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Journal of Molecular Catalysis B: Enzymatic 40 (2006) 58–63
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Direct immobilization of polyphenol oxidases on Celite 545 from ammonium sulphate fractionated proteins of potato (*Solanum tuberosum*)

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Received 13 September 2005; received in revised form 16 February 2006; accepted 1 March 2006

Abstract

In this study an effort has been made to use ammonium sulphate fractionated potato proteins for the direct immobilization of polyphenol oxidase on an adsorbent, Celite 545. The yield of Celite immobilized PPO activity was maximum in the buffer of pH, 7.0 and the support retained 240 units of PPO per gram. The immobilized potato PPO preparation exhibited effectiveness factor 'η' as 0.71. Immobilized enzyme preparation was significantly more resistant to the denaturation induced by pH, temperature, urea, detergents; SDS, Triton X 100, Tween 20 and water-miscible organic solvents; acetonitrile, dimethyl formamide, dioxane and *n*-propanol as compared to its soluble counterpart. The activity of soluble and immobilized polyphenol oxidase was remarkably enhanced in the presence of increasing concentration of non-ionic detergents like Triton X 100 and Tween 20.

Keywords: Adsorption; Celite 545; Immobilization; Polyphenol oxidase; Stability; Water-miscible organic solvents

1. Introduction

Extensive research has been done to investigate the possibilities offered by enzymes in biotechnological and environmental applications [1,2]. However, the use of free enzymes show some significant drawbacks such as thermal instability, susceptibility to attack by proteases, activity inhibition, high sensitivity to several denaturing agents, the impossibility of separating and reusing free catalyst at the end of the reaction. Many of these constraints can be circumvented by using enzymes in immobilized form. The use of immobilized enzymes has proved to be more advantageous than the free enzymes [3]. Since the methods used for the immobilization of enzymes greatly influence the properties of the resulting biocatalyst, the selection of an immobilization strategy determines the process specification for the catalyst [4].

Enzyme immobilization by physical adsorption has the benefit of a wide applicability and may provide a practical convenience of simple regeneration of support by removing the deactivated enzyme and reloading the support with fresh batch of active catalyst [5]. Adsorption of enzymes on insoluble supports is one of the most inexpensive and simple immobilization techniques available. Purified polyphenol oxidases (PPO) from different sources have been successfully immobilized on various supports [6–8]. Immobilized PPO has been successfully used for the treatment of wastewater by removing/transforming the toxic compounds of industrial processes [1,2,9,10]. PPO has proved to be very useful in the analytical determination of cyanide, azide, aromatic amines, phenols and catechols such as neurotransmitter substances and related metabolites [3,11,12]. Several techniques based on the PPO treatment have been developed for the remediation of industrial wastewater but the cost of the processes has limited its use [13,14]. Diatomite carrier, such as Celite, has desirable physical properties. It is quite inexpensive and suitable for the immobilization of enzymes even directly from the partially purified enzyme preparation. The modified Celite has already been used by a number of workers for the immobilization of polyphenol oxidases such laccases [15–17]. However there is a problem of regeneration of the support after the inactivation

Abbreviations: PPO, polyphenol oxidase; S-PPO, soluble polyphenol oxidase; I-PPO, immobilized polyphenol oxidase; SDS, sodium dodecyl sulphate; DMF, dimethyl formamide; MBTH, 3-methyl-2-benzothiazolinone hydrazone

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doi:10.1016/j.molcatb.2006.03.001

of enzymes, therefore direct adsorption of enzymes on the support has been preferred in various biotechnological applications [1–3].

The main purpose of this paper is to obtain an inexpensive immobilized enzyme preparation of potato PPO. In order to reduce the cost of the immobilized enzyme preparation, partially purified potato PPO has been successfully immobilized on Celite 545. The stability of the immobilized potato PPO has been investigated against various forms of denaturants; pH, heat, urea, detergents and water-miscible organic solvents.

