STUDIES ON ENZYMATIC PROPERTIES OF PARTIALLY PURIFIED POLYPHENOL OXIDASE IN CABBAGE (*BRASSICA OLERACEA* L.) AND ITS ANTIMICROBIAL ACTIVITY

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Abstract

Extraction and purification of polyphenol oxidase from *cabbage (Brassica oleracea* L.) were performed by ammonium sulphate precipitation (35-85 %) and gel filtration chromatography on Sephadex G-100. Polyphenol oxidase activity was determined using catechol as a substrate at 420 nm. The protein content was also determined by the Biuret method, using Bovine Serum Albumin (BSA) at 550 nm. The optimum pH and temperature of both crude and partially purified polyphenol oxidase enzymes were found to be 7.0 and 40 °C, respectively. The activation energy of the crude polyphenol oxidase-catalyzed reaction was –9.33 kcal mol^{-1} and that of partially purified enzyme was 6.31 kcal mol^{-1}. The K_m (0.052 M) and V_{max} (3.03×10^{-5} M min^{-1}) of partially purified polyphenol oxidase were determined by using the Lineweaver-Burk plot. The reaction order (n) of the polyphenol oxidase-catalyzed reaction was calculated by using the linear regression method and it was found to be first order for both crude and partially purified polyphenol oxidase. The crude polyphenol oxidase responded the highest antimicrobial activity against the eight microorganisms tested by the agar well diffusion method.

Keywords: cabbage, *Brassica oleracea* L., polyphenol oxidase, ammonium sulphate precipitation, gel filtration chromatography, antimicrobial activity

Introduction

Polyphenol oxidase (EC 1.10.3.1) is a common copper-containing enzyme that is widely distributed in the plant kingdom. and It also known as tyrosinase, catechol oxidase, and monooxygenase. Polyphenol oxidase (PPO) catalyzes the hydroxylation of monophenols to *o*-diphenols, followed by the oxidation of *o*-diphenols to *o*-quinones in the presence of oxygen (Concellon *et al*., 2004). These enzymes are very prevalent in nature and are in charge of the enzymatic browning of plant products. Polyphenol oxidase is present in most higher plants, but also in animals and fungi (Zhang, 2023). Enzymatic browning is one of the most limiting factors in the shelf life of fresh-cut fruits and vegetables (Wong *et al*., 2019). Humans have a polyphenol oxidase enzyme that causes skin pigmentation, including the appearance of freckles. Because polyphenol oxidase activity mechanically ruins food, which results in a browning reaction in the presence of oxygen, many fruits and vegetables, including potatoes, cabbage, lettuce, mushrooms, and eggplants, are lost as a result. These fruits and vegetables include peaches, apricots, apples, grapes, bananas, and strawberries (Guven *et al*., 2017). Numerous researchers have investigated the use of enzymes in the treatment of wastewater (Murniati *et al*., 2010). The main aim of research is to study the enzymatic properties of partially purified polyphenol oxidase extracted from cabbage and its antimicrobial activity.

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Materials and Methods

Cabbage samples were collected from Hledan Market, Kamayut Township, Yangon Region. Then, identification of the sample was done at the Department of Botany, University of Yangon. Sample extraction and purification were performed at the Analytical Chemistry Research Laboratory, Department of Chemistry, University of Yangon. Dipotassium hydrogen phosphate, potassium dihydrogen phosphate from BDH, and ammonium sulphate from Merck were used. The chemicals required and polyphenol oxidase assay reagents were purchased from Sigma-Aldrich, England.

