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COMPARISON OF FUNGAL LACCASE PRODUCTION ON DIFFERENT SOLID SUBSTRATES, IMMOBILIZATION AND ITS DECOLORIZATION POTENTIAL ON SYNTHETIC TEXTILE DYES

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ABSTRACT

Laccases are polyphenol oxidases that require O_2 to oxidize phenols, polyphenols, aromatic amines and different non-phenolic substrates by one electron transfer resulting in the formation of reactive radicals. In the current study 2 laccase producing strains of Aspergillus sp., and Penicillium sp., isolated from natural sources were studied for their optimal production on 5 different solid substrates (wheat bran, rice bran, ground nut cake, coconut cake and sesame cake). Wheat bran was found to be the substrate in which both the fungal strains were able to produce an optimal quantity of laccase enzyme of up to 1.632 IU/ml and 2.0 IU/ml respectively. The enzyme was extracted from the solid substrate having 2.0 IU/ml of enzyme unit and was immobilized on sodium alginate. When this immobilized alginate was allowed to act on the synthetic textile dyes Re Red BSID, Re Yellow merl, Orange merl, Red m5B and indigo carmine a maximum of 92.16% of decolorization was observed for Re Yellow merl after 5 days. All the other dyes were reduced to more than 70 % using the immobilized beads after 5 days.

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KEY WORDS

Laccase enzyme; Azo dye decolorization; Immobilized enzymes; Solid state fermentation.

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[I] INTRODUCTION

Dyes and dyestuffs are widely used within the food, pharmaceutical, cosmetic, textile and leather industries. Over 100,000 commercially available dyes exist and more than $7x10^5$ tonnes of dyestuff are produced annually [1, 2]. The human health impact of dyes used in the textile industry, especially azo dyes and their degradation products, has caused concern for a number of years, with legislation controlling their use being developed in a variety of countries [3]. Increasingly, the environmental and subsequent health effects of dyes released in textile industry wastewater are becoming subject to scientific scrutiny. Wastewater from the textile industry is a complex mixture of many polluting substances ranging from organochlorine-based pesticides to heavy metals associated with dyes and the dyeing process [4].

Microbial decolorization processes offer a complete cleanup of pollutants in a natural way as it reduces the color components to carbon dioxide, ammonia and water by initiating cleavage of the bonds in the dyes rather than creating possible toxic fragments of dyes [5]. Laccases (benzenediol oxygen oxidoreductases, EC 1.10.3.2) are polyphenol oxidases that require O_2 to oxidize phenols, polyphenols, aromatic amines and different non-phenolic substrates by one electron transfer resulting in the formation of reactive radicals [6]. The enzyme has been reported for several dye decolorizations [7, 8].

It was evaluated that laccase played an important role in the decolorization of wide spectrum dyes having diverse chemical structure, which suggested its implications in treating textile effluents as a low-cost and environmentally friendly technology [9, 10]. Nevertheless, laccase is often easily inactivated in waste treatment for the wide variety of treating conditions and is also difficult to be separated from the residual reaction system for reuse, which limits the further industrial applications of laccase. Enzyme immobilization technology is an effective means to make laccase reusable and to improve its stability [11], which is considered as a promising method for the effective decolorization of textile effluents.

The current study is focused mainly in the isolation of Laccase producing fungal strains from various sources. The isolated fungus will then be used in the production of Laccase enzyme on different solid substrates. The activity of the produced enzyme will be characterized under different physical conditions. The crude enzyme and the immobilized crude enzyme would be used in the decolorization of synthetic textile dyes.

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[II] MATERIALS AND METHODS

2.1. Isolation and screening of Laccase producing fungal strain

Fungal cultures were isolated from natural sources (soil samples, fruting bodies). Soil sample and spore suspensions were serially diluted and cultured on potato dextrose agar medium (PDA) and incubated at 30ºC. Selection of laccase producing organisms was done on plates containing following composition (g/l): 3.0 peptone, 10.0 glucose, 0.6 KH_2PO_4, 0.001 $ZnSO_4$, 0.4 K_2HPO_4 0.0005 $FeSO_4$, 0.05 $MnSO_4$, 0.5 $MgSO_4$, 20.0 agar (pH-6) supplemented with 0.02% guaiacol.

