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Characterization and immobilization of purified polyphenol oxidase extracted from banana peel

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Abstract: This study was undertaken to obtain a crude extract of polyphenol oxidase (PPO) from banana peel in addition to purification and characterization to determining the optimal temperature, pH, the storage periods , number of used times and dyes decolrization. A partial purified polyphenol oxidase from banana peel was immobilized by agarose and Calcium Alginates. The entrapment of PPO by the Calcium alginate showed the best method for the immobilization. The immobilized and free PPO enzyme properties were studied. The results of study showed the optimal pH of free and immobilized PPO enzyme activities from banana peel were 7.0; the optimal pH of free PPO enzyme stability ranged from 6.0 - 7.0. The optimal pH of immobilized PPO enzyme stability ranged between7 - 8. The optimal temperatures of free and immobilized PPO activity extracted from banana peel were 35°C and the free enzyme was stabilized in temperature 35 °C then the enzyme activity began to decrease and it was lost at 60°C; the immobilized enzyme remained stable in temperature ranged 35-55 °C. The immobilized PPO activity which incubated for more times with catechol decreased after ninth time of using, the experiment showed that the activity of immobilized enzyme decreased with long storage period. The neutral red, acridine and toluidine were decolorized and showed a change in their absorbance values after the immobilized PPO incubate for a time while no analysis occurred for other dyes.

Keywords: Polyphenol oxidase; Banana peel; Immobilization; Optimization; Decolorization.

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Introduction:

Polyphenol oxidases are defined as oxidoreductase enzymes that contain copper ion that catalyses the oxidation and hydroxylation of phenolic groups in the presence of molecular oxygen. Polyphenol oxidases are a group of enzymes found in animals, plants, bacteria and fungi in a wide range(1). In tissues of plants, PPOs are located in the membrane of chloroplasts in addition to mitochondria; tanning reaction happening after tissue disruption rich in phenol groups which lead to binding the soluble polyphenol oxidase to a particulate fraction(2). With presence of oxygen and PPO; monophenol group is hydroxylated to a diphenol which may be then oxidized to O-quinones and submits the polymerization and yields dark brown polymers(3). PPOs have many applications in the field of medicine, food processes and it is also used in waste waters treatment. Products may lose quality and value between consumption and harvest due to a process of oxidation of phenolic groups giving undesired browning result(4). PPO activity determination has many interests for estimation of the food quality; so that a reliable, rapid method to estimate the PPO activity in products

is desirable(5). PPOs were used in food industries such as flavor improvement in tea, coffee and cocoa production (6). The immobilized enzymes can be used in different fields for an expensive enzymes recovery and it is separated easily from reaction to use again (7). The aim of this study is to extraction, purification and characterization of PPO from banana peel in addition to decolorization of some dyes.

Materials and Methods:

Chemicals:

Polyethylene glycol, Catechol, bovine serum albumin and Coomassie brilliant blue were collected from Sigma CO.; while other chemicals supplied from BDH CO.

Polyphenol oxidase extraction from banana peel:

The green and yellow banana peel were washed by tap water, 25 gm from each dry banana peel was homogenized and dissolved in 100 ml phosphate buffer 0.05 M, pH 7 contain 0.5% polyethylene glycol and ascorbic acid 0.01M using blender for 1 minute only, the extract was filtered and the filtrate was centrifuged at 10000 rpm for 10 min at 4 ^{o}C, the supernatant was used as crude enzyme, the activity and protein concentration of polyphenol oxidase were estimated (8).

Estimation the activity of polyphenol oxidase:

Catechol as a substrate was used to estimate the activity of PPO. The reaction solution contained 5.8 ml of 0.02 M substrate in 0.1M phosphate buffer pH 7.0 and 0.2ml of crude enzyme, 4 ml of catechol solution was used as a blank sample(9). The catechol oxidation was estimated by measuring absorbance at 420 nm after 3 minutes of reaction time. One unit of PPO activity was considered as amount of enzyme that caused the increasing of absorbance value of 0.002/min. Determination of polyphenol oxidase activity was as in the equation:

PPO activity (U/ml): $[(A_2 - A_1) - (B_2 - B_1)] / (0.001\ t)$

A_2: Is the final absorbance value of the sample, A_1 is the initial absorbance value of the sample, B_2 is the final absorbance of the control, B_1 is the initial absorbance value of the control and t is the time of reaction in minutes(10).