Extraction and Purification of Polyphenol Oxidase

Cabbage (*Brassica oleracea* L.) was washed with distilled water and dried at room temperature. Cabbage was cut into small pieces. Cabbage (200 g) was homogenized for 5 min using a blender with 200 mL of 0.1 M potassium phosphate buffer pH (7.0) and centrifuged at 6000 rpm for 30 min. After centrifugation, solid ammonium sulphate (45.86 g) was added to the supernatant to give 35 % saturation. After settling for 2 h, the precipitate was removed by centrifugation for 30 min at 6000 rpm and was discarded. Additional ammonium sulphate (79.59 g) was then added to achieve 85 % saturation. After being kept overnight, the precipitate containing polyphenol oxidase was collected by centrifugation for 30 min at 6000 rpm. Crude polyphenol oxidase (2 g) was dissolved in 4 mL of potassium phosphate buffer (pH 7.0). This solution was applied to a Sephadex G-100 gel filtration column previously equilibrated with the same buffer. The flow rate was adjusted to 1.5 mL per 5 min. A 1.5 mL fraction was collected in a test tube. Protein content in each tube was checked by measuring the absorbance at 280 nm and polyphenol oxidase activity was also measure at 420 nm. The fractions that had the highest polyphenol oxidase activity were pooled. The pooled polyphenol oxidase fraction was stored at 4 °C.

Polyphenol Oxidase Enzyme Assay

A spectrophotometric method was used to determine polyphenol oxidase activity on the basis of the initial rate of the absorbance increase at 420 nm (Guven *et al*., 2017). The reaction mixture consisted of 0.5 mL of potassium phosphate buffer (pH 7.0), 2 mL of catechol (substrate), and 0.5 mL of enzyme extract. The mixture was shaken well and incubated at 37 ℃ for 10 min. One enzyme unit represents the amount of enzyme that produces a rise of 0.001 absorbance in one minute at 420 nm.

Protein Determination

Protein content of the enzyme solution in each purification step was determined by the Biuret method using a spectrophotometer at 550 nm.

Determination of the Optimum pH and Temperature of Polyphenol Oxidase

Polyphenol oxidase activity was determined in a pH range of 6.4-7.6 at 0.2 unit intervals with a 0.1 M phosphate buffer. In this pH range, enzyme activity was measured using the procedure described for the polyphenol oxidase activity enzyme assay.

Furthermore, polyphenol oxidase activity was determined at different reaction temperatures in the range of 20-60 °C using catechol as substrate, and the phosphate buffer (pH 7) was heated to the relevant temperature before the assay.

Kinetic Studies of Crude and Partially Purified Polyphenol Oxidase Enzymes

The Michaelis-Menten constant (K_m), maximum velocity (V_{max}), and reaction order of of crude and partially purified enzymes-catalyzed reactions were determined using catechol as a substrate with a concentration range of 0.005 M to 0.10 M. For the determination of activation energy, the velocities of polyphenol oxidase catalyzed reaction were measured at the temperature range of 20-40 ℃ at 5 unit intervals. It was then determined from an Arrhenius plot of the initial velocity data.

Screening of the Antimicrobial Activity of Crude Polyphenol Oxidase from Cabbage

The antimicrobial activity of the crude polyphenol oxidase from cabbage was studied by the agar well diffusion method at the Chemistry Department, Pathein University. Eight species of microorganisms: *Agrobacterium tumefaciens*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas fluorescens*, *Bacillus pumilus*, *Candida albicans*, *Escherichia coli*, and *Micrococcus luteus* were used for this study.

Results and Discussion

Purification of Polyphenol Oxidase Extracted from Cabbage

The polyphenol oxidase was extracted from cabbage by ammonium sulphate precipitation method and the crude enzyme was obtained. Sephadex G-100 was used to partially purify the crude enzyme. Figure 1 shows the stepwise purification of the polyphenol oxidase on Sephadex G-100 gel. The protein content of the eluate was checked spectrophotometrically at 280 nm, and the enzyme activity was determined at 420 nm. The fractions with the highest activity (20-33 fractions) were collected and pooled. The specific activity of the polyphenol oxidase increased about 3.95 folds over that of the crude extract, and the protein recovery was found to be 18.95 %. The degree of purity of polyphenol oxidase in each purification step is described in Table 1.

