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Application of Immobilized Laccase from *Bacillus subtilis* MTCC 2414 on Decolourization of Synthetic Dyes

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ABSTRACT

Laccases (benzenediol oxygen oxidoreductases, EC 1.10.3.2) are polyphenol oxidases (PPO) that catalyze the oxidation of various substituted phenolic compounds by using molecular oxygen as the electron acceptor. In the present study, *Bacillus subtilis* MTCC 2414 were employed for the production of laccase using guaiacol as a substrate under Submerged Fermentation (SmF) condition. Laccase was partially purified by salt precipitation method followed by dialysis. The synthetic dyes such as Orange 3R, Yellow GR and T-Blue were used for degradation studies using culture filtrate, free and immobilized laccases. The immobilized laccase obtained by entrapment method using sodium alginate was optimized and it exhibited maximum activity at pH 7 (321 U mL^{-1}) and temperature 35°C (317 U mL^{-1}). Similarly, free laccase was also optimized and the maximum activity was observed at pH 9 (309 U mL^{-1}) and temperature 35°C (339 U mL^{-1}). Surprisingly, Yellow GR dye was found to be highly degraded up to 81.72% by immobilized laccase when compared to free laccase (74.69% of T-Blue) and culture filtrate (72.16% of Orange 3R) respectively. The FTIR spectrum showed a spectrum peaks at 2110.12 c/m in control dye and degraded Yellow GR exposed peak at 3406.36 cm^{-1} which represents C-N stretching of aromatic amine group in dyes. Therefore, high degradation ability of laccase from *B. subtilis* MTCC 2414 makes them attractive catalyst for biotechnological applications.

Key words: Laccase, *Bacillus subtilis* MTCC 2414, immobilization, synthetic dyes, guaiacol

INTRODUCTION

Large amounts of chemically different dyes are used for textile dyeing which resulted in significant proportion of dyes that come into the environment as waste water. Most of these dyes remain undegraded without the aid of any physical of chemical processes. The latest and alternating way to this problem would be the use of bacteria or their enzymes, which can oxidize a wide spectrum of synthetic dyes (Robinson *et al*., 2001). Reactive group of azo dyes are commonly used, due to their superior fastness to the applied fabric, high photolytic stability and resistance to microbial degradation (Kalyani *et al*., 2008).

Because of the efficient degradation and non-toxic product generation, the degradation of synthetic dyes through special biocatalysts like laccase has received much attention because of the efficient, eco-friendly and time consuming process (Kunamneni *et al*., 2008; Parshetti *et al*., 2010). Laccase was first discovered in the sap of the Japanese lacquer tree *Rhus vernicifera* and its characteristic as a metal containing oxidase was discovered by Giardina *et al*. (2010). Laccases

(benzenediol: oxygen oxidoreductases, E.C.1.10.3.2) are an interesting group of multicopper enzymes that catalyze the oxidation of a wide range of organic compounds, such as phenols, in the presence of molecular oxygen (Forootanfar *et al*., 2011). Because of their broad specificity for the reducing substrates, laccases from both white-rot fungi and some bacteria have received increasing attention as potential industrial enzymes in several applications such as pulp delignification, wood fiber modification, dye or stain bleaching, chemical or medicinal synthesis and contaminated water or soil remediation (Couto and Herrera, 2006).

All substrates cannot be directly oxidized by laccases, either because of their large size which restricts their penetration into the enzyme active site or because of their particular high redox potential. To overcome this hindrance, suitable chemical mediators such as guaiacol are used which are oxidized by the laccase and their oxidized forms are then able to interact with high redox potential substrate targets (Arora and Sharma, 2010).