2. Experimental

2.1. Materials

MBTH and catechol was obtained from Sigma Chemicals Co. Ltd. (St. Louis, MO) USA. Celite 545 (20–45 μ mesh) was purchased from Serva Labs., Heidelberg, Germany. Acetonitrile, ammonium sulphate, benzoic acid, dioxane, dimethyl formamide, *n*-propanol, sodium dodecyl sulphate, Triton X 100 and Tween 20 were purchased from SRL Chemicals, Mumbai, India. Potato was obtained from the local vegetable market. All other chemicals and reagents employed were of analytical grade and were used without any further purification.

2.2. Extraction and partial purification of potato PPO

Potato (200 g) was homogenized in 400 mL of pre-cooled 50 mM sodium phosphate buffer, pH 7.0 in the presence of benzoic acid (1.8 g/L of buffer) to stop the enzymatic browning. The mixture was filtered through four layers of cheesecloth. The extract obtained was centrifuged at 3000 × *g* for 10 min at 4 °C on a Remi Cooling Centrifuge (C-24). The supernatant obtained was used as a source of enzyme and was subjected to 0–60% ammonium sulphate fractionation with continuous overnight stirring in cold. The precipitated proteins were collected by centrifugation at 10,000 × *g* for 20 min at 4 °C and the pellet obtained was redissolved in 50 mM sodium phosphate buffer, pH 7.0. The dissolved proteins were dialyzed against the same buffer containing benzoic acid (1.8 g/L). This enzyme preparation was stored at freezing temperature for further use [18].

2.3. Adsorption of potato PPO on Celite 545

Celite 545 (20 g) was suspended in 100 mL of distilled water and stirred for 1 h at room temperature. The fine particles present in the suspension were removed by decantation and this procedure was repeated at least three times [19]. The binding of potato PPO on Celite 545 was carried out by incubating 1046 U of PPO/g of the Celite 545 in the buffers of varying pH values; sodium citrate buffer (pH 5.0) and sodium phosphate buffer (pH 6.0–8.0) with overnight stirring at 4 °C. The molarity of each buffer was 50 mM. Celite bound enzyme was centrifuged to remove the unbound proteins. Immobilized enzyme preparation was suspended in 50 mM sodium phosphate buffer, pH 7.0 and was washed four to five times with same buffer. The bound enzyme was finally suspended in 5.0 mL of 50 mM sodium phosphate buffer, pH 7.0 and stored at 4 °C.

2.4. Effect of detergents on soluble and immobilized potato PPO

Soluble and immobilized PPO (4.0 U) were incubated with increasing concentration of ionic detergent; SDS (0.1–1.0%, v/v), non-ionic detergents; Triton X 100 and Tween 20 (0.2–1.0%, v/v) in 50 mM sodium phosphate buffer, pH 7.0 at 37 °C for 1 h. PPO activity was monitored at all the indicated detergent concentrations. The percent activity of soluble and immobilized PPO in assay buffer without any detergent was considered as control (100%) for the calculation of percent activity.

2.5. Effect of water-miscible organic solvents on soluble and immobilized potato PPO

Soluble and immobilized PPO (4.0 U) were incubated with increasing concentrations of water-miscible organic solvents (0–60%, v/v) in 50 mM sodium phosphate buffer, pH 7.0 at 37 °C for 1 h. PPO activity was assayed at all the indicated organic solvent concentrations. The percent activity of soluble and immobilized PPO in assay buffer without any detergent was considered as control (100%) for the calculation of percent activity.

2.6. Assay of the PPO activity

The method of Batra and Gupta [18] with some slight modifications was used to assay the PPO activity. The reaction was initiated by adding a definite quantity of enzyme to a reaction volume containing 1.0 mL of 45 mM catechol and 0.05 mL of 1.0% MBTH (dissolved in methanol) in a total volume of 2.0 mL with 50 mM sodium phosphate buffer, pH 7.0. The reaction mixture was incubated at 37 °C for 1.5 min which was then stopped by adding 1.0 mL of 10% H_2SO_4. The reddish colored appeared due to the complex formation between catechol-generated quinones and MBTH was measured at 500 nm using Cintra 10*e* spectrophotometer.