The isolated fungal strains were inoculated into these plates and the plates were incubated at 30°C for 7 days. Laccase activity was visualized on plates containing 0.02% guaiacol since laccase catalyzes the oxidative polymerization of guaiacol to form reddish brown zones in the medium [12].

2.2. Identification of the screened fungal strains

Based on the reddish brown zones, the efficient strains of fungi selected were identified based on the standard Microscopic tests by lacto phenol cotton blue staining.

2.3. Comparison of Laccase production on different Agro Wastes

About ten agar discs of the two fungal strains were inoculated on 150 gm of 5 different solid substrates after proper sterilization (wheat bran, rice bran, ground nut cake, coconut cake and sesame cake) in a 250 ml Erlenmeyer flask. The flasks were incubated for a period of 10 days at 27^0C after which the enzyme component in the flask was estimated. The efficiency of the agricultural wastes on laccase production was compared statistically by correlation analysis.

2.4. Isolation of crude enzyme from the different solid substrates studied

The contents were extracted with sodium acetate buffer (pH 5.0, 10mm), filtered and centrifuged at 7000 rpm for 20 minutes at 4ºC. The supernatant was thus collected and used as enzyme source for the quantification of laccase enzyme.

2.5. Immobilization of crude enzyme with Sodium alginate

Ten milliliters of crude enzyme extract (Total laccase activity 2 IU) was added with sodium alginate solution (4%w/v) and properly mixed for about 20 minutes. It was added drop-by-drop by means of a Pasteur pipette into 50 ml of 100 mM calcium chloride solution; corresponding metal alginate beads were formed. After 45 minutes of hardening in each solution, the beads (about 3-4 mm in diameter) were separated from the hardening solution by filtration. All immobilized crude laccase alginate enzymes were kept in distilled water at 4ºC [13].

2.6. Characterization of immobilized laccase enzyme

2.6.1. pH stability

The effect of pH on enzyme immobilized beads and crude enzyme were compared and studied by incubating the samples in 0.1 M citrate-phosphate buffers (ph 2.0-6.0) for 24 hrs. Enzyme activity was estimated at the end of 24 hrs for all the different pH ranges as described in 2.9.

2.6.2. Thermostability

Thermal stability was assayed by incubating the enzyme immobilized beads and crude enzyme simultaneously at 60ºC for 240 minutes. At the end of 240 minutes the enzyme activity was measured at 30ºC as described in 2.9.

2.6.3. Storage stability

Immobilized beads were stored in 0.1M sodium acetate buffer (pH – 4.5) at 4ºC for several days. The enzyme activity was measured at 30ºC.

2.7. Decolorization studies of immobilized enzyme on different synthetic textile dyes

Decolorization potential of the immobilized enzyme (2.0 IU/ml) on 5 dyes (Re Red BSID, Re Yellow merl, Orange merl, Red m5B, Indigo carmine) were carried out on flasks containing 10mg/100ml of each dye on distilled water [13]. At the end of every 24 hrs the decolorization of each dye was measured at their absorption maxima. The percentage of decolorization was calculated by the formula

$$D=100(A\ i-A\ t)\ /\ A\ i$$

Where D was the Decolorization of the dye (in %), Ai the initial Absorption of the dye at its absorption maxima and At was the final absorption of the dye. The patterns of decolorization of the various dyes in relation to the time in days in relation to the immobilized laccase enzyme were analyzed using ANOVA.

2.8. Microtoxicity assay

Test cultures (Staphyloccus aureus, E. coli, and Trichoderma sp.) were cultivated on nutrient agar and potato dextrose agar plates for 24 hrs by spread plate technique. Two wells were bored in which the same concentrations (10 µl) of degraded and undegraded dyes were loaded. The plates were incubated for 24 hrs to 48 hrs to observe the zone of clearance around the wells bored [14].

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2.9. Extra-cellular Laccase activity assay

Enzyme activity was assayed at 30ºC by using 10mm guaiacol in 10mm acetate buffer containing 10% (v/v) acetone. The changes in absorbance of the reaction mixture containing guaiacol was monitored at 470nm (ε = 2.6 × 104M–1 cm–1) for 5 min of incubation. One unit enzyme activity was defined as the amount of enzyme that oxidizes 1µ mole of guaiacol per minute at 30ºC. The activities were expressed in IU/ml [15].