Several studies have been reported on the dye degradation ability of laccase, which mainly depends on physiochemical parameters, cell aging, dye concentration, immobilization of whole cells etc (He *et al*., 2004; Kalyani *et al*., 2008). Immobilization methods have been proved to be most effective and straight forward technique(s) in order to improve the efficiency of oxidative enzymes for continuous application (Sheldon, 2007; Pang *et al*., 2011). Selection of immobilization techniques depends on certain properties of the biocatalyst which includes overall catalytic activity, effectiveness of catalyst utilization, deactivation and regeneration kinetics and cost (Duran *et al*., 2002).

Laccase immobilization can be performed through various methods which includes adsorption, entrapment, covalent binding, cross-linking etc. (Wang *et al*., 2008). In the present study, immobilization of laccase was carried out by entrapment method using sodium alginate. This was well supported by Mogharabi *et al*. (2012) where they immobilized *Pycnoporus sanguineus* laccase by alginate-gelatin mixed method and *Trametes versicolor* laccase by sodium alginate in the presence of copper sulphate or calcium chloride solution (Freire *et al*., 2001; Swamannan *et al*., 2012).

Most bacterial laccases are highly thermo-tolerant and maintain high levels of activity in neutral to alkaline conditions whereas at high temperature and pH, fungal laccases usually drop their activities. Thus, the aim of the present work is to ascertain guaiacol as the suitable synthetic substrate for the production of laccase from *B. subtilis* MTCC 2414. Moreover, optimization, partial purification and characterization of enzyme were also carried out followed by immobilization of laccase for efficient degradation of synthetic dyes, such as Orange 3R, Yellow GR and T Blue.

MATERIALS AND METHODS

Microorganism and substrate: *Bacillus subtilis* MTCC 2414 was procured from Microbial Type Culture Collection Center (MTCC), Chandigarh, India. The strain has been tested for laccase producing ability through plate test method (Rayner and Boddy, 1988). The culture was maintained on nutrient agar slants at 4°C and sub-cultured every three weeks. Guaiacol procured from Hi-Media Laboratory, Mumbai was employed as a raw material for the production of laccase by *B. subtilis* MTCC 2414.

Production of enzyme under SmF condition: Exactly 1 mL of guaiacol was added to a 250 mL Erlenmeyer flasks and was moistened with Mineral Basal Salt Solution (MBSS) containing (g L^{-1}): peptone 3, dextrose 10, K_2HPO_4 0.4, KH_2PO_4 0.6, $MnSO_4$ 0.5, $FeSO_4$ 0.0005 and $ZnSO_4$ 0.01. The contents of the flask(s) were sterilized, cooled to room temperature and inoculated with 1 mL

(3.5×10^6 CFU) of inoculum and incubated at optimum incubation period of 72 h, temperature 40°C and pH 7.0 (results of previous experiments done by authors). After incubation, the contents of flask was centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was examined for enzyme activity.

Partial purification of laccase: The supernatant obtained after centrifugation was concentrated by fractioned precipitation of 80% ammonium sulphate saturation. Flasks were re-suspended in 100 mL of 50 mM glycine-NaOH buffer (pH 9.0) and centrifuged at 10,000 rpm for 10 min. After centrifugation, the sample having maximum enzyme activity was extensively dialyzed against phosphate buffer (50 mM, pH 8.0) and used for immobilization studies. The experiments were carried out at room temperature (28±2°C). Laccase activity of the dialyzed sample was measured by monitoring the oxidation of 1 mM Guaiacol (Hi-Media, Mumbai, India) buffered with 0.2 M sodium phosphate buffer (pH 4.5) at 420 nm for 1 min. The reaction mixture (900 μL) contained 1 mM guaiacol, culture filtrate and 0.2 M sodium acetate buffer (pH 4.5) solution (300 μL each). One unit of enzyme activity was defined as the amount of enzyme that oxidized 1 μmol of guaiacol per minute. The enzyme activity was expressed in U mL^{-1} (Das *et al*., 1997).

Determination of molecular weight: To determine the molecular weight of the protein, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed on 12% polyacrylamide gel using protein markers, bovine serum albumin and oval albumin and the protein bands were visualized with Coomassie Brilliant Blue R (Laemmli, 1970).

