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Immobilization of Partially Purified Fungal Dye Degrading Laccase Enzyme

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ABSTRACT

In the modern world many industries different kinds of polluting agents are spoiling our mother nature. 100000 dyes have been generated worldwide with an annual production of over 7×10^5 metric tons. Laccase is a copper-containing polyphenol oxidase that acts on a wide range of substrates. This enzyme is found in many plant species and is widely distributed in fungi. Because of its importance in bioremediation, potential dye degrading fungal cultures were screened for laccase enzyme production by plate test method using the indicator compound crystal violet. Alginate-gelatin mixed gel was applied to immobilized laccase for decolonization of some industrial dyes including crystal violet.The immobilization procedure was accomplished by adding alginate to a gelatin solution containing the enzyme and the subsequentdropwise addition of the mixture into a stirred CaCl2 solution. The obtained data showed that both immobilizedand free enzymes acted optimally at 45◦C for removal of industrial dye, but the entrapped enzyme showed higher thermal stability compared to the free enzyme. The immobilized enzyme represented optimum decolorization at pH 7. Reusability of the entrapped laccase was also studied and the results showed that 71% activity was retained after five successive cycles. The best removalcondition was applied for decolorization of several other industrial dyes. Results showed that the maximum and minimum dye removal was related to gentian violet and direct black 19, respectively. Potential fungal dye degrading laccase enzyme is as an important source for bioremediation of toxic dye removal.

***KEYWORDS**:* Industrial dye, *Aspergillusspp,* immobilized enzyme bioremediation.

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INTRODUCTION

Dyes are released in to the environmental through industrial effluents from three major sources such as textiles, dyestuff manufacturing and paper industrial [1]. Green technologies to deal with this problem include adsorption of dyestuffs on bacterial and fungal biomass [2, 3] or low-cost non-conventional adsorbents[4, 5]. In the recent years, enzymes have gained great importance in Industries; Laccases are one among them which are widely present in the nature. Laccases are the oldest and most studied enzymatic systems. These enzymes contain 15–30% carbohydrate and have a molecule mass of 60–90kDa [6]. Laccases (EC 1.10.3.2; benzenediol: oxygen oxidoreductases) are multicopper enzymes belonging to the group of blue oxidases that catalyses oxidation of a wide variety of organic and inorganic compounds including diphenols, polyphenols, diamines, and aromatic amines. One electron at a time is removed from the substrate and molecular oxygen is used as the electron acceptor [7]. Enzymes action at mild conditions of pH, pressure and temperature proposes them as candidates for suitable catalysts in industries where low cost, energy savings and simplicity are important[8, 9]. Enzyme entrapment uses natural and synthetic polymers such as agarose, agar and gelatin through thermo reversal polymerization alginate, polyvinyl acetate, acrylic acid and β-carrageenan by ionotropic gelation [10]. Laccases are an interesting group of multicopper enzymes produced by higher plants and fungi that catalyze the oxidation of a wide range of organic compounds such as phenols in the presence of molecular oxygen [11, 12]. The most important applications discussed for laccases include pharmaceutical and food industries, textile effluent transformation and wastewater detoxification biosensors. The unique properties of laccases such as high stability in solution, mild reaction conditions and selectivity for phenolic structure make them attractive for use in chemical synthesis [13]. Alginategelatinmixed gel to immobilize laccase which is further employed in the decolorization of some synthetic dyes, such as crystal violet in aqueous solutions. Optimum pH, temperature, and proper enzyme content for decolorization by the immobilized laccase were also studied. To our knowledge, the use of alginate-gelatin mixed gel has not been examined previously for the purpose of enzyme entrapment [14, 15]. The aim of the present paper is to screen and isolate potential dye degrading fungi from dye accumulated soil and water samples and identifying a new source of extracellular laccase. The extracellular fungal laccase in the culture medium was subjected to purification, characterization and immobilization studies.

MATERIALS & METHODS

All other reagents and chemicals were of the highest purity available. Various industrial dye accumulated soil and water samples were collected for isolate a potential dye degrading microorganisms (fungi). Different types of industrial dyes are collected from the industry from 4

different places in Thirupur {Tamil nadu,India} in 2 summer and winter climatic conditions. Isolation of dye decolourizing fungal strain was selected and further Production of Extracellular laccaseenzymewas partially purified from the following of Mikiashvili*et al*., (2006) [16] procedure was applied for the potential dye degrading fungal isolate.

A. *Enzymatic plate assay*

Laccase activity was determined by the oxidation of ABTS method [17]. The laccase plate assay allowed rapid determination of the presence of laccase in the extracellular fluid. 15mL of sterile agarose (0.5%) medium containing 0.5mM of ABTS per mL in sodium acetate buffer (pH 4.5, 0.1M) was placed on a petriplate (5 × 5 cm). The development of an intense bluish-green color around the wells was considered as a positive test for laccase activity [18].