One unit of PPO activity is defined as the amount of enzyme protein that catalyzes the formation of MBTH–quinone complex with 0.05 increases in optical density per minute at 500 nm.

In case of immobilized enzyme the procedure for assaying activity was same except that the reaction mixture was continuously stirred during the progress of reaction. After the reaction, the mixture was centrifuged at 3000 × *g* for 5 min before measuring absorbance [19].

2.7. Determination of protein concentration

Protein estimation was done according to the procedure described by Lowry et al. [20]. Bovine serum albumin was used as a standard.

Table 1
Immobilization of potato PPO on Celite 545

Enzyme loaded (X) (U)	Enzyme activity in washes (Y) (U)	Activity bound/g of Celite 545 (U)			% Activity yield ($B/A \times 100$)
		Theoretical ($X - Y = A$) (A)	Actual (B)	Effectiveness factor (η) (B/A)	
1046	708	338	240	0.71	71

Each value represents the mean for three-independent experiments performed in duplicate, with average standard deviation < 5%.

3. Results and discussion

PPOs from potato are the most potential enzymes with respect to its availability and cost. Simple ammonium sulphate precipitated total proteins were considered for PPO activity. Salt fractionated proteins exhibited 96 units of PPO activity per gram of intact potato.

3.1. Adsorption of potato PPO on Celite 545

The diatomite carriers have been used as a support for the immobilization of enzymes [21,22]. The extraordinary selectivity of Celite for tyrosinase and other blue proteins suggested that this material might be useful for the isolation of copper containing proteins [23]. PPOs from potato are copper containing enzymes [24]. Simple ammonium sulphate precipitated total proteins were considered for PPO activity. Salt fractionated proteins exhibited very high enzymatic activity. These proteins were immobilized on Celite 545. The binding of PPO on Celite 545 was significantly affected by altering the pH of the buffer. The enzyme was maximally adsorbed at pH 7.0 and retained 240 U of PPO/g of Celite 545 with an effectiveness factor 'η' of 0.71 (Table 1). Above and below this pH, the binding of PPO on Celite decreased. At pH 5.0, the binding of enzyme activity was only 68% as compared to the maximum binding at pH 7.0 (Fig. 1). Several earlier workers have also reported the pH-dependent adsorption of PPO on various other supports [18].

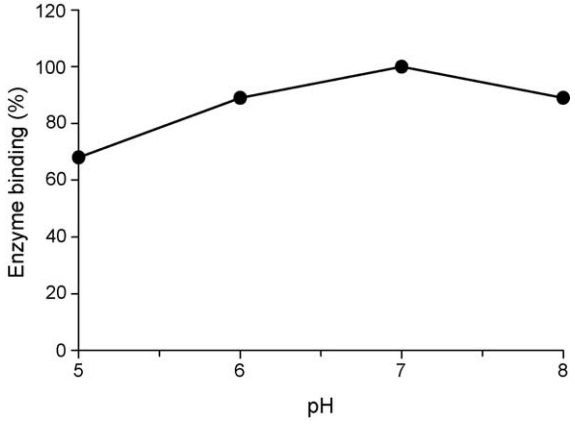

Fig. 1. Effect of pH on the adsorption of potato PPO on Celite 545. Soluble potato PPO was stirred with Celite 545 in the buffers of varying pH values 5.0–8.0 and stirred at 4 °C overnight. Unbound proteins were removed by centrifugation. Enzyme adsorbed matrix was washed three to four times with 50 mM sodium phosphate buffer, pH 7.0. The activity of potato PPO in supernatants, washes and bound to the Celite at each pH was measured by the procedure described in the text. Symbol (●) indicates the % binding of potato PPO on Celite at a particular pH.

3.2. Stability studies of immobilized polyphenol oxidases

In order to check the compatibility of the immobilized enzyme preparation for the treatment of various aromatic compounds present in industrial effluents/wastewaters, the stability of the adsorbed preparation was compared with its soluble counterpart.

