Chapter 2

Immobilized Enzymes – Characteristics and Potential Applications in Synthetic Dye Color Removal

Farrukh Jamal and Sangram Singh

Additional information is available at the end of the chapter

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Abstract

In recent times, enzymatic approaches have been used in treatment of colored wastewater/industrial effluents. Peroxidases are very useful in removal of recalcitrant toxic compounds or transforming them into innocuous products. Although much attention has been paid in the utilization of biocatalysts in several fields, their involvement in solving the environmental problems has gained support. Enzymes in soluble states have limitations of catalytic ability and stability. As the complexity of the effluents increases, the ability of the enzymes to execute its catalytic potential decreases. Therefore, one of the approaches to improve stability, catalytic ability, reusability and shelf life of enzymes is by immobilization. Work in the area of enzyme technology has provided significant clues that facilitate using enzymes optimally at large scale by cross-linking, entrapping and immobilizing. The current article presents an insight into the use of peroxidases immobilized on several different supports for the dye color removal of synthetic dyes as well as several different contaminants.

Keywords: Peroxidases, Immobilization, Dye decolorization, Continuous reactor

1. Introduction

Technological innovations have contributed immensely in wastewater treatment especially with respect to removal of synthetic dye color. Enzymatic approaches have immense potential and consequently have gained much attention in recent times. These biomolecules are not only ecofriendly but exhibit specificity in targeting and reducing hazardous wastes. Peroxidases (EC 1.11.1.7) are a key to wastewater treatment and distributed in a variety of plants, animals and microorganisms. These are heme-containing enzymes with specificity towards a wide spectrum of substrates. Essentially, these enzymes require hydrogen peroxide to act on a variety of aromatic compounds. The enzyme in its presence is oxidized to a catalytically active

form which subsequently reacts with phenolic contaminants. During their course of action they get inactivated which is due to the formation of free radicals which are adsorbed on the enzymes catalytic site and blocks the binding of the substrate molecules.

Peroxidases are very useful in removal of recalcitrant toxic compounds or transforming them into innocuous products [1, 2]. The characteristic of a given waste upon treatment with peroxidases is sufficiently changed so that it is more amenable to subsequent treatments. Being enzymes, they have higher reaction rates, milder operating conditions (relatively low temperature and in the entire aqueous phase pH range) greater stereo-specificity, and consequently in comparison to chemical catalysts are selective and efficient. Although, biocatalysts have been exploited in several areas, their use in dealing with environmental problems is the need of the time. Soluble form of enzymes has inherent limitations of stability and the complexity of effluents make them vulnerable to denaturation and consequently loss of catalytic potential. To a certain extent these limitations can be overcome by immobilizing these enzymes on suitable supports which would improve their catalytic parameters.

Peroxidases in conjunction with hydrogen peroxide have been extensively used in removal of aromatic compounds present in effluent discharge. Enzymatic treatment of wastewater has certain merits over conventional strategies. While there is simplicity in controlling the enzymatic processes, they can be exploited in a wide range of pH, temperature, salinity, contaminant concentration, recalcitrant materials. Nevertheless, the effective use of these enzymes was limited due to their non-reusability, high purification cost and sensitivity to the denaturants in wastewater. In order to overcome some of these constraints immobilization of these enzymes on various supports opens up new avenues for exhaustive utilization. With the advancement of technology, enzymes can be immobilized optimally at large scale on several different supports by cross-linking and entrapping. The current article provides an insight of using simple supports to immobilize peroxidases and testing it for dye color removal of synthetic dyes

2. Enzyme Immobilization: Characteristics and applications

2.1. Immobilization of polyphenol oxidase on Celite 545

Achieving immobilization of enzymes by simple adsorption has a practical advantage of regeneration of support and consequently easy replacement of deactivated enzymes with a fresh batch of active catalysts [3]. An insoluble support is not only inexpensive but the relatively simple adsorption techniques make the approach interesting. Polyphenol oxidases (PPO) finds application in the analytical determination of cyanide, azide, aromatic amines, phenols and catechols such as neurotransmitter substances and related metabolites [4-6]. PPO have been immobilized on various supports and used for the treatment of wastewater [7]. Although, 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 [8, 9].

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Celite is an inexpensive diatomite carrier with properties desirable for the immobilization of enzymes. It has been used as a support even while using partially purified protein preparations [10]. Celite exhibits preferential selectivity for tyrosinase and other blue proteins indicating that this material is useful for isolating copper containing proteins [11]. PPOs from potato were immobilized on Celite 545 [12]. The adsorption of PPO on Celite 545 was significantly affected by varying the pH of the buffer [13]. Maximum binding was recorded at pH 7.0 whereas above and below this pH, the binding decreased. For instance upon altering the pH to 5.0, the bound enzyme activity was only 68% as compared to the maximum at pH 7.0. Similar variation has also been documented for immobilization of PPO on various other matrices [14]. While studying the influence of pH on soluble and Celite-PPO, immobilized preparation exhibited wider pH-activity profile with no difference in pH-optima between pH 5.0 and pH 6.0. Also, immobilized PPO retained significantly higher fraction of enzyme activity in the acidic range [15]. Celite bound PPO from brinjal shows higher pH-optima as compared to potato PPO [7].

