasing. Above 60°C, further increment in temperature decreases the activity. A sharp

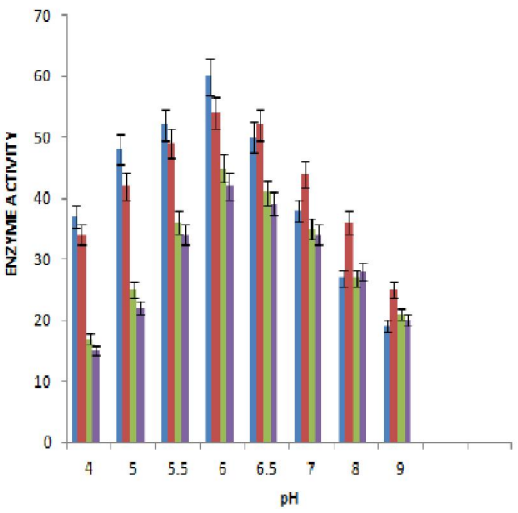

Figure 3a : Effect of pH in ATP

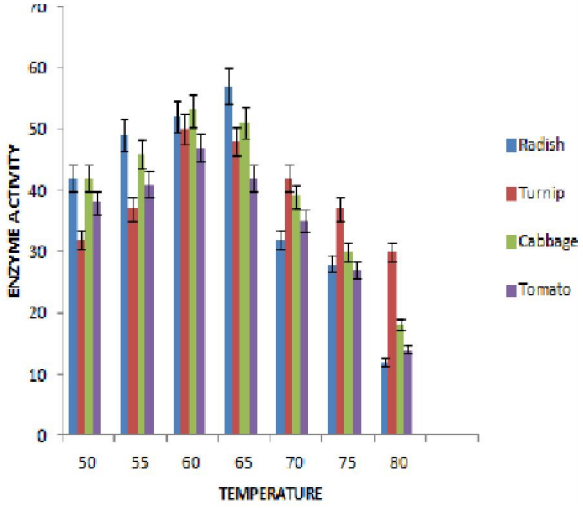

Figure 3b : Effect of temperature in ATP

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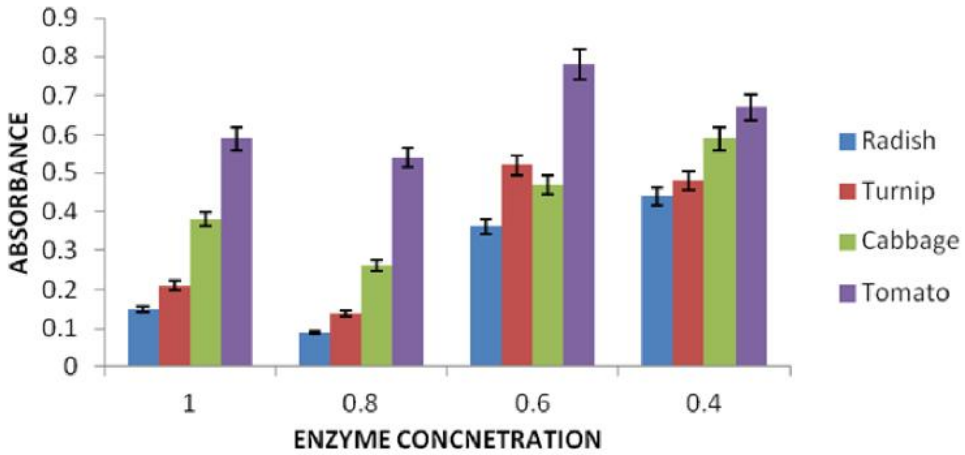