Immobilization of laccase: The immobilization of laccase enzyme was carried out by entrapment method as described previously by Niladevi and Prema (2007) using sodium alginate as the efficient carrier material. Laccase (100 U) was added to the sodium alginate solution (2.5% w/v) and mixed thoroughly by mild shaking on a rotary shaker. The viscous alginate-enzyme mixture was taken in a syringe fitted with Luer-lock needle and the solution was added drop wise into 0.05 M copper sulphate under magnetic stirring to produce copper alginate beads. The beads were kept for hardening at 4°C for 2 h. After 2 h, the beads were filtered and washed thoroughly until there was no detectable protein in the wash out solution.

Characterization studies: The morphological characterization of immobilized laccase enzyme was evaluated by Scanning Electron Microscope (SEM, Model-JEOL-JSM 6390, Japan) and Fourier Transform Infrared Spectroscopy (FTIR, Model-SHIMADZU-IRTracer-100, Japan) was carried out for samples which showed maximum dye degradation and also for control dye.

Effect of pH and thermal stability: The pH profiles of free and immobilized laccase were measured in acetate buffer (0.05 M, pH 4.0-6.0), phosphate buffer (0.1 M, pH 6.0-8.0) and glycine-NaOH buffer (0.05 M, pH 8.0-11.0) at optimal temperature (30°C). Effect of temperature on the activities of free and immobilized enzymes were also conducted over the temperature range of 25-55°C for 24 h at optimum pH. Laccase with maximum activity at a desired pH and temperature was set as optimum for further experiments.

Dye degradation studies: For dye degradation studies, the dyes used were Orange 3R, Yellow GR and T-Blue. Twenty four hour old culture broth and partially-purified laccase of *B. subtilis* MTCC 2414, free and immobilized enzyme were used for dye degradation experiments. Initially,

the dyes were pre-heated with 40% sulfuric acid at a constant concentration of 0.01% (w/v). The final volume of culture filtrates (5 mL; 2.5×10^6 CFU mL^{-1}), free enzymes (50 U) and immobilized laccase (100 U) were analyzed and experiments were carried out under shaking conditions (150 rpm) at 40°C for different time intervals from 0-120 h. The residual dye concentration was monitored spectrophotomertrically (Hitachi U-2800 double beam spectrophotometer) at the maximum visible wavelength of each dye (Orange 3R-493 nm, T-Blue-664 nm, Yellow GR-430 nm) (Murugesan *et al*., 2007). Dye degradation was expressed in terms of percentage. The residual activity was measured using following equation:

$$\text{Residual activity (\%)} = \frac{AF}{AI}\times100$$

Where:
AI = Initial absorbance
AF = Absorbance of decolourized medium

RESULTS

Partial purification of laccase: The maximum laccase activity was observed in pellets (375 U mL^{-1}) when compared to supernatant sample (235 U mL^{-1}). The pellet sample was further dialyzed and enzyme activity was found to be 490 U mL^{-1}. The molecular weight of partially-purified laccase from *B. subtilis* MTCC 2414 was determined by SDS-PAGE with molecular mass of approximately 37 kDa (Fig. 1).

Immobilization of laccase: Spherical and regular shaped sodium alginate beads were obtained by using entrapment method and the diameter of the beads were measured to be 1.5-2.0 mm. The prepared immobilized beads were morphologically examined using SEM which showed dispersed and uniform structure (Fig. 2).