Temperature activity profile is an important parameter to assess the functional suitability of the enzyme which may be subjected to varying thermal conditions. Celite - PPO retained greater fraction of the catalytic activity at higher temperatures. A complete loss in activity was noticed at 80°C with soluble PPO fractions whereas the immobilized counterpart retained 27% of the original PPO activity [7]. Similar alteration in temperature optima was reported for fig tree latex ficin immobilized on Celite [15]. The retention of biological activity in thermal denaturation studies suggested that Celite bound preparation was significantly more stable as compared to soluble counterpart. Celite bound potato PPO has better stability as compared to the Celite bound brinjal PPO [7].

As compared to soluble counterparts, immobilized states of enzymes were superior in stability towards denaturants. Immobilized enzymes exhibited an enhancement in catalytic activity as against soluble states which were able to exhibit half of its initial activity after an incubation of 2 h. Treatment with 4.0 M urea for 2 h under similar conditions did not cause much loss in catalytic potential. Effluent discharge is also loaded with several detergents. It was observed that soluble form of peroxidases in 1.0% SDS for 1 h lost 61% of its original activity, the immobilized counterparts retained over 83% of initial activity under similar experimental conditions. Potato PPO on incubation with increasing concentration of Triton X 100 and Tween 20 (0.2 – 1.0%, v/v) underwent catalytic stimulation. Immobilized preparations of PPO were more activated as compared to soluble states of enzymes. Findings are suggestive of existence of potato PPO in its partially active form, which was fully activated in presence of lower concentrations of various detergents [16, 17]. 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.

The behavior of soluble and immobilized enzyme preparations upon exposure to various water-miscible organic solvents like (dimethylformide) DMF, dioxane, n-propanol and acetonitrile suggested that the immobilized enzyme preparation exhibited retention of very

high enzyme activity even when the strength of the organic solvent was very high whereas the soluble enzyme preparation lost its activity rapidly when exposed to water-miscible organic solvents. The immobilized enzymes exhibited over 38% of its initial activity as compared to soluble form which could retain only about 24% activity under identical exposure to 60% dimethylformide. In the presence of 60% n-propanol immobilized enzymes showed enhanced activity whereas soluble form underwent a decrease in activity, manifesting only 75% of its initial activity. An exposure of these two states of enzymes to 60% (v/v) acetonitrile suggested better performance of immobilized preparation expressing 40% in comparison to soluble forms with only 19% of the original activity. The activity of Celite bound PPO and free form enzymes underwent stimulation in enzymatic activity at concentrations of organic solvent up to 30% (v/v). In case of immobilized PPO, the stimulation was more pronounced. Several investigators have 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 [3, 18].

2.2. Immobilization of bitter gourd (*Momordica charantia*) peroxidase on DEAE cellulose

An anion exchanger, diethyl aminoethyl (DEAE) cellulose has been used to immobilize the salt fractionated and dialyzed bitter gourd proteins (BGP) [19]. DEAE-BGP preparation was compared with its soluble counterpart for its stability against pH, heat, urea, detergents, water-miscible organic solvents and proteolytic enzyme (trypsin). BGP was immobilized in very high yield on DEAE cellulose and it bound 590 units of BGP/g of the ion exchanger. The preparation was highly active and exhibited very high effectiveness factor. Effectiveness factor of an immobilized enzyme is a measure of internal diffusion and reflects the efficiency of the immobilization procedure. In this case, the yield of immobilization was quite superior over other methods used for the immobilization of peroxidases [20, 21]. BGP was tightly associated with DEAE cellulose as treatment with 0.5 M NaCl did not resulted in any significant detachment of enzyme molecules. This matrix bound BGP exhibited high degree of stabilization against pH, thermal and denaturation with urea. These findings suggested the use of DEAE cellulose as matrix for high yield and stabilization of enzymes and proteins that have also been supported by other workers [22, 23]. The immobilized BGP preparation exhibited broadening in pH–activity profiles as was the case with Con A-Sephadex bound BGP. DEAE cellulose and Con A-Sephadex bound BGP was remarkably stable against trypsin mediated proteolysis [24]. DEAE cellulose bound BGP was quite resistant against denaturation induced by detergents. The native conformation of enzymes is unaffected in the presence of lower concentration of detergents. Lower concentration of detergents improves the catalytic activity of enzymes more so in immobilized states which also exhibit remarkable stability against higher concentrations of detergents [4].

The structure of enzymes can be affected by the presence of organic solvents and several aromatic compounds present as pollutants. Therefore, it is essential to assess the stability of enzymes against such solvents. BGP immobilized on DEAE matrix are stable on exposure to dioxane, DMSO and propanol. Immobilization involving multipoint confers protection against organic solvents in co-solvent mixtures [25, 26]. Several workers have shown that potato

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polyphenol oxidase adsorbed on chitin exhibited variation in their properties as compared to free form in aqueous-organic co-solvent mixtures. They have also dwell on polyphenol oxidase, peroxidase, trypsin and acid phosphatase and showed that within a specific concentration range of water-miscible organic solvent in the medium the activity of enzymes are stimulated [3]. It has also been shown that enzymes immobilized on protein supports were also quite resistant to denaturation induced by various water-miscible organic solvents [27, 28].

BGP preparation has enhanced stability against pH, heat, urea, proteolysis, detergents and water-miscible organic solvents when immobilized on DEAE matrix. This aspect of improved stability to various form of denaturing conditions upon immobilization has been described elsewhere [22, 29, 30]. The resistance to protease is an additional attribute to the adsorption of BGP on anion exchanger. Looking at the convenience of immobilization and retention of catalytic activity, DEAE bound BGP may open up new avenues in treatment of organic pollutants in effluents from industrial units. As only non-covalent interactions hold the BGP to DEAE cellulose, desorption of enzyme from support cannot be ruled out. Additionally, these enzymes could cross-linked by bi-functional or multifunctional reagents to prevent the leakage of peroxidases from matrix.