Figure 4 : Phenolic degradation action on peroxidase

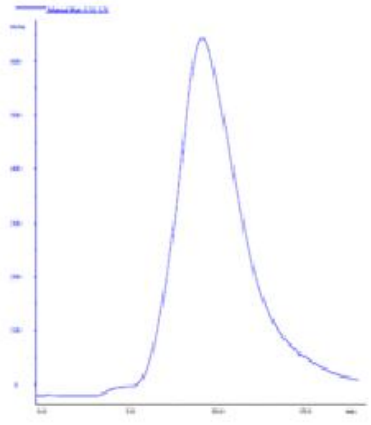

Figure 5a : Gel filtration chromatography of radish

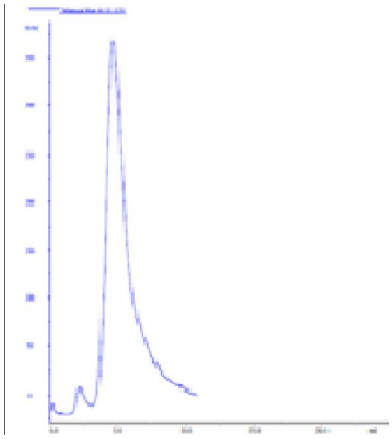

Figure 5b : Gel filtration chromatography of turnip

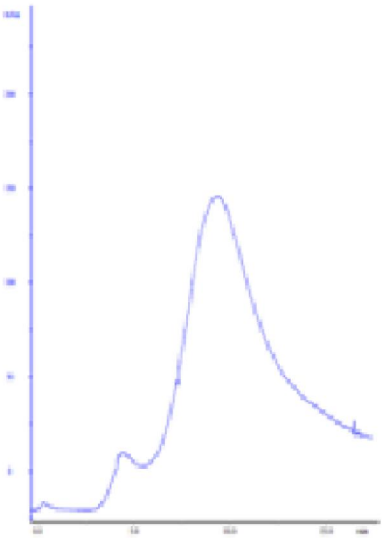

Figure 5c : Gel filtration chromatography of cabbage

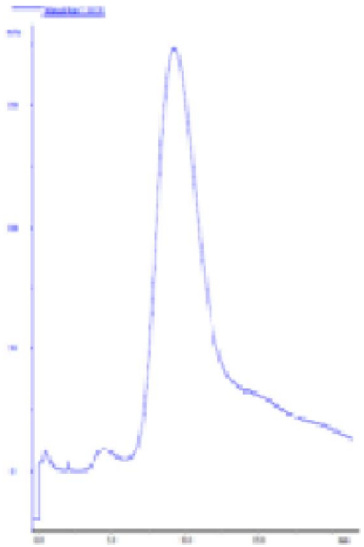

Figure 5d : Gel filtration chromatography of tomato

decrease in the relative activity was found after 80°C thus suggesting us that the optimum temperature is around 60°C and anything before or after that has lower enzyme activity.

Effect of pH

pH values were optimized by evaluating its enzyme activity at various pH. Peroxidase was found to be stable over wide range of pH between 4-9, but it was found

TABLE 2 : Purified Peroxidase in Gel filtration chromatograpgy

Source	Enzyme Activity (U/mg)	Total Protein (mg/ml)	Specific Activity (U/ml)
Radish	10.94	0.32	34.18
Turnip	12.43	0.29	42.86
Cabbage	7.34	0.41	17.90
Tomato	6.83	0.38	18.01

to be more active in the acidic environment when compared to the basic one. The optimum pH was found to be around 6-6.5 as seen in Figure 3a and the value of enzyme activity decreased before and after that pH.

CONCLUSION

Activity of peroxidase was inversely proportional to the amount of phenol i.e. activity of phenol decreased on increasing the concentration of enzyme extract. Figure 4 shows that the estimation of phenol, after the treatment of 0.8ml (80mg/ ml) phenol with four different of concentration of crude enzyme (0.2ml, 0.4ml, 0.6ml and 0.8ml) for turnip and next two for cabbage and tomato. Maximum degradation of phenol was observed with high concentration of enzyme extract. This shows that phenol degradation occurred due to enzyme action. Phenol's maximum degradation was seen with high concentration of enzyme, and source which degraded maximum phenol was found to be turnip. As the concentration of phenol increased, reduction in phenol degradation efficiency was observed. From this experiment it can be concluded that enzymatic treatment using peroxidase can surely be a viable option for the degradation of phenol.

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