3.2.1. Effect of pH on soluble and Celite bound potato PPO

Both soluble and Celite bound potato PPO preparations exhibited pH-optima at pH 6.0. However, immobilized enzyme showed broadening in pH–activity profile as compared to the soluble enzyme. There was no difference in pH-optima between pH 5.0 and pH 6.0 for the immobilized potato PPO. The immobilized potato PPO preparation retained significantly higher fraction of enzyme activity in the acidic range (Fig. 2). This observation was in agreement with other earlier published work [21,25]. In a recent study we have shown that the brinjal PPO bound to Celite had higher pH-optima as compared to potato PPO [19]. Celite bound brinjal PPO also exhibited a shift in pH-optima from pH 8.0 to 9.0 while the pH-optima for both soluble and immobilized potato PPO was at pH 6.0.

3.2.2. Effect of temperature on soluble and Celite bound potato PPO

Fig. 3 illustrates the result of temperature–activity profiles of soluble and Celite bound potato PPO. Soluble form of potato PPO exhibited its temperature-optima at 40 °C whereas the

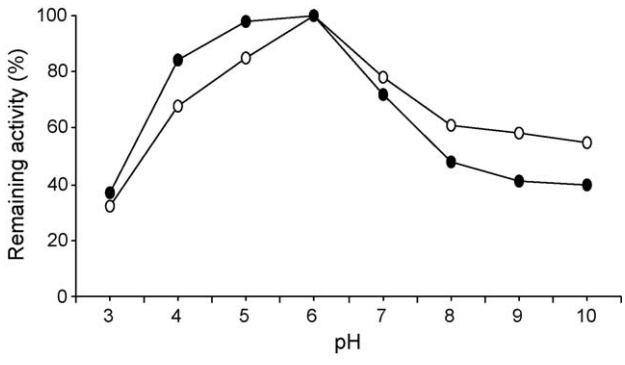

Fig. 2. pH–activity profiles of soluble and Celite bound potato PPO. An appropriate and equal amount of soluble and immobilized PPO was taken for determining the activity at all the indicated pH. The buffers used were glycine–HCl (pH 3.0 and 4.0), sodium acetate (pH 5.0 and 6.0), sodium phosphate (pH 7.0) and Tris–HCl (pH 8.0–10.0). The activity was determined in each buffer at 37 °C for 1.5 min. The molarity of each used buffer was 50 mM. The symbols indicate the soluble (◯) and immobilized (●) enzyme.

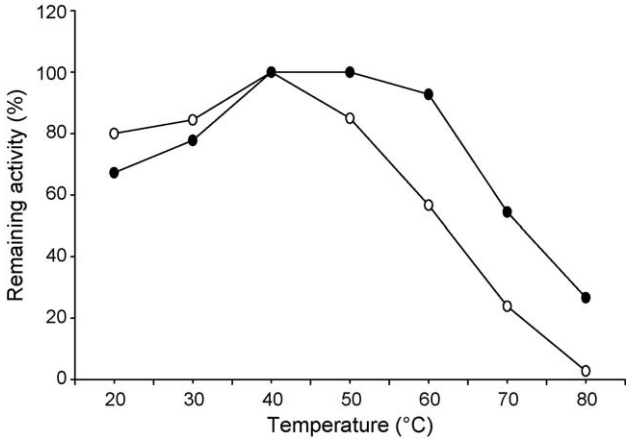

Fig. 3. Temperature–activity profiles for soluble and immobilized potato PPO. Soluble and immobilized PPO preparations (4.0 EU) were taken to assay the activity at various indicated temperatures. Activity expressed at 40 °C was taken as control for the calculation of percent activity. For symbols refer to Fig. 2 legend.

immobilized preparation had no difference in maximum activity between 40 and 50 °C. Celite bound PPO retained greater fraction of the catalytic activity at higher temperatures as compared to their soluble counterpart. Soluble potato PPO completely lost its activity at 80 °C whereas the immobilized potato PPO preparation retained 27% of the original PPO activity. Some earlier investigators have also reported an alteration in temperature-optima, of fig tree latex ficin immobilized on Celite, from 60 to 80 °C [21].