2.3. Immobilization of turnip (*Brassica rapa*) peroxidase on bioaffinity support

Polysaccharides could be used for bioaffinity based purification of Con A from the extract of jack bean. Bioaffinity based media can be prepared for immobilizing enzymes obtained from crude preparations. Turnip peroxidase (TP) was immobilized on Con A-cellulose support and it retained 672 U of TP/g of the matrix [24, 31, 32]. Con A-cellulose bound TP exhibited very high stabilization against the inactivation induced by pH, heat, urea and guanidinium-HCl denaturation.

Several earlier investigators have also reported about the use of Con A support for high yield and stable immobilization of glycoenzymes [31, 33]. Lower concentrations of various detergents enhanced activity of soluble and immobilized TP. Immobilized TP preparation was sufficiently stable against high concentration of several detergents. Such immobilized systems could be used for treatment of hazardous aromatic substances. Enzymatic structure are affected by pollutants in wastewater. Con A cellulose bound TP was remarkably more resistant against the inactivation mediated by DMF, dioxane and n-propanol. Akhtar et al. [24] have demonstrated that bitter gourd peroxidase immobilized on Con A-Sephadex support behaved differently compared to soluble enzyme in aqueous–organic co-solvent mixtures.

Fernandes et al. [34] improved HRP organic solvent tolerance by immobilization. The immobilized soybean seed coat peroxidase exhibited full activity in the presence of organic solvent of concentration range (5-70%, v/v). The soluble form of enzyme was almost inactive in 50% (v/v) of solvents assayed. Enzyme catalysis in organic solvents is possible if structural losses are kept minimum in organic solvents and to achieve such performances from enzymes the Con A- cellulose associated TP seems feasible [35, 36]. Stability to heat, pH, urea, guanidinium-HCl, detergents and several water-miscible organic solvents is remarkable on using Con A-cellulose as matrix to immobilize TP. Immobilization of glycoenzymes on Con A lectin support

improves the stability against several denaturants as reported elsewhere and consequently holds potential in wastewater treatment [31, 37].

2.4. Concanavalin A-wood shaving immobilized turnip peroxidase in dye color removal

Wood shaving (WS) is an inexpensive support that finds use in immobilizing enzymes. Bioaffinity support has been prepared using concanavalin A from Jack bean. Salt fractionated turnip peroxidase was immobilized on Con A-WS support. This immobilized protein under varying internal conditions was compared with soluble form in its ability to decolorize direct dyes and its mixtures in batch and continuous reactor system [38]. Con A-WS immobilized TP retained an activity of about 67% as that of initial value. The non-covalent binding of glycoprotein to bioaffinity column adsorbed with Con A is a successful strategy to enhance glycoprotein immobilization. While studying its effectiveness it was observed that immobilized TP (ITP) exhibited a higher dye color removal as compared to soluble forms. Con A-WS served as an excellent bioaffinity column that conferred additional resistance to enzymes in extremes of pH [39]. DR23 and dye mixtures were effectively decolorized by ITP in batch processes at elevated temperatures where as performances were limited to soluble TP. At elevated temperatures the thermal stability improved due adsorption of enzymes with lectin [24, 40].

Dye solutions with sodium chloride (0.5 M) were effectively decolorized by ITP up to 70%. Textile effluents are often contaminated with heavy metals [41, 42]. ITP exhibited better performance in dye color removal of dye solutions containing metal ions suggesting its importance in removal of aromatic pollutants from industrial discharge. The potential of ITP in dye color removal in the presence of 10% and 30% dioxane is excellent. Although soluble enzymes are susceptible to denaturation, ITP showed better catalytic potential as multipoint attachment stabilized the protein structure and prevents the ease of denaturation [39]. Enzymatic catalysis in organic solvents is possible if the organic solvent does not substantially disturb the active site structure [36]. As immobilized enzymes are reusable the processes becomes cost effective. Over 40% of decolorizing activity was still retained when DR23 was treated with eighth repeated use of ITP.

One of the important advantages of immobilized enzyme is its reusability, which influences the cost of industrial applications [24]. Further, the efficiency of ITP was assessed at large scale using two-reactor system [38]. The performance of the reactors was quite efficient as they fulfilled the following requirements: (i) limited accessibility to the immobilized enzyme does not seem to play a role since decolorization by free enzyme was never better than with immobilized preparation [43], (ii) retention of the immobilized enzyme in the reactor in order to maintain an effective operation [44], (iii) maintenance of a good flow rate in order to ensure efficient dye decolorization/degradation [45], and (iv) the performance of the continuous reactor was improved in terms of dye degradation by using activated silica in the second reactor, which helped in the adsorption of toxic reactive species [46].

Water free of dyes (DR 23 and mixture) was obtained when applied on to a double reactor system. ITP present in the first column acted on the dyes which were adsorbed in the second column containing activated silica. The reactors functioned efficiently for over 90 days. In the

presence of redox mediator, 1-hydroxybenzotriazole (HOBT); ITP caused significant dye color removal of DR 23 and its mixtures. While studying the absorption spectra it was noticed that the disappearance of peak in the visible region was either due to the removal of pollutants in the form of insoluble products or due the degradation of chromophores in the dyes [24, 47, 48].