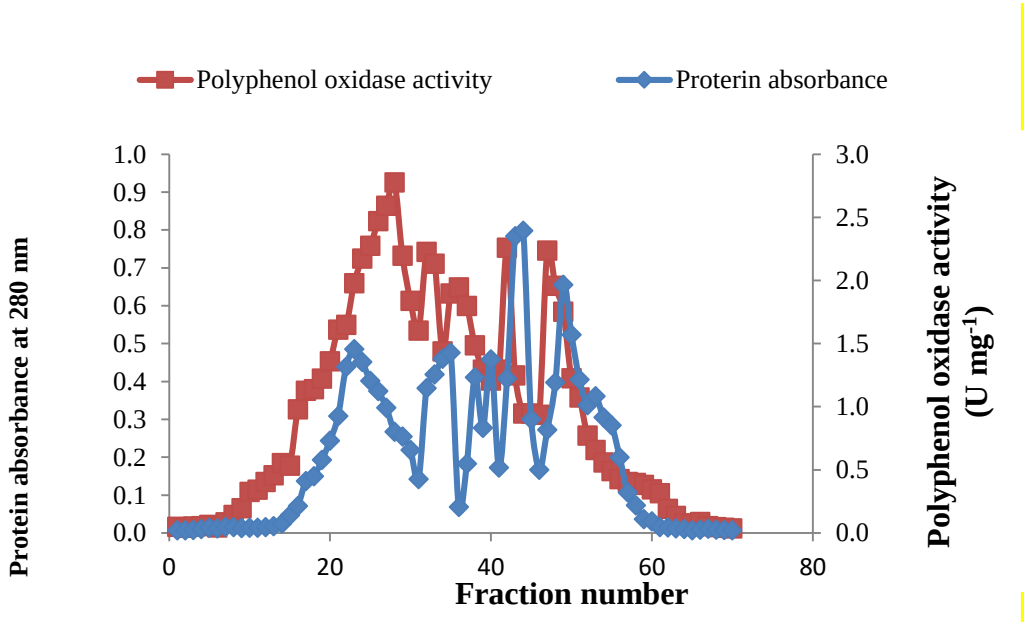


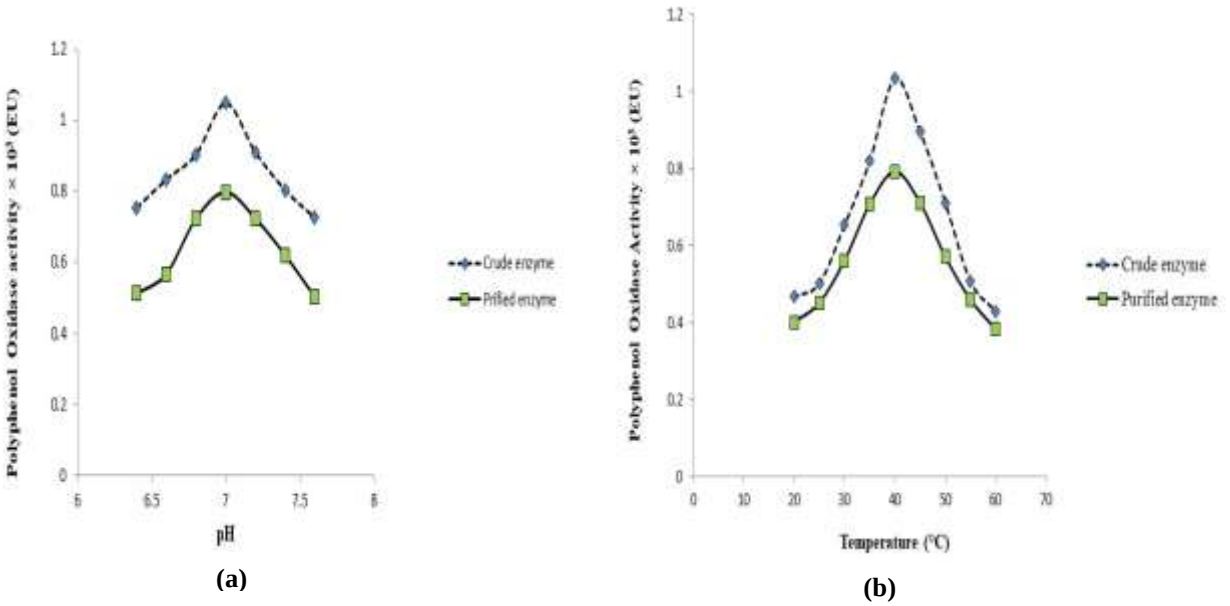
Figure 1. Chromatogram of crude polyphenol oxidase on Sephadex G-100 column