Determination of the concentration of protein:

The concentration of protein was estimated by depending on the binding of Bradford dye (11) using bovine serum albumin (Standard solution).

Partial purification of polyphenol oxidase by ion exchange chromatography:

DEAE-cellulose column was prepared according to a method of (12). A crude PPO enzyme was concentrated using dialysis tube against sucrose then ion exchange chromatography was done for the concentrated sample using DEAE -cellulose column. 10 ml of the concentrated enzyme was loaded on the ion exchanger column (15.5×2.5 cm) using pasture pipette. The separated fractions were collected at a flow rate of 36 ml/h. The wash step was achieved using 0.005M Tris-HCl buffer (pH 8), while the elution step was achieved using the same buffer with a gradient

concentration of sodium chloride salt ranged between (0.1-1) N. After collection of a fraction, the absorbance of each fraction was estimated at a wavelength of 280 nm for the wash and elution fractions, the enzyme activity was calculated in the fractions then the activation parts were collected gently, then the ppo activity and the protein concentration were calculated.

Polyphenol oxidase immobilization
Entrapment using agarose:

The entrapment of enzyme using agarose experimented by dissolved 3gram of agarose in 100ml distilled water under heating then cooling it and mixing the purified polyphenol oxidase in 1:0.25(v:v), the solution was poured in a plate until solidify and cut it as square pieces then was store in $NaCH_3COO$ buffer (20mM pH 6.0), activity of immobilized ppo was determined(13).

Entrapment using Calcium alginate:

It was prepared by dissolving 5 gram of Calcium Alginate in 100ml distilled water under heating then cooling it and mixing with PPO in 1:0.25(v/v) ,then adding the mixture 200 mM Calcium chloride, the bead was washed with 0.02M $NaCH_3COO$ buffer (2M, pH 6.0). The activity and remaining activity % of immobilized enzyme were determined (5).

Free and immobilized enzymes characterization:

Estimation pH effect into PPO Activity:

Various kinds of buffer were chosen (0.1M of Sodium Acetate solution pH 4.0-6.0; 0.1M of Phosphate solution pH 7.0and 0.1M Tris HCl solution pH8.0 – 9.0) in the substrate preparation, the catechol substrate was incubated with the free and immobilized enzyme and measured their activity (14).

Estimation pH effect into PPO Stability:

Various kinds of buffer (0.1M Sodium Acetate solution pH 4.5-6.0, 0.1M phosphate solution pH 7.0 and 0.1M Tris HCl solution pH8.0-9.0) were chosen and incubated with free enzyme and immobilized enzyme in avolume1:1 for 30 minutes, the remaining activity of enzyme was determined (15).

Estimation temperature effect on PPO Activity:

Immobilized and Free enzyme incubated with Catechol solution using water bath at temperatures (35, 40, 45, 50, 55, 60 and 65 ^{o}C) for 3 min., the activity was determined for each treatment (14).

Estimation temperature effect on PPO Stability:

Free and Immobilized enzyme were incubated at various temperature values ranging (35, 40, 45, 50, 55, 60 and 65 ^{o}C) for 30 min then transferred into cold water bath directly and the remaining activities were calculated for each temperature (15).

Determination number of times using enzyme:

This experiment was achieved by incubate enzyme with catechol and

measured its activity then the PPO washed using 100mM Potassium Phosphate solution and repeated the incubation of enzyme with substrate for other times until no enzyme activities were recorded(16).

Detection of the effect of storage period on the enzyme activity:

Weight was about 1gm of immobilized PPO and measuring the enzyme activity by incubating the immobilized enzyme with the substrate, then storaged enzyme in 5 ^{o}C for 21 days and determining the enzyme activity after each 3 days (17).

Polyphenol oxidase activity in dye decolorizing:

Different types of dyes (Giemsa stain, Bromophenol blue, brilliant green, Toluidine, Acridine orange, Indigocarmine and Neutral red) were dissolved in distilled water and diluted to 10 times .A milliliter of immobilized PPO enzyme incubated with 2 ml of each one dyes solution for 3 minuites; The absorbance of dyes was determined respectively. Decolorization efficiency of PPO was noted by recording the absorbance decreasing (16).