The decolorization efficiency of DR 23 and mixtures were different. As compared to individual phenol/dye, biodegradation of complex mixtures of dyes and phenolic compounds were quite slower [24, 49]. One of the possible explanations could be the competition between various phenols/dyes for the enzyme catalytic site or the compounds were recalcitrant and underwent slow transformation. Argument supports the view that industrial effluents containing dyes and its mixtures could be subject to treatment by ITP.

In the presence of ITP the treated polluted water had significantly lower level of TOC (total organic carbon). ITP treated dye solutions exhibited greater loss of TOC. Immobilized HRP removed ~88% of TOC from simulated wastewater containing mixture of chorophenols [50]. Significant amount of TOC from polluted water containing dyes/dye-mixtures and dyeing effluent was removed when treated with soluble and immobilized bitter gourd peroxidase [24]. These findings are suggestive that ITP could be successfully used in removal of dye from effluents of textile, printing and etc.

The support did not showed any signs of physical adsorption of direct dye or mixtures. The dye solution was stable when exposed to bioaffinity support, silica gel, H_2O_2 or enzyme alone. If this is so, than decolorization was an outcome H_2O_2-dependent enzymatic reaction which may involve formation of free radical followed by polymerization and precipitation. The loss of dye color in batch processes was due to removal of aromatic compounds by precipitation. The decrease in aromatic pollutants from the dye solutions was because of adsorption of initial product, free radical compound on the activated silica present in second column.

2.5. β-cyclodextrin-chitosan-horseradish peroxidase (β-CD-chitosan-HRP) complexes for dye color removal

Cyclodextrins (CDs) are formed from the enzymatic degradation of starch by bacteria. These cyclic oligosaccharides containing 6 (α-CD), 7 (β-CD) or 8 (γ-CD) linked with α-(1, 4) linked glucose units. The most important structural features of these compounds include their toroidal shape with an interior cavity that is hydrophobic and exterior hydrophilic [51]. CDs are capable of forming inclusion compounds both in solution and in the solid state with a variety of guest molecules, whereby the guest molecules are placed in their hydrophobic interior cavity [52]. To improve the chemical stability, absorption, bioavailability and controlled release of some drugs the CDs find wide applications in pharmaceutical science [53]. In addition, CDs because of their low production cost have attracted considerable attention in many others fields (e.g., agriculture, nanocomposites, chromatography, biotechnology and bioremediation).

With the progressive adoption of industrial-based lifestyle and the increasing global population have increased the anthropogenic impact on the biosphere. Textile industry discharges hazardous compounds that can seep into aquifers and subsequently contaminate under-

ground water or surface waters. These pollutants of various natures have far reaching impact on aquatic life and human health [54]. These dyes are difficult to remove by conventional wastewater treatment as they possess highly complex chemical structures [55, 56]. The development of enzyme based strategy to treat dyes from wastewater has received much emphasis in recent times [24, 57, 58].

Peroxidases can be used in the detoxification and biotransformation of several aromatic pollutants present in wastewater [4, 59]. HRP was immobilized in very high yield with β-CD-chitosan. The immobilized preparation was highly active and showed a very high effectiveness factor. This method of immobilization was far superior as compared to other strategies [20, 21, 60]. The immobilization of different enzymes with CDs involves forces that are responsible for the stability were ionic, hydrogen, Van der Waals and hydrophobic interactions [61].

At pH 8.0, maximum binding was achieved for HRP with β-CD-chitosan. The adsorption of enzymes on different types of support is pH dependent [35, 62]. Zhu et al. [63] reported a novel immobilization approach based on the supramolecular function between β-CD polymer and HRP in which maximum binding of the enzymes on the support was obtained in a phosphate buffer solution of pH 7.0. It was concluded that the optimization of the enzyme concentration is also of most importance because higher enzyme concentrations shows no significant influence on dye decolorization. The dye color removal by soluble HRP increased as the incubation time was enhanced with maximum achievement after 2 h. There was no significant effect on decolorizing ability on prolonging the time further. On treatment with fenugreek (*Trigonella foenum-graecum* L.) seed peroxidase the textile effluent from the carpet industry decolorized to 68% of original in 2.5 h [64].

At concentrations above 0.6 mM H_2O_2 soluble HRP was inactivated. Buchanan and Nicell [65] had earlier reported the inactivation of peroxidases by higher concentrations of H_2O_2. Once, the substrate is fully consumed, most of the enzyme is expected to react with residual H_2O_2. Therefore, if a treatment process is to be designed in order to reuse residual enzyme activity, it will be necessary to limit the quantity of H_2O_2 supplied during the treatment [66, 67]. The decolorization of the textile effluent is pH dependent because it affects the solubility and concentrations of the counter ions, with subsequent impact on the functional groups of the used reagents and the degree of ionization during reaction. The decolorization of an effluent by soluble calcium alginate-entrapped bitter gourd (*Momordica charantia*) peroxidase; 48% and 71% removal of color were recorded at pH 5.0, by the soluble and immobilized peroxidase respectively [68].

Color removal efficiency by the free and immobilized enzyme preparations increased with increasing temperature; the soluble enzyme exhibits a lower optimal temperature than the immobilized samples. The improvement in thermal stability of the complex may be an outcome of a multipoint complexation of peroxidase with the support. A similar rationale was made to explain why lectin bound enzymes were more stable than the soluble form [68]. The color removal efficiency in a batch process was lower for the soluble form of HRP than the bound enzyme. This may be due to the fact that the immobilized enzyme preparation would be less sensitive to the inactivating metabolites produced from the catalytic reaction, which accumulate in batch processes [69].