B. Preparation of mixed gels, immobilization of laccase, and decolorization of crystal violet

The gelatin-alginate mixed gel was prepared according to the method of Panouille and Larreta-Garde [19], with some modifications. Briefly, entrapment of laccase in gelatin-alginate mixed gel was performed by adding 0.1 g sodium alginate to 10mL of a solution containing 10% (w/v) gelatin and laccase in the range of 5–50mg (1mg enzyme is equal to 1.25 U) under continuous stirring at room temperature. The mixture was syringed into a stirring $CaCl_2$ solution (200 mM), and the resulting beads were left to be hardened for 1 h under the same conditions, washed three times by deionized water, and then stored at 4◦C prior to being used in the decolorization study. The amount of bound protein was determined by Lowry's method.

C. Optimum temperature, pH, and enzyme concentration of the immobilized laccase

The effect of pH on the enzymatic decolorization was monitored with a dye concentration of 0.25mg/ml at a 3–9 pH range adjusted by citrate or ammonia buffers. To determine the effect of temperature on the enzymatic decolorization, the reaction mixture was incubated at a thermal range of 30–70◦C, in steps of 10◦C. In order to assess the effect of enzyme quantity on decolorization, the reaction was started with different enzyme amounts, from 0.25 to 0.5mg/ml, in 0.05mg/ml increments. The experiments were performed in triplicate and the results shown are means standard deviation.

D. Reusability

Reusability of the gelatin-alginate immobilized enzyme was investigated in acetate buffer solution 0.1M at pH 8 and 45◦C. The used beads were filtered at the end of each cycle and washed three times with the same buffer to treat in the next fresh colored solution. The reusability study was performed in triplicate.

Relative Decolorization (%) = (*A*initial− *A*final)/*A*initial× 100, where *A*initial was the initial absorbance and *A*observed was the final absorbance at the given wavelength.

RESULT AND DISCUSSION

The potential dye degrading fungal (*Aspergillusspp*) isolate to produce a partially purified laccase enzyme was applied for further analysis of ABTS plate assay and immobilized laccase was optimized.

A. Enzymatic Plate Assay

The laccase plate assay showed the presence of laccase activity in the partially purified sample. The heat-treated sample showed less intense green color than that of the partially purified sample as shown in (Fig. I). The development of an intense bluish-green color zone the wells was considered as a positive test for laccase activity

Figure: I –ABTS plate assay for demonstration of laccase activity in potential fungal sample were compared with control and test petriplates.

B. Preparation of mixed gels, immobilization of laccase, and decolorization of crystal violet

Spherical and regular-shaped gelatin-alginate beads were obtained by using ionotropic gelation; the diameters of the beads were 2.0–5mm (Fig. II). The results indicated the rupture force increase with the rise in alginate concentration. The prepared beads containing 5% alginate exhibited

the highest mechanical stability. Alginate could provide a very strong network that required 0.980kgf (kilogram-force) forces to rupture and the bound protein level 0.149 µg/ml.

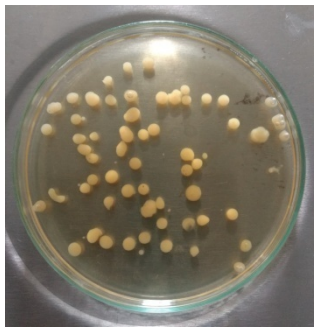


Figure: II – Preparation of Mixed Gels, Immobilization of Laccase enzyme

C. Optimum temperature, pH, and enzyme Concentration of decolorization of industrial dyes of the immobilized laccase enzyme

Among this analysis the effect of temperature on dye decolorization was investigated by changing the reaction mixture temperatures in a 30–70◦C range. The results obviously showed that decolorization increased as the temperature increased up to 45◦C; at higher temperatures, decolorization efficiency remarkably decreased, which normally attributed to denaturation of enzyme. However, the comparison of decolorization results indicated high thermal stability of immobilized enzyme and confirmed the protective role of alginate-gelatin mixed gel for maintaining enzyme activity. At 45◦C, the crude and immobilized enzymes exhibited more activity. Similarly, Forootanfar and colleagues (2011) reported that the optimum temperature for laccase activity was 45◦C. Other research studies have reported an optimum temperature range of 45–50◦C for (Table.I)laccase activity immobilized by both covalent binding and adsorption [19, 20].