Results and discussions:

Effect of extracted media on PPO action:

Green and yellow banana peels were used in the extraction of PPO and the results showed different specific activities. PPO from yellow banana peels gave high specific activity that reached to 1256 U/mg, while green banana peels gave 1220 U/mg. A banana peel was benefit as a source of PPO due to its considered inexpensive source and available waste. (18) found the apple was a good source for PPO production while (19) found that banana waste was the best.

Purification of PPO by ion exchange chromatography:

Ion exchange technique was experimented to the crude enzyme after concentrated by sucrose, DEAE–cellulose used as ion exchanger in the presence of 0.02M Tris-HCl buffer, pH 8.0. The results showed two peaks of protein in wash step and only one peak of them showed low enzyme activity in fractions (17-28). Three peaks appeared in elution step and only one peak showed high enzyme activity in fractions (69-84). The purification fold of this step was 2.5, yield 70 % and gave a specific activity of 1972 U/mg. Ion exchange chromatography used in many studies of PPO purification from the plant tissues. (6) used DEAE-cellulose in PPO purification from *celery root* and the purification fold of his experiment was 5.21with the yield 57.93%. This enzyme was also purified from *Pouteria sapota* by DEAE–cellulose exchanger resulted in purification fold 6.89 and enzymatic yield 34.56% (1).

Immobilization of PPO:

Enzyme Immobilization using Agarose:

The enzyme entrapment by agarose and cut into small squares, the amount of the enzyme was 46 % and gave activity 10 U/g.

Enzyme Immobilization using Calcium Alginate:

This method showed the best result for enzyme entrapment. The PPO activity was 13 U/g and the amount of free enzyme in this method was 84%. Immobilization process is depended on a polymeric network that allows the substrate and products to pass a long and enzyme retaining (13).

Optimum pH of free PPO activity:

In order to estimate the optimal pH of PPO activity, different values of pH were experimented to characterize their effect on PPO action as showed in figure (1), the pH 7.0 was the optimum pH and the activity decreased in pH value below and above 7.0. (17) found the best pH for free PPO extracted from quince was 8.0, while (20), found the optimal pH for PPO activity from potato was 7.0.

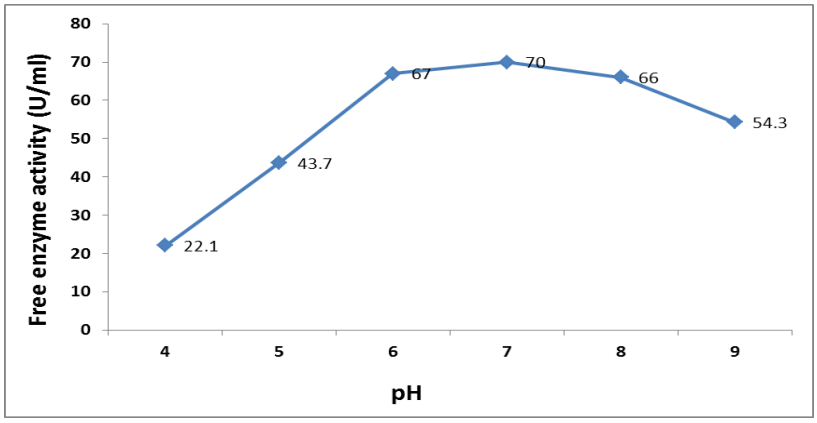

Figure (1): Optimum pH for free purified PPO Activity

Optimum pH of free PPO stability:

The optimal pH of enzyme stability was considered an important factor in order to estimate suitable conditions for enzyme storage. Results in figure (2) showed that PPO was stable at PH from 6.0 - 7.0, and it was less stable at pH 4.0 and 9.0. This decreasing was due to changes enzyme residues in the secondary and tertiary structure (21). (17) found that the pH stability for free PPO extracted from Quince was 7.0