Table 1. Polyphenol Oxidase Activities, Protein Contents and Specific Activities of the Enzyme Solutions at Different Purification Steps

Purification steps	Total volume (mL)	Total protein content (mg)	Total PPO activity (U)	Specific activity (U/mg)	Protein recovery (%)	Degree of purity (fold)
crude	240	530.06	20640	38.94	100.00	1.00
after 35% $(NH_4)_2SO_4$	223	400.41	21138	52.79	75.54	1.36
after 85%v$(NH_4)_2SO_4$	10	160.33	10600	66.11	30.25	1.69
after passing the Sephadex G-100	20	100.43	15444	153.93	18.95	3.95

Optimum pH and Temperature of Crude and Partially Purified Polyphenol Oxidase

As shown in Figures 2 (a) and (b), optimum pH and temperature of polyphenol oxidase-catalyzed reaction were observed as 7.0 and 40 °C, respectively, using catechol as a substrate. The previous studies also reported that optimum pH was 7.0 for parsley (Lin *et al*., 2016), artichoke (Dogan *et al.*, 2005), and jackfruit (Tao *et al*., 2013). Although optimum temperatures of polyphenol oxidase varies among plants depending on the extraction methods and types of substrates used, the optimum temperature is mostly obtained between 30 and 50 °C (Aydin *et al*., 2015, Sun *et al*., 2010, Palma-Orozco *et al*., 2011).



(a) **(b)**

Figure 2. Plots of the crude and partially purified polyphenol oxidase activity as a function of (a) pH and (b) temperature of the solutions

Effect of Substrate Concentration on Polyphenol Oxidase-Catalyzed Reaction

The velocities of crude polyphenol oxidase-catalyzed reactions measured at varying levels of catechol concentration and their reciprocal values are shown in Table 2. In the

Michaelis-Menten plot shown in Figure 3, the velocity of an enzyme-catalyzed reaction is directly proportional to the percentage of the enzymes reacting with the substrate to form an enzyme-substrate complex (Yilmaz., 2020). At this point, the enzyme was said to be saturated with substrate, and further increases in the concentration of the catechol would not increase the velocity of the reaction. The enzyme could have worked no faster, and thus the maximum velocity, V_{max}, can be obtained. The concentration of substrate at which the reaction reaches half its maximum velocity is equal to K_m. For a more accurate estimation of K_m and V_{max} values, these were computed from Lineweaver-Burk (Figure 4) and Eadie-Hofstee plots (Figure 5). Similarly, Table 3 shows the velocities of partially purified polyphenol oxidase-catalyzed reactions at different catechol concentrations. Michaelis-Menten, Lineweaver-Burk, and Eadie-Hofstee plots are depicted in Figures 6, 7, and 8, respectively.

Table 2. Relationship between Velocity of Crude Polyphenol Oxidase-Catalyzed Reaction and Substrate Concentration

[S] (M)	-[S] (M)	1/[S] (M^{-1})	V $\times 10^5$ (M min^{-1})	1/V $\times 10^{-5}$ (M^{-1} min)	V/ [S]$\times 10^5$ (min^{-1})	[S]/V $\times 10^{-5}$ (min)
0.005	-0.005	200.0	0.300	3.330	60.00	0.017
0.010	-0.010	100.0	0.550	1.800	55.00	0.018
0.020	-0.020	50.00	0.920	1.086	46.00	0.022
0.030	-0.030	33.33	1.250	0.800	41.60	0.024
0.040	-0.040	25.00	1.490	0.673	37.30	0.027
0.050	-0.050	20.00	1.630	0.613	32.60	0.031
0.060	-0.060	16.64	1.720	0.581	28.60	0.035
0.070	-0.070	14.29	1.760	0.568	25.10	0.039
0.080	-0.080	12.50	1.800	0.555	22.50	0.044
0.090	-0.090	11.11	1.850	0.541	20.55	0.049
0.100	-0.100	10.00	1.870	0.534	18.70	0.053