Soluble and immobilized potato PPO preparations were incubated at 60 °C for various time intervals. Incubation of soluble enzyme at 60 °C resulted in a loss of nearly 42% activity after 2 h whereas immobilized enzyme retained about 77% of original activity under similar incubation conditions. These observations demonstrated that Celite bound preparation was significantly more stable as compared to soluble counterpart when thermal denaturation studies were done at 60 °C (Fig. 4). There were reports about the heat sensitivity of mushroom tyrosinase, soluble mushroom PPO rapidly lost its activity even after 10 min incubation at 55 °C [8]. Contrary to these results our findings demonstrated that the Celite bound PPO was remarkably more stable to heat inactivation. However, Celite bound potato PPO also proved its better stability as compared to the Celite bound brinjal PPO [19].

3.2.3. *Effect of urea on soluble and Celite bound potato PPO*

Soluble and immobilized PPO preparations were incubated with 4.0 M urea for varying times at 37 °C. Immobilized enzyme was found to be far superior in stability as compared to its soluble counterpart. Soluble enzyme retained half of its initial enzyme activity after 2 h of incubation while the immobilized enzyme exhibited an enhancement in enzyme activity. There was only a marginal loss in its original activity after incubation with 4.0 M urea for 2 h under other similar incubation conditions (Fig. 5).

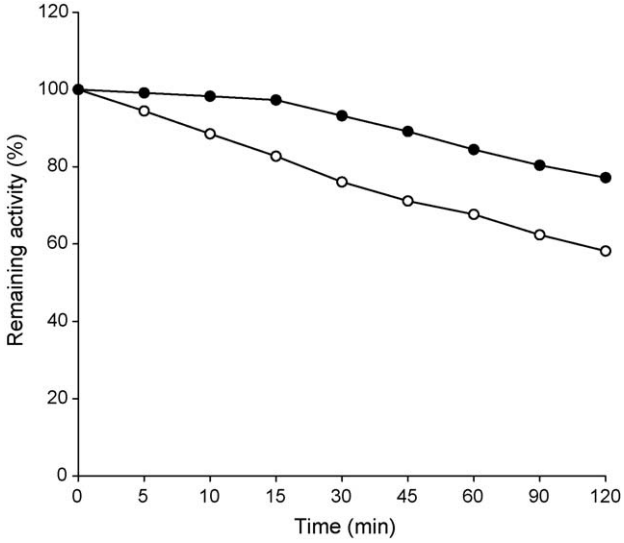

Fig. 4. Thermal denaturation of soluble and immobilized potato PPO. The soluble and immobilized PPO preparations were incubated at 60 °C in 50 mM sodium phosphate buffer, pH 7.0. Aliquots of each preparation were taken at various time intervals and enzyme activity was determined. Samples not exposed to heat were considered as control for the calculation of percent activity. For symbols refer to Fig. 2 legend.

3.2.4. *Effect of detergents on soluble and Celite bound potato PPO*

Several detergents are also present in wastewaters; therefore we have evaluated the stability of Celite bound potato PPO against a pure anionic detergent, SDS and some non-ionic detergents, Triton X 100 and Tween 20. Soluble and immobilized potato PPO preparations were exposed to various concentrations of SDS (0.1–1.0%, w/v). Incubation of soluble enzyme with 1.0% SDS for 1 h resulted in a loss of 61% of its original enzyme activity whereas the immobilized enzyme retained more than 83% of the initial activity under identical incubation conditions (Fig. 6). However, when the potato PPO was incubated