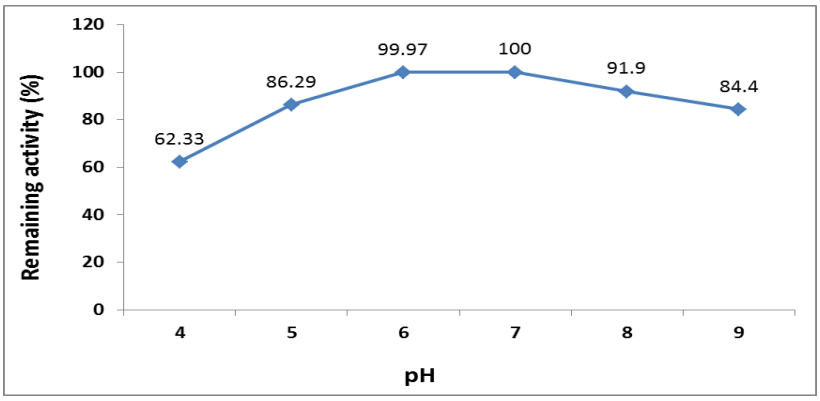

Figure (2): Optimum pH for free purified PPO Stability

Optimum pH of immobilized PPO activity:

The pH of immobilized PPO activity was estimated; the pH 7.0 was the optimal pH for immobilized PPO and the enzyme activity in pH less or more than 7.0 begin to decrease (figure 3). The pH can change the ionization state of acidic or basic groups in amino acids as a result the 3-dimention structure of enzyme (22).

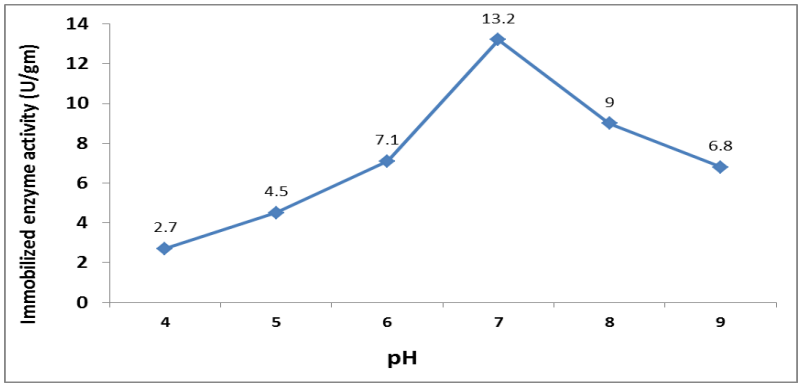

Figure (3): The Optimal pH of Immobilized PPO Activity

Optimum pH for immobilized PPO stability:

The pH of immobilized PPO stability was studied because of its importance in estimation of storage conditions of enzymes. Figure (4) shows the range of immobilized PPO stability was from 7.0 - 8.0 and the activity was very low at pH 4 and pH 9.

The pH of the enzyme environment may affect its stability and the enzyme may be denatured at an extreme level of alkalinity or acidity, in addition to the pH of the reaction solution that may lead to the substrate dissociation by its effect on the substrate which changes the character of the curve of pH activity (23).

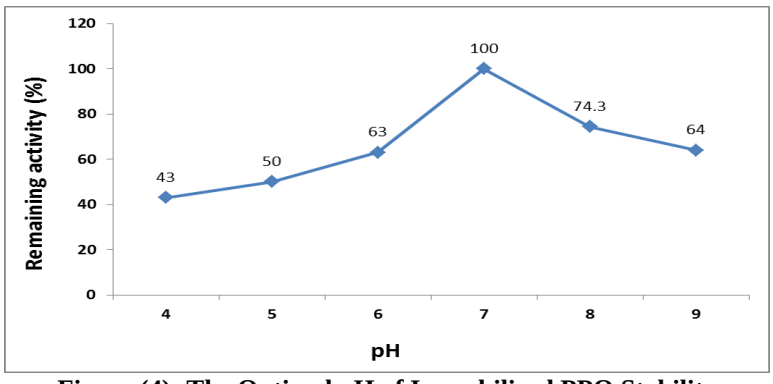

Figure (4): The Optimal pH of Immobilized PPO Stability

Effect of temperature on free PPO action:

The free Polyphenol oxidase was incubated with catechol in different temperature values that ranged between 35-70 ^{o}C for 3 min., results in figure (5) showed that 35 ^{o}C was the optimum temperature for free PPO action from banana peel and gave activity that reached to 68.5 U/ml, and the activity was lost completely at 65 ^{o}C.