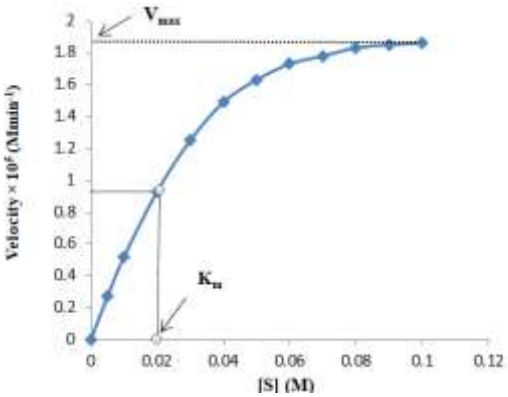


Figure 3. Michaelis-Menten plot of the velocity of crude polyphenol oxidase-catalyzed reaction as a function of substrate concentration

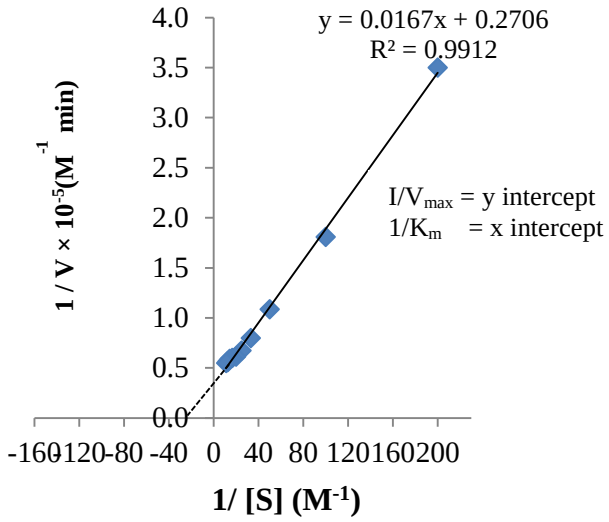


Figure 4. Lineweaver-Burk plot of 1/V *vs*. 1/[S] used for graphic evaluation of V_{max} and K_m for crude polyphenol oxidase

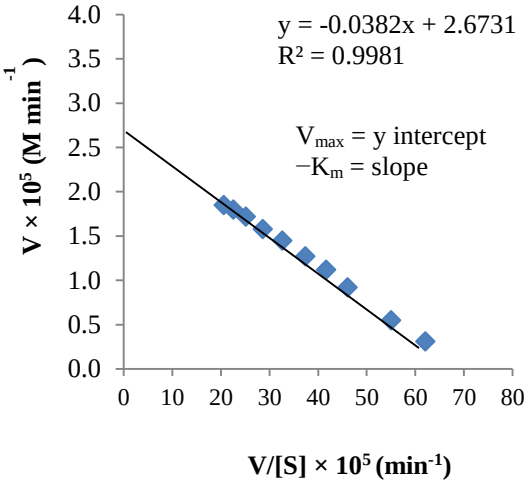


Figure 5. Eadie-Hofstee plot of V *vs*. V/[S] used for graphic evaluation of V_{max} and K_m for crude polyphenol oxidase

Table 3. Relationship between Velocity of Partially Purified Polyphenol Oxidase-Catalyzed Reaction and Substrate Concentration

[S] (M)	-[S] (M)	1/[S] (M^{-1})	V ×10^5 (M min^{-1})	1/V ×10^{-5} (M^{-1} min)	V/ [S]× 10^5 (min^{-1})	[S]/V ×10^{-5} (min)
0.005	-0.005	200.0	0.280	3.600	56.00	0.018
0.010	-0.010	100.0	0.510	1.960	51.00	0.020
0.020	-0.020	50.00	0.860	1.160	43.00	0.023
0.030	-0.030	33.33	1.120	0.890	37.33	0.027
0.040	-0.040	25.00	1.340	0.746	33.50	0.030
0.050	-0.050	20.00	1.550	0.650	31.00	0.032
0.060	-0.060	16.64	1.650	0.606	27.33	0.036
0.070	-0.070	14.29	1.740	0.574	24.71	0.040
0.080	-0.080	12.50	1.830	0.546	22.87	0.044
0.090	-0.090	11.11	1.940	0.515	21.56	0.047
0.100	-0.100	10.00	2.030	0.493	20.30	0.049