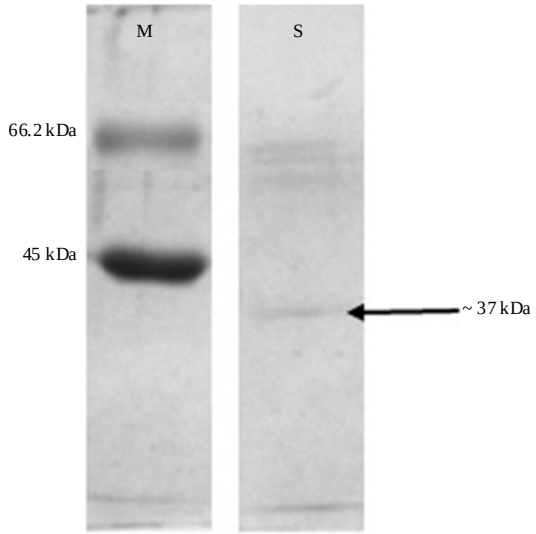

Fig. 1: SDS-PAGE of partially purified laccase, M1: Bovine serum albumin marker, S1: Laccase from *B. subtilis* MTCC 2414, M2: Oval albumin marker

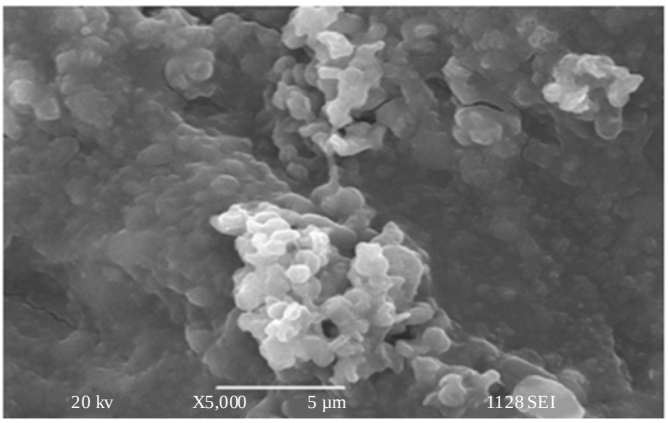

Fig. 2: SEM image of immobilized laccase enzyme from *Bacillus subtilis* MTCC 2414

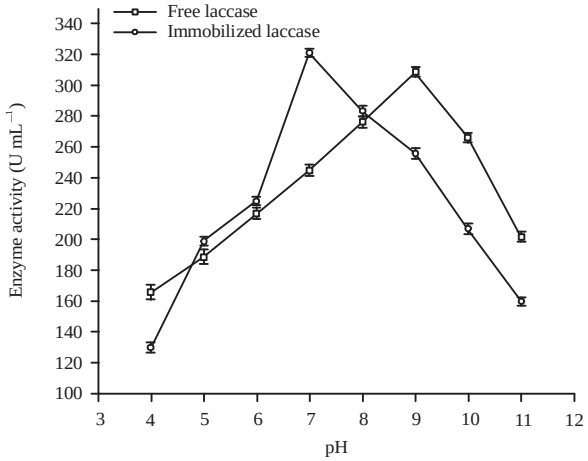

Fig. 3: pH profile of free and immobilized laccase from *Bacillus subtilis* MTCC 2414

Effect of pH and thermal stability: Among the investigated parameters, pH plays a key role in enzyme production by influence of hydrogen ion concentration. The effect of pH was studied in the range between 4.0-11.0, using acetate (4.0-6.0), phosphate (6.0-8.0) and glycine-NaOH (8.0-11.0) buffers. In case of free enzyme, the maximum laccase activity was observed at pH 9 (309 U mL^{-1}), whereas the minimum activity was found at pH 4 (166 U mL^{-1}). The immobilized laccase exhibited maximum activity at pH 7 (321 U mL^{-1}) and minimum activity at pH 4 (130 U mL^{-1}) (Fig. 3). Upon comparing the results, immobilized laccase exhibited maximum activity when compared to free laccase from *B. subtilis* MTCC 2414.