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Two continuous bed reactors containing both of the immobilized preparations were operated simultaneously for the continuous oxidative removal of azo dye from textile effluent. These reactors had high color removal efficiency. A similar observation reported that the oxidative degradation and removal of endocrine disrupting compound, bisphenol A, in a spiral bed reactor [69]. The spiral bed reactor at operational parameters was functional in oxidative removal of bisphenol A for approximately a month. Immobilized preparations of enzymes have dual advantages of stability as well as reusability. In comparison to uncrosslinked enzymes, the cross-linked preparation retained a remarkable high decolorizing activity. Calcium alginate-starch-entrapped cross-linked bitter gourd peroxidase was shown to polymerize and remove benzidine from model wastewater. Even after its sixth repeated use, the immobilized enzyme was able to catalyzed oxidation and polymerizes 58% of the benzidine [70].

2.6. Immobilized cauliflower (*Brassica oleracea*) bud peroxidase (CBP) on calcium alginate gel beads in dye color removal

Synthetic dyes are difficult to remove from effluents by conventional biological processes as they are highly stable and resistant to microbial attack [71]. Although physico-chemical methods are available for dye color removal but being expensive finds limited application [72]. Another concern of such approaches are the generation of intermediates and end products that are carcinogenic and mostly more toxic than the dyes *per se*. Approaches utilizing biodegradative abilities of some white rot fungi and peroxidases from vegetable sources seems promising [73, 74]. Owing to their extracellular nonspecific free radical-based enzymatic system, they can completely eliminate a variety of xenobiotics as well as synthetic dyes, giving rise to nontoxic compounds [75, 76].

B. oleracea popularly known as cauliflower is widely planted in tropical areas and consumed as vegetables and has earlier been shown by our group to be significantly effective in decolorizing synthetic recalcitrant dye. Salt fractionated immobilized CBP with lectin Con A was entrapped into calcium alginate-pectin beads [77]. Peroxidases in conjunction with hydrogen peroxide can act on specific recalcitrant pollutants to remove them either by precipitation or transformation to often innocuous products. They can change the characteristics of a given waste rendering it more amenable for treatment [78]. Their catalytic action is extremely efficient and selective as opposed to chemical catalysts due to higher reaction rates, milder reaction conditions (relatively low temperature and in the entire aqueous phase pH range) and greater stereo-specificity [79]. Though much attention has been paid in the utilization of biocatalysts in several fields, their involvement has been felt very recently in solving the environmental problems [80, 81]. Soluble enzymes have inherent limitations of losing catalytic potential due structural variations influenced by the complexity of effluents. Immobilization is a way out to improve their catalytic characteristics with enhancement in their reusability. Different polymeric materials have been employed for encapsulation of enzymes and their ability in treating the effluents containing pollutants [82, [83]. However, appropriate selection of encapsulation material specific to the enzyme and optimization of process conditions is still under intensive investigation.

Immobilizing enzymes directly from crude homogenate is relatively a much cheaper approach [84]. Although the immobilized form of bio-molecules holds commercial importance, protocols available for such preparations are limited. Immobilization by adsorption is an effective procedure for binding enzymes directly from partially purified preparations or even from crude homogenates [29]. Calcium alginate mediated entrapment is a simple, economical, effective and sustainable approach for using enzymes either from crude extract or directly from partially purified preparations for detoxification and degradation of phenolic compounds in waste water [24, 39, 77].

Immobilization turns out be an articulated approach as the enzyme activity must not be severely compromised. Where enzymes are glycosylated proteins, the glycosyl moieties can be exploited for immobilization as they are generally not engaged in catalysis. While lectins are useful in characterizing glycoproteins, glycoenzymes have been immobilized on concanavalin A affinity column or as Con A-glycoenzyme complexes [37, 39]. Peroxidase from cauliflower bud is a better choice than other vegetable peroxidases as it is sufficiently thermostable, operates in a wide range of pH, economic and effective with low concentration of redox mediators in decolorizing recalcitrant synthetic dyes [85].

2.6.1. Activity of Soluble and Immobilized CBP

The specific activity of peroxidase preparation increased to 3.5 fold over crude enzyme which exhibited an initial specific activity of 98 U/mg of protein. This enzyme preparation was used for direct immobilization as enzyme–Con A complex. Peroxidases in free form have a better chance to leak out of calcium alginate pectin gel and therefore the leaching of enzymes from porous beads is checked by complexing peroxidases and using the insoluble CBP-Con A complex for entrapping into calcium alginate pectin gel. By selecting these concentrations, encapsulation efficiency increases to 93% and leakage decreases to lower than 6%. With 0.2 ml of Con A, the CBP–Con A complex expressed an activity of 84% which on entrapment into calcium alginate–pectin gel resulted in further decrease (64%) of peroxidase activity. Further, the effect of enzyme loading on entrapped activity was evaluated by entrapping increasing concentration of enzyme. Optimum concentration (512 U/ml) was sufficient for maximum expression of peroxidase activity by entrapped preparation. Pre-immobilized or cross-linked enzymes that remain inside polymeric matrices for longer duration than their soluble counterparts provide higher mechanical and operational stability [19, 86]. It also indicated that enzymes with high molecular mass stay for longer duration inside polymeric matrix. Pre-immobilization increases molecular dimensioning of the enzyme and thereby prevents its leaching from alginate beads.