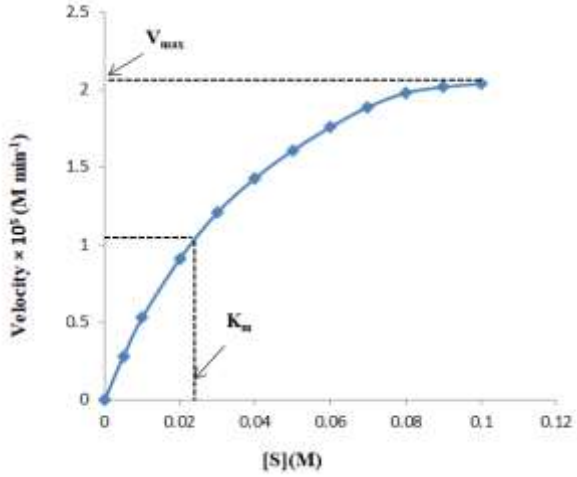


Figure 6. Michaelis-Menten plot of the velocity of partially polyphenol oxidase-catalyzed reaction as a function of substrate concentration

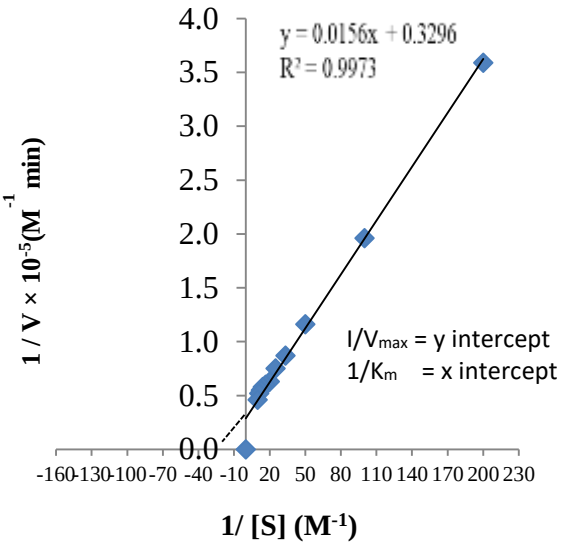


Figure 7. Lineweaver -Burk plot of 1/V *vs*. 1/[S] used for graphic evaluation of V_{max} and K_m for partially purified polyphenol oxidase

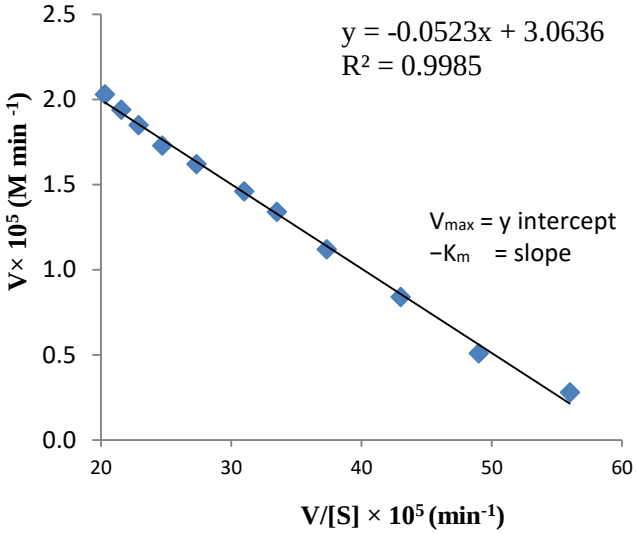


Figure 8. Eadie-Hofstee plot of V *vs.* V/[S] used for graphic evaluation of V_{max} and K_m for partially purified polyphenol oxidase

Table 4 shows the K_m and V_{max} values of crude and partially purified polyphenol oxidase-catalyzed reactions obtained from graphical methods and linear regression methods.

The K_m and V_{max} values of these two enzymes are not significantly different.