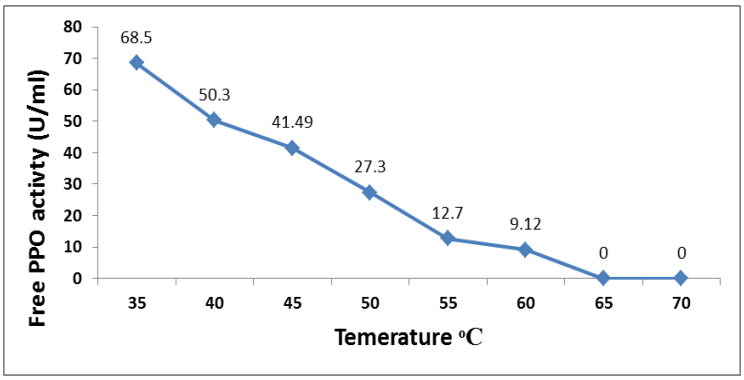

Figure (5): Optimal temperature of free purified PPO

Thermal stability of free purified enzyme:

PPO from banana peel incubated with different temperatures ranged from 35°C to 70 °C for 30 min. The result in figure 6 showed that stability of enzyme was at 35 °C and the activity was completely lost in 60 °C. The temperature values can change the enzyme structure by breaking the bonds that stabilize the protein's structure (22).

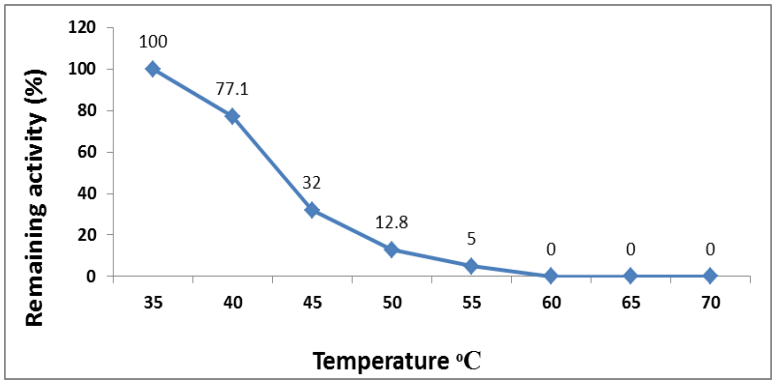

Figure (6): Thermal stability of free PPO

Optimal temperature for immobilized PPO action:

Immobilized PPO was incubated with catechol at different temperature values that ranged 35-70 °C, Results in figure (7) showed that 35 °C was the best temperature for immobilized PPO action and gave 13.4 U/g while activity was lost completely at 65.

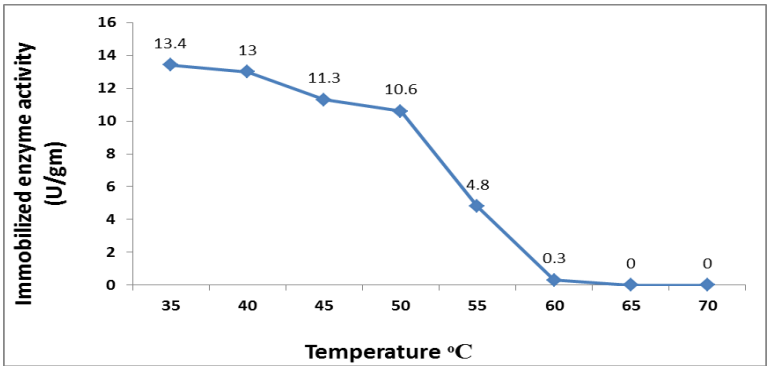

Figure (7): Effect of temperature on immobilized PPO action

Thermal stability of immobilized purified PPO:

The immobilized purified PPO from banana peel was incubated with catechol in different temperatures that ranged between 35 – 70 °C, result in figure (8) showed that the immobilized enzymes were in temperatures between 35-55°C maintained their stability and at 70 °C they were completely lost.