Immobilized peroxidase preparations exhibited maximum peroxidase activity at 40°C which was true even for soluble counterpart of CBP. However, CBP–Con A and entrapped CBP–Con A complex retained greater fraction of catalytic activity at higher temperatures. A comparative analysis showed that entrapped CBP-Con A expressed 79.6% activity whereas CBP-Con A complex with 62.6% and soluble state 48.4% were relatively poor performers under identical experimental conditions. The peroxidase activity was sufficiently high in the first 1 h for different immobilized forms (79.6% & 89.6% for CBP–Con A complex and entrapped states

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respectively) whereas a relatively progressive decline was observed with the soluble CBP on prolonging the time duration.

2.6.2. Activity of CBP under different operational conditions

2.6.2.1. Temperature and pH

Immobilization enhanced the resistance/stability of enzyme to high temperatures. At reasonably high temperatures of 60°C the enzyme expressed 66.6% and 83.7% peroxidase activity as CBP–Con A complex and calcium-alginate entrapped CBP–Con A complex respectively. On increasing the temperature further to 80°C the entrapped immobilized form of CBP exhibited 46.5% peroxidase activity which declined to 31.2% at 90°C. The entrapment of CBP-Con A complex conferred higher retention of its molecular structure and as a result the catalytic activity was improved at elevated temperatures. Such systems could prove useful where operational temperatures are high. As peroxidases are glycoprotein in nature, lectin association immobilizes the molecular conformation by multipoint attachment contributing to stability and thus serves even better when entrapped in calcium-alginate pectin gel [28]. pH activity profile of soluble CBP, CBP–Con A and entrapped CBP–Con A was evaluated by incubating these preparations in the buffers of varying pH values (2.0–9.0). The pH range 4.0–6.0 was sufficient for optimum enzyme activity. While comparing the activity in varying pH medium, it was noticed that immobilized preparations were more stable at pH 4; whereas soluble CBP exhibited a maximum activity (96%) at pH 5.0 which declined in alkaline medium. Interestingly, immobilized CBP could withstand wide alkaline medium in comparison to soluble forms. This broadening in pH-activity suggested that entrapment of enzymes in gel beads provides a microenvironment for enzyme, which may play an important role in the state of protonation / deprotonation of protein molecules [29]. Formation of CBP–Con A complex promotes retention of molecular structure and consequently confers additional resistance to enzyme against extreme conditions of pH [24, 87].

2.6.2.2. Denaturants

Urea (4.0 M) is a strong denaturant of some proteins and it irreversibly denatures soluble CBP [88]. Upon subjecting the soluble and immobilized CBP preparation to urea entrapped CBP-Con A complex retained profound activity with progressive increase in the incubation time. The free enzyme lost almost 78% of initial activity, whereas in case of CBP-Con A complex the activity diminished by 35% under identical experimental conditions.

The mechanism of urea induced denaturation is not completely understood. However, it is suggested that protein molecule unfolds by direct interaction of urea molecule via non-covalent interactions with the peptide backbone. The peptide backbone essentially contributes to the structure of the molecule and therefore the loss of structure results in the loss of catalytic activity [24, 29]. Fatima and Husain [89] have reported the enhancement of resistance to denaturation by complexing glycoenzymes with lectin like Con A. In the presence of lower concentration of dioxane the free and immobilized states of enzymes showed over 60% of peroxidase activity. However, the activity manifested by immobilized counterparts was

sufficiently higher. With higher concentration of dioxane (60% v/v) soluble enzyme retained only 29.8% while CBP–Con A complex and entrapped CBP– Con A retained nearly 41.6% and 54.1% of their actual activity, respectively. It was interesting to note that at even higher concentration of dioxane (80% v/v) soluble form of CBP lost 92% peroxidase activity as compared to immobilized and entrapped CBP-Con A complex exhibiting 29.9% and 48.2% respectively.

Enzyme catalysis is affected by the presence of water miscible organic solvents. As the wastewater is often contaminated by organic pollutants hence the stability of CBP preparations against some water miscible organic solvents needs to be explored. It was observed that entrapped CBP–Con A complex retained remarkably high stabilization against inactivation caused by dioxane and DMF as compared to soluble CBP and CBP–Con A complex. This is substantiated by earlier findings that bioaffinity bound enzymes were significantly more stable likely due to decrease in flexibility and increased molecular rigidity against exposure to water miscible organic solvents [24].

Immobilized peroxidases are reported to be significantly stabilized against denaturation induced by some commonly used detergents (Triton X 100, Tween 20, SDS) [29]. The immobilization of protein by attachment to matrix is sufficient enough to accords protection from denaturation mediated by organic solvents. Moreover, the stabilization of immobilized enzymes against various forms of water-miscible organic solvents could perhaps be due to low water requirement or enhanced rigidity of the enzyme structure. In organic solvents enzymes can perform catalytic activity if the organic solvent does not adversely alter the active site conformation [36].

A number of studies have already been performed on the inhibitory effect of such compounds such as horseradish peroxidase where sodium azide has been shown to be a potent inhibitor of many heme protein-catalyzed reactions [90]. While the effect of sodium azide, on enzyme was adverse, ethylenediamine tetra-acetic acid showed no significant effect on the activity of soluble and immobilized CBP even when its concentration was raised 30 mM. Peroxidase in the presence of sodium azide and H_2O_2 mediates one electron oxidation of azide ions forming azidyl free radicals which bind covalently to the heme moiety of peroxidase, thus inhibiting the enzyme activity [91]. EDTA did not have any significant effect on the activity of CBP and such an observation on enzyme activity has already been reported [92].