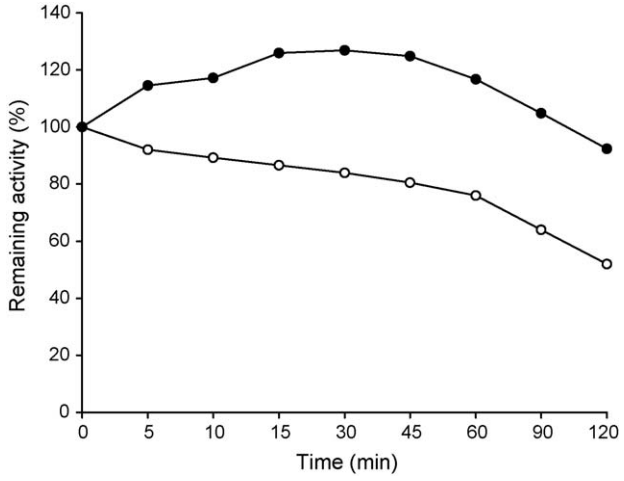

Fig. 5. Effect of 4.0 M urea on soluble and immobilized potato PPO. The soluble and immobilized potato PPO preparations were incubated with 4.0 M urea in 50 mM sodium phosphate buffer, pH 7.0 at 37 °C. Aliquots of an appropriate and equal amount were withdrawn from both the preparations at varying times and enzyme activity was determined. For symbols refer to Fig. 2 legend.

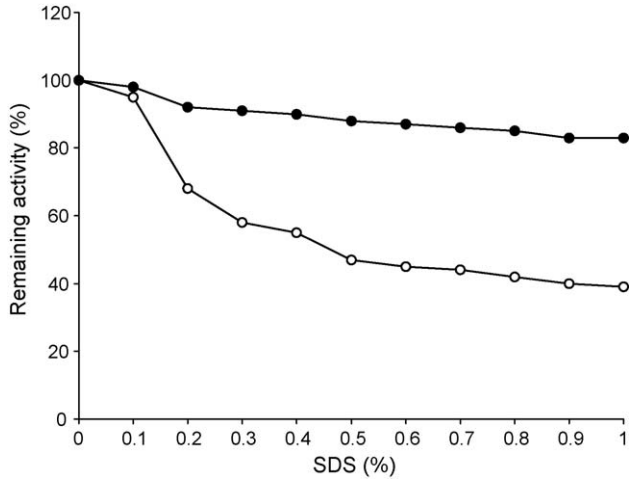

Fig. 6. Effect of SDS on soluble and immobilized potato PPO. Soluble and immobilized PPO preparations (4.0 U) were incubated with SDS (0–1.0%) prepared in 50 mM sodium phosphate buffer, pH 7.0 at 37 °C. Enzyme activity was determined after 1 h of incubation. For symbols refer to Fig. 2 legend.

with increasing concentration of (0.2–1.0%, v/v) Triton X 100 and Tween 20, there was a remarkable stimulation in the activity of soluble and immobilized PPO preparations. However, the soluble PPO preparation showed less activation as compared to immobilized preparation. Soluble PPO showed an activation of 206% with 1.0% Triton X 100 while the immobilized preparation was activated to 351% by the same amount of Triton X 100 (Table 2).

The activity of soluble potato PPO was stimulated to 206% when exposed to 0.2% Tween 20 for 1 h at 37 °C. On further exposure to increased concentration of Tween 20 there was a decrease in activation but still retained 138% of the initial activity after 1.0% Tween 20 exposure under other similar conditions. In case of immobilized potato PPO the activation by Tween 20 exposure was remarkably more pronounced and this preparation exhibited 544% of the original enzyme activity after exposure with 1.0% Tween 20 (Table 2). These results suggested that potato PPO was in its partially active form, which was fully activated in presence of lower concentrations of various detergents. There were various reports regarding the stimulation of PPO activity by means of detergent treatments [26,27]. The activation of immobilized potato PPO activity by the presence of some pure detergents suggested that this behavior is significantly fruitful for using such preparations for the treatment of organic pollutants even in the presence of such type of detergents.

Table 2
Effect of Triton X 100 and Tween 20 on soluble and immobilized potato PPO

(%) Detergent	(%) Observed activity in the presence of detergents			
	Triton X 100		Tween 20	
	S-PPO	I-PPO	S-PPO	I-PPO
0.2	86	66	206	222
0.4	157	153	172	303
0.6	201	218	141	370
0.8	203	263	136	482
1.0	206	351	137	544

PPO activity was assayed at all the indicated detergents concentrations and other assay conditions were the same as mentioned in the text. Each value represents the mean for three-independent experiments performed in duplicate, with average standard deviation < 5%.