Table: 1. Enzyme production on different solid substrates

Fungal strains	Agro wastes used	Laccase activity (IU)
***Aspergillus* sp.**	Wheat Bran	1.632
	Rice bran	1.072
	Groundnut cake	0.973
	Coconut cake	1.433
	Seasame cake	1.267
***Penicillium* sp.**	Wheat Bran	2.0
	Rice bran	1.335
	Groundnut cake	1.299
	Coconut cake	1.351
	Seasame cake	1.063

[III] RESULT AND DISCUSSION

3.1. Isolation and screening of Laccase producing fungal strain

About 35 different fungus were isolated from natural sources and only 2 strains were found to be Laccase positive strains forming a reddish brown zone around the colonies inoculated.

3.2. Identification of the screened fungal strains

The two efficient fungal strains were identified as Aspergillus sp. and Penicillium sp. based on their microscopic observation. The first strain showed a septate and dichotomous hyphae, at 45o angle branching. Conidial heads are radiate to loosely columnar. Conidiophores are coarsely roughened, uncolored, vesicles spherical, metulae covering nearly the entire vesicle in biseriate species. Conidial heads radiate, uni- and biseriate; however, some isolates may remain uniseriate, producing only phialides covering the vesicle which are the characteristic features of Aspergillus sp. [16]. For the second fungus the hyphae were terverticillate and the conidia were spherical to elliptical in shape. Conidia were smooth and had a green color reflection in the mass. These microscopic features were found to be that of Penicillium sp. [17].

3.3. Comparison of Laccase production on different agro wastes

Wheat bran was found to be the effective substrate inducing a maximum enzyme production on both the isolated fungus. Aspergillus sp. produced up to 1.632 IU/ml of enzyme whereas Penicillium sp. produced the maximum of 2.0 IU/ml of enzyme after 10 days of incubation. On comparison rice bran and coconut cake were also found to be effective next to wheat bran. The Laccase enzyme production of both the organisms on different solid substrates was given in **Table–1**. Enzyme produced by Penicillium sp. (Maximum) was used in the immobilization and decolorization experiments. Similarly enhanced laccase production (>2 IU/ml) by S. psammoticus, was estimated when grown on wheat bran [18]. The value of the correlation coefficient for the laccase enzyme produced through the two fungus used was found to be + 0.67 (r value) which implies that the there is a positive correlation between the agricultural source and the enzyme produced i.e., any change in the feed used has an equal effect on the enzyme production.

3.4. Characterization of immobilized laccase enzyme

3.4.1. pH stability

The immobilized laccase was stable in the pH range 5–7 with over 80% residual activity, while the free laccase was stable in the pH range 4–5 **[Figure–1]**. This indicated that the immobilization appreciably improved the stability of laccase over a wide pH range. Similar results were observed for Adinarayana Kunamneni et al., 2008 [19] where the immobilized laccase enzyme exhibited a more stability over a wide pH range than that of the crude free laccase enzyme.

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3.4.2. Thermostability

The residual activity of the immobilized laccase enzyme reduced with time when incubated at 60ºC for 240 minutes. A maximum activity which was observed initially reduced from 100 % to 80% in the first hour of incubation. The enzyme activity was reduced continuously over the period of incubation. The residual activity at the end of 240 minutes of incubation was found to be about 37 % on compared to the 3 % of the crude extract **[Figure–2]**.

For Adinarayana Kunamneni et al., 2008 [19] where the immobilized laccase enzyme retained their activity over a longer period of time than that of the crude free laccase enzyme. Similar results were observed for Wang ping et al., 2008 [20] where the Thermostability of the immobilized enzyme was higher than that of the crude free laccase enzyme. The range of the immobilized laccase enzyme was over 80% even after incubating the immobilized enzyme at 60ºC for 60 minutes.