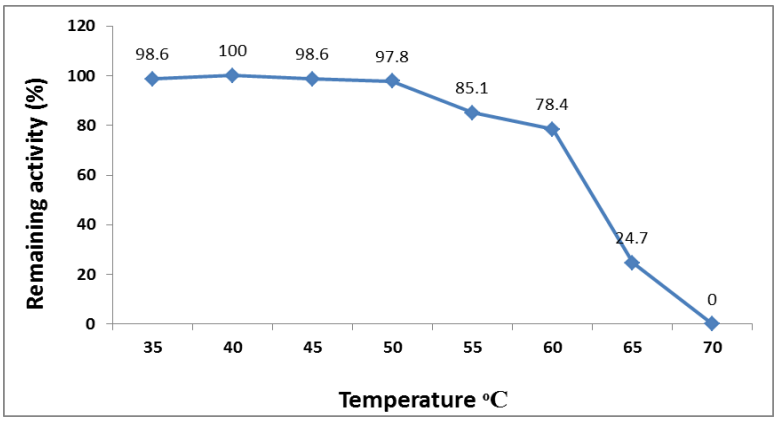

Figure (8): Thermal stability of immobilized purified PPO

Estimation number of immobilized PPO reusing:

Immobilized PPO was incubated for many times with Catechol, the result of this study showed that the enzyme activity decreased after ninth time of reuse. (17) found that the activity of immobilized PPO extracted form *Cydonia Oblonga* was lost after the fourth using.

The effect of storage time on immobilized PPO activity:

The effect of storage time on immobilized enzyme by Ca-alginate was experimented by incubation of immobilized PPO with substrate then storage immobilized enzyme in 5°C for 3 days and repeated this process every 3 days, the result of this experiment showed that the immobilized enzyme activity decreased with long storage period as in figure (9).

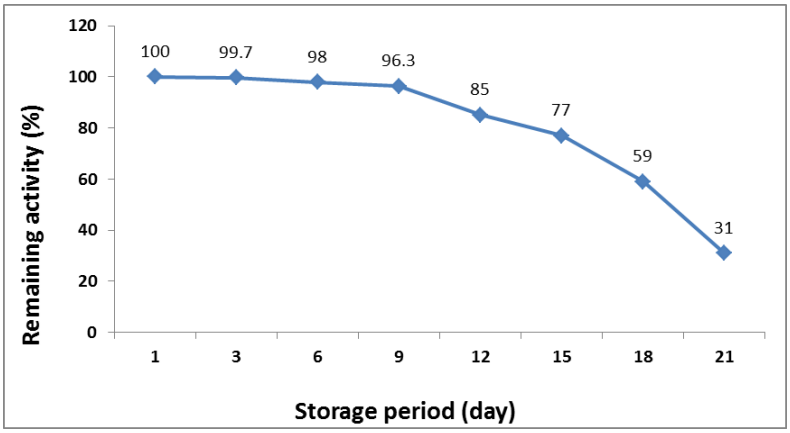

Figure (9): Effect of storage period on immobilized PPO purified from banana peel.

Dye decolorization by immobilized PPO enzyme:

The immobilized PPO was tested for their ability to decolorizing different types of dyes in order to demonstrate their importance in wastewater treatment. Results in table (1) show that immobilized purified PPO showed decolorizing of Toluidin, Neutral red and Acridine dye and reducing in absorbance in contrast to other dyes.

Table (1): Effect of immobilized purified PPO in some dyes decolorization

Relation between Dye types and Absorbance during certain time	Wave length at nm	Absorbance over a time period (minute)						
		zero	3	6	9	12	15	18
The green Brilliant	625	2.20	2.20	2.20	2.20	2.20	2.20	2.20
Bromophenol blue	590	2. 43	2.43	2.43	2. 43	2. 43	2. 43	2. 43
Giemsa stain	530	1.49	1.49	1.49	1.49	1.49	1.49	1.49
Toluidine	630	2.81	2.47	2.15	1.81	1.40	1.10	0.64
Neutral red	545	1.36	1.12	1.04	0.93	0.88	0.54	0.40
Acridine dye	495	1.39	0.97	0.82	0.71	0.60	0.56	0.50
Indigo dye	495	2.06	2.06	2.06	2.06	2.06	2.06	2.06

Conclusion:

It is possible to purify PPO enzyme partially from banana peel by ion exchange chromatography. The optimal pH for free and immobilized purified PPO was 7.0 and the stability pH for free and immobilized purified PPO of banana peel were 6.0-7.0 and 7.0-8.0 respectively.It is useful to PPO immobilization by Ca-alginate and the ability of immobilized PPO to decolorization of some dyes.

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