3.2.5. *Effect of organic solvents on soluble and Celite bound potato PPO*

The soluble and immobilized enzyme preparations were treated with increasing concentrations of various water-miscible organic solvents like DMF, dioxane, *n*-propanol and acetonitrile. The immobilized enzyme preparation exhibited retention of very high enzyme activity even when the strength of the organic solvent was very high as 60% (v/v) whereas the soluble enzyme preparation lost its activity rapidly when exposed to water-miscible organic solvents (Table 3).

Table 3 demonstrates that the soluble enzyme had lost about 39% of its initial enzyme activity upon exposure to (50%, v/v) DMF while the immobilized enzyme exhibited more than 90% of its original activity under the similar incubation conditions. Furthermore the effect of dioxane (0–60%, v/v) on the activity of soluble and immobilized PPO was examined. Soluble enzyme retained about 24% of its initial activity after exposure with 60% DMF whereas the immobilized enzyme showed over 38% of its initial activity under identical exposure (Table 3). The activity of soluble enzyme was decreased by the exposure of the 60% *n*-propanol and exhibited 75% of its initial activity while the immobilized enzyme showed an enhancement (140%) of its original activity.

Table 3
Effect of water-miscible organic solvents on soluble and immobilized potato PPO

(%)Organic solvent	Remaining activity (%)							
	Dioxane		DMF		*n*-propanol		Acetonitrile	
	S-PPO	I-PPO	S-PPO	I-PPO	S-PPO	I-PPO	S-PPO	I-PPO
10	100	101	110	129	108	125	121	122
20	90	106	111	141	107	165	89	90
30	73	104	101	141	89	150	67	70
40	66	89	86	122	75	140	50	55
50	62	92	61	90	75	140	40	48
60	60	87	24	38	75	140	19	40

PPO activity was assayed at all the indicated organic solvents concentrations. Each value represents the mean for three-independent experiments performed in duplicate, with average standard deviation < 5%.

In presence of 60% (v/v) acetonitrile soluble enzyme expressed only 19% of its initial activity while the immobilized enzyme retained over 40% of its original activity. At lower concentration of organic solvents up to 30% (v/v) the activity of soluble and Celite bound PPO was stimulated. Moreover this stimulation was more pronounced in case of immobilized PPO preparation.

The observations regarding the stabilization of PPO activity against several forms of physical and chemical denaturants are in agreement with earlier published data. Several investigators have already shown the immobilization of potato PPO via adsorption on chitin, chitosan, and Eudragit S-100, etc. supports resulted in the stabilization of PPO activity against water-miscible organic solvents [5,18].

4. Conclusion

Here an attempt has been made to obtain a simple, inexpensive, stable and reusable immobilized potato polyphenol oxidase preparation. Simple ammonium sulphate precipitated total proteins of potato were taken for immobilization of PPO on Celite 545. This work indicates that the Celite adsorbed potato polyphenol oxidase preparation exhibited very high stability against heat, pH, urea, water-miscible organic solvents and detergents. The soluble and immobilized potato PPO activity was markedly stimulated by the exposure of non-ionic detergents such as Triton X 100 and Tween 20. Immobilized enzymes have advantages due to their high stability against several forms of chemical and physical denaturants such as temperature, pH, urea, SDS and water-miscible organic solvents. The use of cheaper source of enzyme and support will definitely minimize the cost of immobilization of enzyme and it will provide a suitable system for the treatment of huge volume of wastewater in batch processes as well as in continuous reactors.

Acknowledgements

Council of Scientific and Industrial Research, New Delhi, India is gratefully acknowledged for financial assistance in the form of project entitled “Decolorization and detoxification of textile and some other industrially important dyes from the wastewater by using immobilized polyphenol oxidases”. The authors are also thankful to the University Grants Commission and DST, New Delhi, India for providing special grants to the Department in the form of DRS and FIST, respectively for developing infrastructure facilities.

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