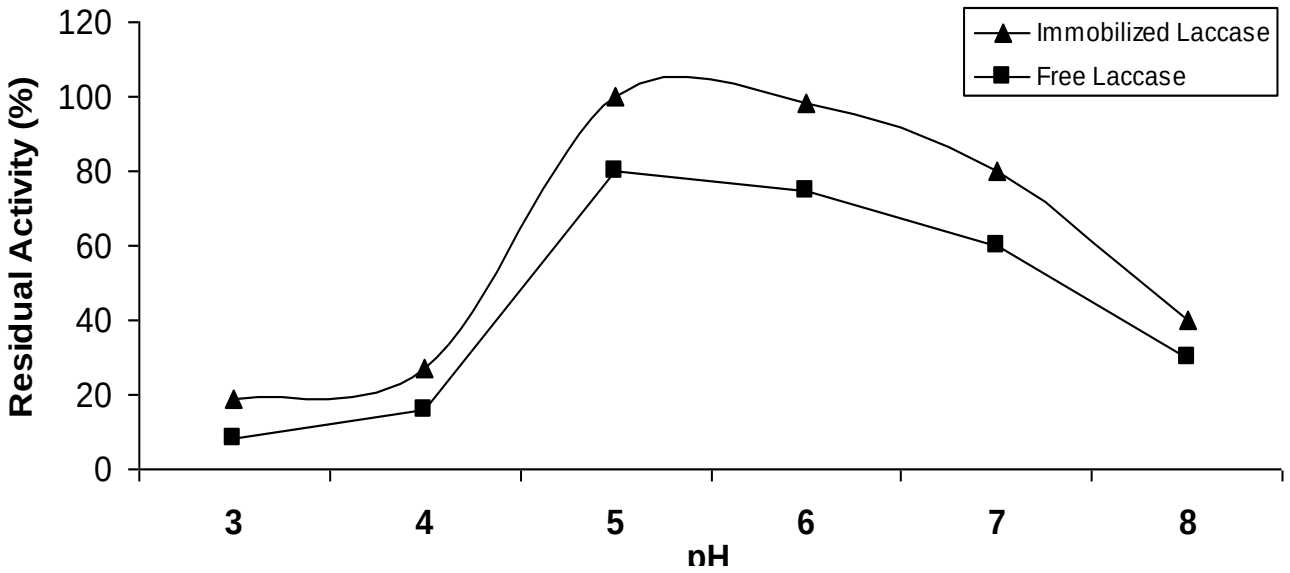

Fig: 1. Effect of pH on immobilized and free Laccase enzyme activity

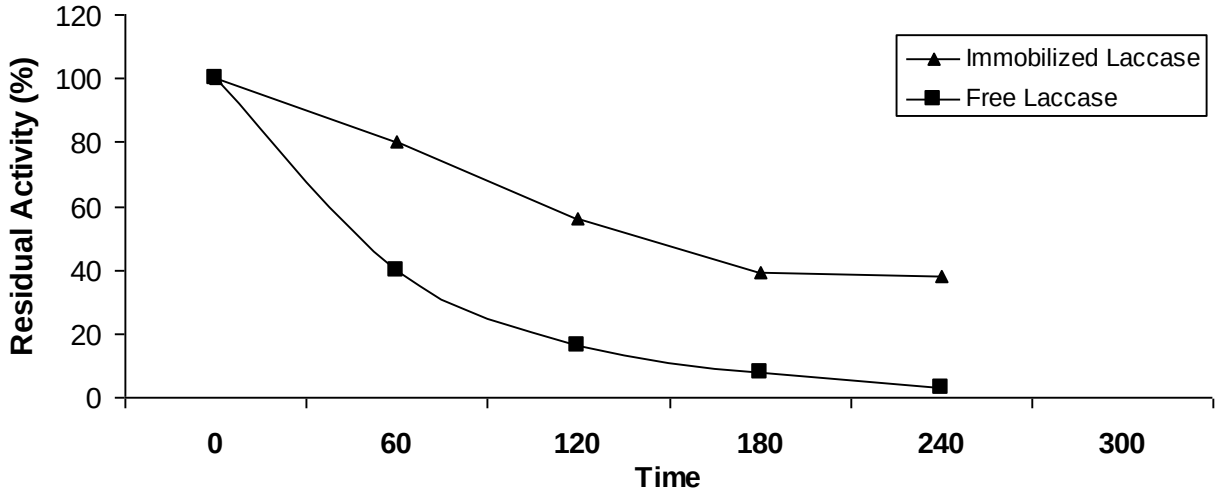

Fig: 2. Thermostability of immobilized and free Laccase

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3.4.3. Storage stability

During the first 2 months storage period there was a 2.5% drop in activity. Thereafter, the activity declined more slowly loosing an additional 1.3% in 2 months, equivalent to an approximate loss of 0.03% per day over the latter period and to a total loss of 3.8% in 4 months. Under the same storage conditions, the recovered activity of the soluble laccase amounted to 95 and 91%after 2 and 4 months storage, respectively. Leonowicz et al., 1988 [21] reported an increase in storage stability of laccase from Trametes versicolor immobilized on glutaraldehyde-activated aminopropyl porous glass.