The effect of temperature had much significance in the SmF conditions because at fermentation, temperature might increases due to respiration in fermenting mass concentration. In the present study, *B. subtilis* MTCC 2414 exhibited maximum laccase activity at 35°C (339 U mL^{-1}) and minimum activity was observed at 55°C (154 U mL^{-1}) over free enzyme. Similarly, immobilized laccase showed higher activity at 35°C (317 U mL^{-1}), whereas lower activity was observed at 55°C (170 U mL^{-1}) (Fig. 4). The results revealed that, the thermal stability of laccase was higher in immobilized laccase than free enzyme.

Dye degradation of synthetic dyes: The most important biotechnological applications of laccase was degradation of synthetic dyes. To support this aspect, laccase producing bacterial strain

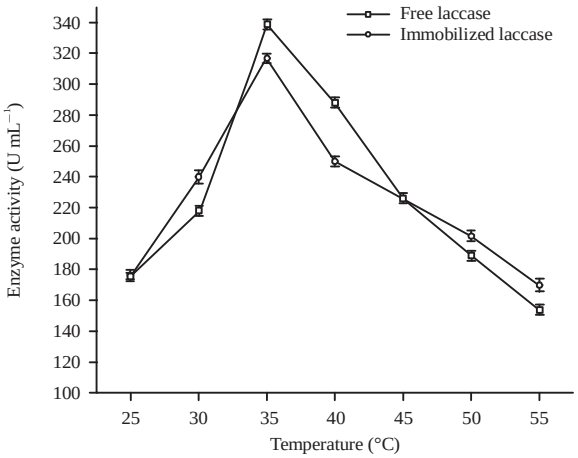

Fig. 4: Temperature profile of free and immobilized laccase from *Bacillus subtilis* MTCC 2414

B. subtilis MTCC 2414 was employed to efficiently degrade three selected synthetic dyes namely Orange 3R, Yellow GR and T Blue. A standard dye concentration of 0.1% (w/v) was used for the treatments by culture filtrate, free enzyme and immobilized laccase. The culture filtrate showed maximum degradation (72.16%) towards Orange 3R at 120 h followed by 69.52% of T-Blue and 69.34% of Yellow GR in similar period of time (Fig. 5). But, the degradation efficiency of culture filtrate against synthetic dyes was found to be less at initial incubation time of 24 h (i.e., 37.72% of T-Blue, 39.32% of Orange 3R and 43.27% of yellow GR).

Although free enzyme exhibited highest degradation efficiency of 74.60% against T-Blue dye followed by Yellow GR (72.72%) and Orange 3R (71.22%) (Fig. 5). Also, minimum degradation of 46.39% of Orange 3R was observed at early incubation time of 24 h followed by 47.64% of Orange 3R and 48.47% of Yellow GR. But, when compared to culture filtrate, free enzyme showed higher degradation ability on synthetic dyes. Surprisingly, the immobilized laccase displayed maximum degradation efficiency of 81.72% towards Yellow GR followed by 78.55% of T-Blue and 77.2% of Orange 3R (Fig. 5). But the lowest degradation efficiency of immobilized laccase was observed to be 54.2% of T-Blue, 55.73% of Yellow GR and 57.15% of Orange 3R at 24 h (Fig. 5). Upon comparing the overall results, the dye degradation percentage of Yellow GR dye was found to be efficiently degraded by immobilized laccase from *B. subtilis* MTCC 2414 when compared to other dyes employed in the present study. However, the current results suggested that, the immobilized laccase exhibited higher degrading ability over free enzyme and as well as culture filtrates *B. subtilis* MTCC 2414.

FTIR analysis: The FTIR spectral analysis for control dye and the treated dye (after decolourization at 120 h by immobilized laccase) was compared for structural difference after degradation process (Fig. 6). Immobilized laccase from *B. subtilis* MTCC 2414 showed maximum degradation activity towards the Yellow GR dye. In control dye, spectrum peaks at 2110.12 cm^{-1} represent C-N stretching of aromatic amine group in Yellow GR and the free N-H stretching structure was found to be at 3406.36 cm^{-1}. The presence of sulfonic acid was confirmed by asymmetric S = O stretching at 1429.25 and 837.11 cm^{-1}.