Table 4. Comparison of Kinetic Parameters of Crude and Partially Purified Polyphenol Oxidase from Different Methods

Enzyme	Method	Linear regression method		Graphical method	
		K_m (M)	$V_{max} \times 10^5$ (M min^{-1})	K_m (M)	$V_{max} \times 10^5$ (M min^{-1})
crude	Michaelis-Menten	-	-	0.020	1.87
	Lineweaver- Burk	0.040	2.77	0.040	2.80
	Eadie-Hofstee	0.040	2.73	0.038	2.67
partially purified	Michaelis-Menten	-	-	0.025	2.03
	Lineweaver- Burk	0.049	3.03	0.052	3.03
	Eadie-Hofstee	0.050	3.03	0.052	3.06

Reaction Order of Polyphenol Oxidase-catalyzed Reaction

Depending on the substrate concentrations, the kinetics of an enzyme–catalyzed reaction may be described by the first-order rate equation (Bergmeyer, 1983). In this study, the 'n' value was determined from the plot of Log $V/(V_{max} - V)$ *vs.* Log [S] for polyphenol oxidase activity using the linear regression method (Table 5 and Figure 9). The reaction order (n) values of crude and purified polyphenol oxidase were calculated to be 0.98 and 1.001, respectively, and thus, it is a first-order reaction.

Table 5. Relationship between Log [S] and Log V/ (V_{max}-V) for the Determination of Reaction Order of Polyphenol Oxidase-Catalyzed Reaction

[S] (M)	Log [S]	Crude enzyme		Partially purified enzyme	
		$V \times 10^5$ (M min^{-1})	Log $V/(V_{max}- V)$	$V \times 10^5$ (M min^{-1})	Log $V/(V_{max}- V)$
0.005	-2.301	0.30	-0.908	0.280	-0.979
0.01	-2.000	0.55	-0.607	0.510	-0.714
0.02	-1.700	0.92	-0.304	0.086	-0.402
0.03	-1.520	1.25	-0.085	1.120	-0.232
0.04	-1.400	1.49	-0.065	1.340	-0.100
0.05	-1.301	1.63	0.155	1.550	0.020
0.06	-1.222	1.72	0.214	1.650	0.072
0.07	-1.160	1.76	0.241	1.740	0.124
0.08	-1.110	1.80	0.268	1.830	0.183
0.09	-1.050	1.85	0.303	1.940	0.250
0.10	-1.000	1.87	0.317	2.030	0.307

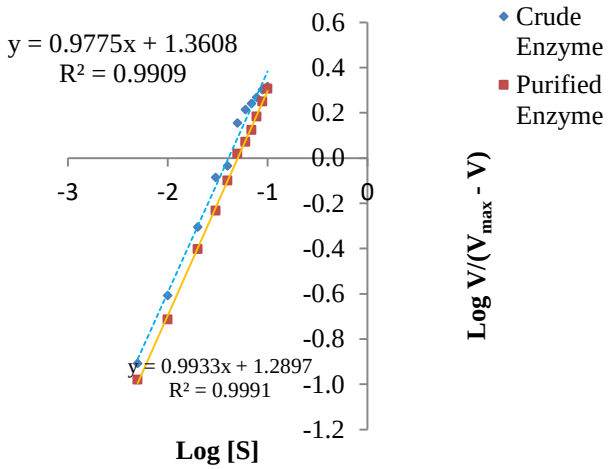


Figure 9. Plot of log V/(V_{max}-V) *vs* log [S] of polyphenol oxidase- catalyzed reaction

The activation energy (E_a) for enzyme-catalyzed reactions was determined by assaying the enzyme at different temperatures and constructing an Arrhenius plot (Table 6 and Figure 10). The activation energies of crude and partially purified polyphenol oxidase were calculated to be 9.33 kcal mol^{-1} and 6.31 kcal mol^{-1}, respectively. Moreover, the Arrhenius constants of crude and partially purified polyphenol oxidase were determined to be 3.4824×10^7 and 2.7561×10^5, respectively.