3.5. Decolorization studies of immobilized enzyme on different synthetic textile dyes

A maximum of 92.16% of reduction was observed for Re yellow merl dye after 5 days of incubation with the immobilized enzyme **[Table-2]**. However, there exists a significant difference between the reduction percentage of the dyes upon the days of incubation (ANOVA, $p>0.05$). For all the dyes the percentage of reduction increased from the first day onwards till the 5th day. All the dyes under study were reduced by more than 80% with indigo carmine showing maximum resistance showing a reduction of up to 72.63% **[Table-2]**. There is no significant difference between the reduction percentage of the different dyes used (ANOVA, $p<0.05$).

RBBR decolourization by the immobilized copper-alginate enzymes, a rapid dye removal about 90% at 60 min [13] methyl red decolourization by the immobilized zinc-alginate enzymes, slightly increased up to 94% after 150min of the treatment [22]. In case of Indigo carmine decolourization was about 96% after 150min treatment with sodium-alginated immobilized enzyme. Bromophenol blue decolourization by immobilized calcium-alginate was about 84% within 150 minutes. The purified laccase could efficiently decolourize the indigo dye upto 80% within 4-5 days of incubation [13].

3.6. Microtoxicity assay

Microbial toxicity study on all the synthetic textile dyes showed the formation of zone and proved to be toxic to test organisms used (Staphylococcus aureus, E. coli, Aspergillus sp. Trichoderma sp. and Penicillium sp.) on compared to that of the degraded dye intermediates **[Table– 3]**. Among the dyes studied the azo dyes (Re Red BSID, Re Yellow merl, Orange merl and Red M5B) were found to be more toxic than the vat dye indigo carmine. This proves that the toxicity of the dye intermediates have reduced significantly on comparison with that of the dyes used in the study.

Table: 2. Reduction percentage of the synthetic dyes by immobilized Laccase enzyme

Dye used	Days of Incubation				
	Day 1	Day 2	Day 3	Day 4	Day 5
Re Red BSID	25.84	38.69	59.89	75.6	89.26
Re yellow merl	22.15	37.15	63.26	81.5	92.16
Orange merl	37.63	38.59	56.43	78.19	84.26
Red M5B	30.12	44.15	58.72	77.26	85.32
Indigo carmine	39.26	45	51.14	63.47	72.63

Table: 3. Microtoxicity studies of the synthetic dyes against standard test organisms

Test Organisms	Re Red BSID		Re yellow merl		Orange merl		Red M5B		Indigo carmine	
	S	T	S	T	S	T	S	T	S	T
S. aureus	+	-	+	-	-	-	+	-	-	-
E. coli	-	-	+	-	+	-	-	-	+	-
Trichoderma sp.	+	-	-	-	+	-	+	-	-	-

S – Synthetic dyes; T-Treated synthetic dyes; + Zone of clearance; - No zone of clearance

According to the work of Mane et al., 2008 [14] the dye Navy blue RX was toxic for the growth of agriculturally important microorganisms Azotobacterium sp and Pseudomonas aeruginosa. This made a conculsion that the dyes metabolites are not only non-toxic but stimulatory to agricultural crops and microorganism.

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[IV] CONCLUSION

From the current study it was concluded that immobilized laccase enzyme could potentially be used as a replacement for the conventional dye decolorization using microorganism. The main advantage in the immobilized enzyme treatment was that the time required by the organism to produce the enzyme was neglected and the decolorization rate will be faster in terms of the number of days or hours that the dyes need to be treated.

CONFLICT OF INTERESTS

I do hereby inform you that there is no conflict of interest with regard to the financial commitments, location at which the work done and with the number and order of co-authors in publishing this article in your esteemed journal.

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