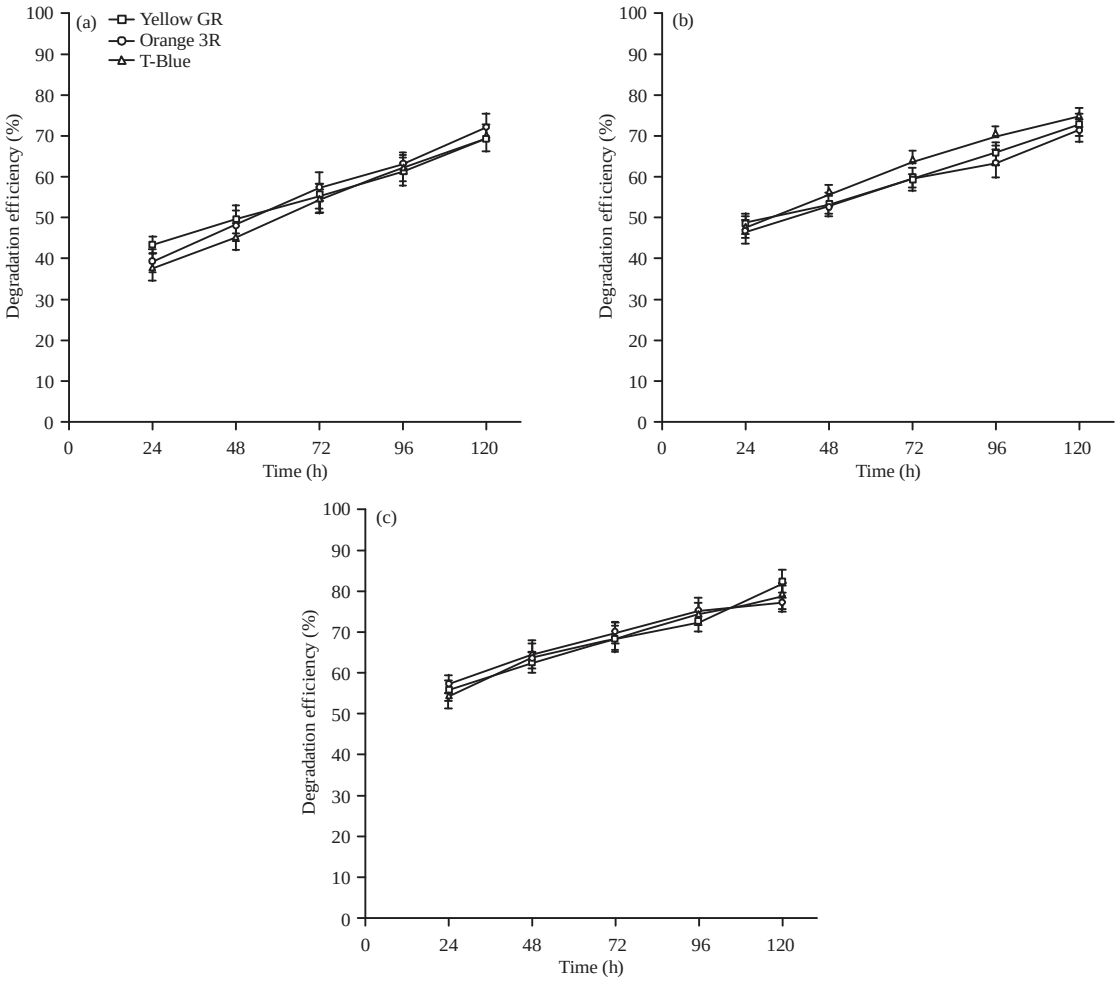

Fig. 5(a-c): Profiles of dye degradation using (a) Culture filtrates, (b) Free laccase and (c) Immobilize laccase from *Bacillus subtilis* MTCC 2414