Table 6. Relationship between Temperature and Velocity of Crude and Purified Polyphenol Oxidase-Catalyzed Reaction

Temperature (°C)	Temperature (K)	1/T× 10^3 (K^{-1})	Crude enzyme		Partially purified enzyme	
			Velocity × 10^6	Log V	Velocity × 10^6	Log V
20	293	3.41	5.9	0.750	5.6	0.748
25	298	3.36	6.9	0.830	6.3	0.800
30	303	3.30	9.0	0.950	8.1	0.900
35	308	3.25	11.4	1.056	9.9	0.980
40	313	3.30	14.7	1.166	10.8	1.030

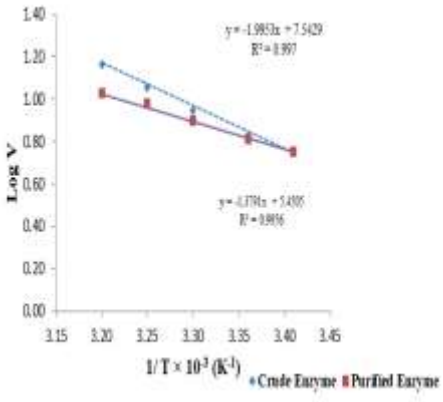


Figure 10. Plot of log V *vs* 1/T for crude and purified polyphenol oxidase activity

Antimicrobial Activities of the Crude Polyphenol Oxidase Enzyme

The antimicrobial activities were investigated against eight species of microorganisms such as gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Bacillus pumilus*, and *Micrococcus luteus*), gram-negative bacteria (*Agrobacterium tumfaciens*, *Pseudomonas fluorescens*, and *Escherichia coli*), and gram-positive fungus (*Candida albicans*) by using the agar well diffusion method. In this investigation, the inhibition zone diameters ranged between 26 and 28mm (Figure 10 and Table 7). Crude polyphenol oxidase showed very high antimicrobial activity on all eight microorganisms.

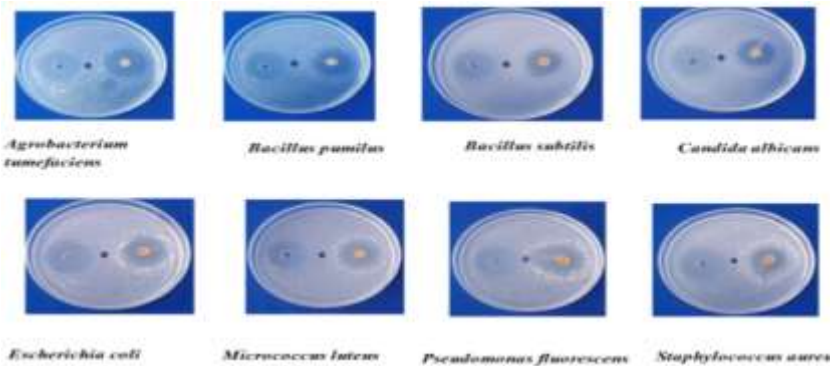


Figure 11. Screening of antimicrobial activity of crude polyphenol oxidase by agar well diffusion method

Table 7. Antimicrobial Activity of Crude Polyphenol Oxidase by Agar Well Diffusion Method

No.	Microorganisms	Inhibition zone diameters (mm)	
		Standard	Enzyme
1	*Agrobacterium tumefaciens*	28	28
2	*Bacillus pumilus*	28	27
3	*Bacillus subtilis*	28	26
4	*Escherichia coli*	28	26.
5	*Micrococcus luteus*	29	27
6	*Pseudomonas fluorescens*	29.	27
7	*Staphylococcus aureus*	28.	27
8	*Candida albicans*	29	26

diameter of agar well = 8 mm
10-14 mm = low activity
15-19 mm = good activity
20 mm above = very high activity

standard = chloramphenicol (for bacteria)
standard = nystatin (for fungus)
control = potassium phosphate buffer

Conclusion

Polyphenol oxidase from cabbage (*Brassica oleracea* L.) was successfully extracted by using the ammonium sulphate precipitation method (salting out) and partially purified by gel filtration chromatography using Sephadex G-100. The specific activity and relative purity of the enzyme increased by about 3.95 folds. The optimum pH and temperature of polyphenol oxidase were 7 and 40 ℃, respectively. The kinetic parameters, K_m and V_{max} of crude and partially purified polyphenol oxidase were determined as 0.040×10^{-5} M and 2.8×10^{-5} M min^{-1}, and 0.052 M and 3.03×10^{-5} M min^{-1}, respectively. The reaction order (n) for both crude and purified polyphenol oxidase was found to be first order. The activation energy of partially purified polyphenol oxidase was lower than that of the crude enzyme. Crude polyphenol oxidase showed very high antimicrobial activity.

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