The chemical contamination of water by a wide range of toxic derivatives, particularly, heavy metals are a serious environmental problem owing to their potential human toxicity. In view of their presence in wastewater, it became necessary to evaluate the effect of some heavy metals on the activity of CBP. Our results revealed that CBP exhibited more resistance to heavy metal induced inhibition; a concentration-dependent gradual inhibition of CBP activity by $HgCl_2$ was observed. Some recent reports indicated that horseradish peroxidase was remarkably inhibited by heavy metal ions [42, 93].

However, the inhibition of immobilized CBP by $HgCl_2$ was quite low as compared to the soluble enzyme. Although, metals induce conformational changes in enzymes, however peroxidases remain active even in the presence of a number of metal ions, as a part of their

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detoxifying role. The effect of different metal ions on the activity of different enzymes is related to their affinity to different functional groups present in the enzymatic structure [94, 95]. The stability of immobilized CBP against several metal compounds showed that such enzyme preparations could be exploited for the treatment of aromatic pollutants even in the presence of heavy metals.

2.6.2.3. Kinetics

The kinetic parameters of soluble and immobilized CBP were determined using different concentrations of o-dianisidine HCl. The plot of initial enzyme activity versus different concentrations of o-dianisidine HCl for both the enzyme preparations followed a hyperbolic pattern as expected according to the Michaelis–Menton kinetics. Lineweaver–Burk plots of soluble and immobilized CBP were also found to be linear and Michaelis– Menton constant, K_m, for soluble and entrapped CBP–Con A complex was 0.076 and 0.089 mM, respectively. The Vmax values for soluble and immobilized CBP were found to be 22.4 and 16.6 mM/min, respectively. Vmax of soluble enzymes was more than that of immobilized states, although K_m values were closely related. Thus, the immobilization of CBP-Con A complex by entrapment using calcium alginate pectin did not affect the conformation of enzyme, however the accessibility for the substrate was slightly altered.

Glutaraldehyde crosslinking enhances the structural rigidity of protein and also maintains the native structure [96]. The structural rigidity and consequently the native structure of a protein is enhanced by crosslinking [96]. Immobilization by entrapment serves similar functions due to which the CBP becomes more resistant to environment. Since the entrapment of CBP–Con A complex is non-specific and masking of certain amino acids at or near the active site contributed towards lesser formation of enzyme-substrate complex, a decrease in V_{max} was observed in the case of immobilized enzyme preparation. It is well documented that the K_m values of several immobilized enzymes were either unaltered or exhibited minor alteration as compared to those of their respective soluble counterparts.

2.6.2.4. CBP and dye decolorization

Entrapped immobilized CBP decolorized 93.7% and 88.2% of DR 19 and mixture of dyes (DR19+DB9) after 2 h of incubation, respectively. Immobilized CBP was more effective as compared to its soluble counterpart in the decolorization of both DR 19 and mixture of dyes. The decolorization achieved by entrapped Con A-CBP complex was reasonably higher for DR19 and dye mixture (DR19+DB9), respectively after 20 days. Even after operation of the two-reactor system for 120 d and 80 d decolorization achieved for DR19 and disperse dye mixture was 64.8% and 56.8% respectively. In the presence of redox mediator, riboflavin the loss of color was significant for both DR19 and dye mixtures. The enzyme catalyzed the breakdown of chromophoric groups present in dyes [47].

In case of continuous reactors TOC decreased significantly on treatment of polluted water with immobilized CBP. The significant loss of TOC from wastewater indicates that the major toxic compounds may have been eliminated from the treated samples. Akhtar and Husain [97] have

reported that significant amount of TOC was removed from water polluted with dyes/dye-mixtures and dye effluents on treatment with soluble and immobilized bitter gourd peroxidases. These evidences strongly proved that immobilized CBP–Con A complex could be successfully used for the removal of dye effluents loaded with recalcitrant synthetic dyes.

2.6.2.5. Reusability of Immobilized Enzymes

The immobilized enzyme could be easily removed and assessed for its left over catalytic activity. To demonstrate the reusability of encapsulated enzyme, capsules were separated after 120 min of reaction time and then rinsed thoroughly with distilled water. After 5 times of repeated use test, the dye removal efficiency was reduced to 67.9% for DR 19 and 63.5% for dye mixture (DR 19+DB 9). This efficiency showed progressive decrease with the increase in the cycle numbers. This downfall in the catalytic efficiency might be an outcome of the plugging of the pore membrane and accumulation of radicals in the interior of each capsule which entraps the enzyme active site that leads to the inactivation of enzyme molecules.

While inorganic compounds require acidic conditions to be effective, they may increase costs associated with initial pH of waste waters, corrosion of hardware during treatment and pH neutralization of wastes prior to their release. Enzyme based catalysis reflects preference over intact organisms (containing a multitude of enzymes) because the isolated enzymes act with greater specificity, thereby allowing specific group of pollutants to be targeted for treatment; their potency can be better standardized, they are easier to handle and store and enzyme concentration is not dependent on bacterial growth rates. Enzymes are naturally occurring chemical species and our source comprises of readily renewable resources. Moreover, in contrast to many other enzymes, CBP retains its catalytic activity over wide ranges of temperature, pH, contaminant concentration, consequently making it particularly suitable for application in industrial environment. In addition unlike other enzymes with similar functions CBP is relatively non-specific in terms of its organic substrates, making it applicable for treatment of large variety of aromatic pollutants.