Extremely high or low pH values generally result in complete loss of activity for most enzymes. pH is also a factor in the stability of enzymes. As with activity for each enzyme there is also a region of pH optimal stability.pH plays the key role in dye decolorization. The effect of pH on dye decolorization was examined at pH ranging from 3 to 9, using buffers. The free enzyme significantly displayed higher decolorization activity in pH 5 (table. II). Immobilized enzyme beads are sharp increase at pH values above 7 to 9. The optimal pH for the immobilized enzyme activity was 8. In addition to temperature and pH there are other factors, such as ionic strength, which can affect the enzymatic reaction. Each of these physical and chemical parameters must be considered and optimized in order for an enzymatic reaction to be accurate and reproducible.

To determine the proper amount of enzymes required for maximum decolorization, the effect of enzyme quantity on dye decolorization was also studied. As shown in table.III, decolorization increased as enzyme quantity increased, from 0.5 to 5 mg/mL. However, the results demonstrated that the minimum enzyme quantity to obtain maximum decolorizationwas0.35 µg/mL.

TABLE: I Optimum temperature for dye degradation of the immobilized laccase enzyme

S.No	20◦C.	30◦C.	*40*◦C.	45◦C.	50◦C.	60◦C.	*70*◦C.
Laccase	1.256	1.038	0.947	0.307	0.529	1.077	1.298

TABLE: II Optimum pH for dye degradation of the immobilized laccase enzyme

S.No	3	5	7	8	9
Laccase	1.298	0.934	0.541	0.299	0.359

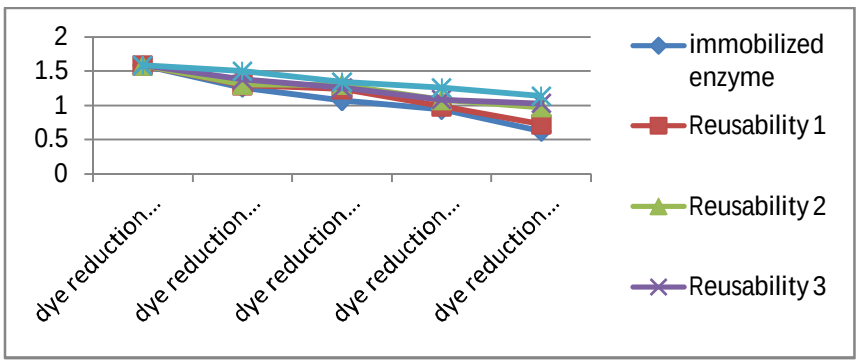
TABLE: III Optimum enzyme concentration for dye degradation of the immobilized laccase enzyme

S.No	0.5 mg/ml	0.3 mg/ml	0.35 mg/ml	0.4 mg/ml	0.45 mg/ml
Laccase	1.042	0.563	0.187	0.185	0.187

D. Reusability

Reusability of immobilized enzymes exhibits the most important aspect for industrial applications, because immobilization of enzymes decreases the cost of production due to their repeated continuous uses. In this work, reusability of the immobilized enzyme was investigated up to five cycles; the residual activities (Figure. III). During enzymatic reactions, the alginate-gelatin mixture may cause a decrease in the pore sizes of the network, leading to difficulties in the diffusion of the substrate and product in the matrix of the gel. This restriction may cause a decrease in the efficient activity of laccase entrapped in gel after repeated use. In the literature, there are reports of successful reuses of various immobilized laccase systems, such as 60% activity after ten cycles for covalently immobilized laccase on activated polyvinyl alcohol [21, 22, 23.]

Figure: III– Reusability of immobilized enzyme for dye degradation



Hence, this study/analysis bears significance for the dye decolourization and this potential isolate *aspergillusspp* has a high potential to decolourize the different types of industrial dyes. The use of these fungi, thus could offer a much cheaper and efficient alternative treatment of waters contaminated heavily with textile dyes [24].

CONCLUSIONS

The potential fungal isolate *Aspergillusspp* was capable of decolorizing the different industrial dyes. But the % of decolourization will be varying from one to another. Minimum 55.7% to maximum 90.9% of dye decolourization was detected. That responsible fungal laccase enzyme was monitored further [25]. Immobilization of partially purified laccase enzyme has received increasing attention in dye removal of different types of industrial dyes. Immobilized laccase enzyme Gelatin-alginate beads were prepared as a matrix system for laccase entrapment; the immobilized enzymes exhibited more stability during operation compared to free enzymes, a result that can be considered an advantage in wastewater treatment. To determine the proper amount of enzymes required for maximum decolorization, the effect of immobilized laccase enzyme beads quantity on dye decolorization was also detected. In addition, the reusability of gelatin-alginate beads provides economic benefits when used in largescale applications. Future investigations may focus on the decolorization of different types of dyes that are widely used in the textile industries.

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