A critical aspect that governs and probably limits the industrial applications of enzymes is inactivation through mechanical, chemical and thermal processes that interact to influence enzyme activity. In particular inactivation of CBP can result from oxidation of the enzyme to inactive forms, phenoxy radical inhibition and adsorption and/or entrapment of the enzyme in precipitating polymers. Therefore reducing the cost of the catalysts has been the focus of much attention. The catalytic lifetimes of pointed gourd peroxidase as well as CBP improves significantly when the reaction is conducted at the optimal pH, temperature while maintaining a low instantaneous enzyme concentration [85]. The efficiency of the crude enzyme preparation was independent of its purity as earlier reported by Alberti and Klibanov [98]. By immobilization using entrapment on calcium alginate pectin beads, the apparent rate of enzyme inactivation was reduced which allowed a significant reduction in enzyme requirements for treatment. This increase in enzyme lifetime represents a very significant saving in terms of treatment costs.

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2.7. Diethylaminoethyl Cellulose Immobilized Pointed Gourd (*Trichosanthes dioica*) Peroxidase (PGP) in Decolorization of Synthetic Dyes

PGP cross-linked with glutaraldehyde and adsorbed on DEAE cellulose was efficient in catalyzing the dye color removal of disperse dyes viz., DR19 and dye mixture (DR19+DB9) [99, 100, 101]. Immobilized PGP (I-PGP) was reusable up to seven times to decolorize DR19 and dye mixture to over 50% in comparison to free enzymes. The immobilized states of enzymes are relatively easier to handle and can be stored for longer durations. Immobilized states of PGP have better storage stability and can be used in reactors for the treatment of effluents containing phenolic and other aromatic pollutants including dyes which are predominantly present in textile effluents. The findings on reusability and storage ability suggests that the use of cheaper source of enzyme and support will definitely minimize the cost of immobilization and would provide a suitable strategy for treatment of huge volumes of wastewater in continuous as well as batch processes.

The peroxidases functions better in the presence of redox mediator. Thus it's essential that the enzyme catalytic activity is effective in conjunction with redox mediators and optimum concentration of hydrogen peroxide [102, 103]. The enzymes work well in acidic pH generally in the range of 3 to 6. The findings indicate that immobilized enzymes at operational pH catalyze sufficient dye color removal. The presence of redox mediators enhances the enzymatic dye color removal. Perhaps a correlation exists between redox potential and dye reduction rates. The closer the redox potential is between dye and redox mediator, the faster is dye reduction, because electron transfer is facilitated due to the low potential difference. Such behavior explains the better catalytic properties of riboflavin. However, dye reduction rate is not only determined by redox potential, but also by other factors such as chemical structure, environmental conditions and anaerobic sludge affinity and concentration. The chromophore cleavage by PGP in conjunction with redox mediators was favorable for azo dyes, because the reduction occurs in the nitrogen bonds, which have more affinity to receive electrons, based on electronegative properties, as compared to carbon-carbon bond chromophore of the anthraquinone dyes. Therefore, the effect of redox mediators on dye reduction is related to the molecular structure, being more evident for azo dyes with low decolorization rates in the absence of these compounds, and ineffective for anthraquinone dyes because of the structural stability of the latter [102].

Immobilized peroxidase was much more effective in removing dye color as compared to soluble enzyme in a batch process. A possible reason could be that immobilization shielded the number of reactive free amino groups, which are not protected in soluble case and hence, were more susceptible to reaction with the reactive products like free radicals [50]. Our findings are in accordance to our earlier studies using immobilized PGP-Concanavalin A complex on calcium alginate pectin gel in decolorization of synthetic dyes [29]. To evaluate the efficiency of immobilized PGP on a large scale for the removal of dye color, a vertical continuous reactor system was designed and operated continuously with a flow rate of 15 mL h^{-1}. The reactors worked for more than 60 d approximately, thus explaining their efficiency towards dye decolorization. A significant loss of color appeared when DR19 or mixture of dyes was treated with I- PGP in the presence of redox mediator, riboflavin in a continuous reactor system.

3. Conclusion

The preparation and application of immobilized enzymes has received greater attention in recent times. The experimental results obtained revealed the effectiveness of the immobilized peroxidases in sustainable dye color removal and remediation of other toxic pollutants. The immobilized preparations exhibited adequate storage stability and protein content. The immobilization confers a shielding effect on peroxidases against inactivation and/or inhibition and therefore, higher dye color removal can be reached with the same concentration of immobilized preparation as soluble peroxidases. The performance of dye color removal was found to be highly dependent on enzyme dose, hydrogen peroxidases, temperature and aqueous pH. The encapsulated enzyme activity shows higher relative activity in acidic solutions over a broader range which are the most common conditions appeared in waste stream. Enzyme retention activity, encapsulation and leakage percentage of enzymes are influenced by gel preparation condition and finding a proper value for above quantities totally depends on the used support. The reusability experiment showed that these biocatalysts can be used up to several cycles without serious deficiency in their catalytic performance. A two-reactor system with simple operational protocol for decolorization /degradation of disperse dyes can be designed for the potential future use of immobilized peroxidases. Interestingly, the described system is developed with a cheaper biocatalyst and support matrix that is quite effective in treating dyes continuously. Thus, immobilized peroxidase preparations could be exploited for developing bioreactors for the treatment of phenolic and other aromatic pollutants including synthetic dyes present in industrial effluents.

Author details

Farrukh Jamal* and Sangram Singh

*Address all correspondence to: farrukhrmlau@gmail.com; journal.farrukh@gmail.com

Department of Biochemistry, Dr. Ram Manohar Lohia Avadh University, Faizabad, U.P., India

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