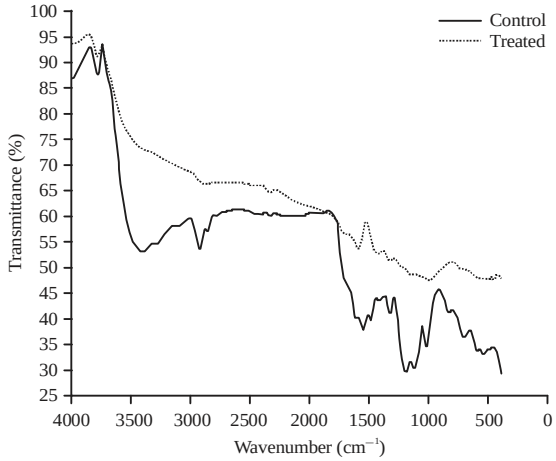

Fig. 6: FTIR spectrum of control dye: Yellow GR and treated dye by immobilized laccase from *Bacillus subtilis* MTCC 2414

DISCUSSION

In the present study, *B. subtilis* MTCC 2414 produced laccase activity in the presence of guaiacol as a substrate under optimized condition. The laccase activity was found to be maximum at pH 7.0, temperature 40°C and incubation time of 72 h (results for previous experiments done by authors). Whereas in case of *Schizophyllum commune* laccase, rice straw showed maximum production after 72 h of incubation (Adejoye and Fasidi, 2010). Also, *Lentinus crinitus* laccase showed enhanced production due to copper and phenolic compounds under optimized condition (Valle *et al*., 2014). Laccase from *Ascomycete mauginiella* was found to be optimum at 40°C (Palonen *et al*., 2003) and 30°C for *B. subtilis* WD23 laccase (Wang *et al*., 2013) which was consistent with current results. Moreover, the laccase produced from *B. subtilis* MTCC 2414 was found to be active at pH 7, which indicates that the results is well accordance with laccase produced from *R. praticola* (Murugesan *et al*., 2007).

Laccase from *B. subtilis* MTCC 2414 was partially purified by ammonium sulphate precipitation at 80% saturation and increase the activity only up to 1.3 fold purification which is due to substrate inhibition. Moreover, laccase are low molecular weight proteins, which can be eluted completely between 40-80% saturation. This was well supported with laccase produced from *Ganoderma* sp. MK05 which was purified at 70% saturation and attained 3.07 fold purification (Khammuang and Sarnthima, 2009), *S. lavendulae* laccase (Suzuki *et al*., 2003) purified at 50% saturation showed 9 fold increase in activity. Also, laccase from *S. psammoticus* exhibited 4.3 fold purification with 60% saturation (Niladevi and Prema, 2007).

The molecular mass of purified laccase from *Azospirillum lipoferum* was found to be 16.3 kDa suggesting that the enzyme is composed of catalytic polypeptide chain (Diamantidis *et al*., 2000). Many studies on purified laccase showed different molecular mass of 39 kDa for *Pseudomonas putida* laccase (McMahon *et al*., 2007), 29 kDa for S. *glaucescens* and laccase and the partially purified laccase form *B. subtilis* MTCC 2414 was found to be 37 kDa. The discrepancy between the current results and those reported previously could be due to the source of species from which the laccase was obtained.

Immobilized gelatin-mixed gel beads loaded with laccase from *B. subtilis* MTCC 2414 enzyme were characterized using SEM which showed spherical and oval shaped structure with average diameter of 2-2.5 nm, which revealed that the increase in alginate concentration due to rise in rupture force (Mogharabi *et al*., 2012). The optimal pH for immobilized laccase from *Shiraia* sp. SUPER-h168 was pH 6 (Yang *et al*., 2013) and laccase immobilized from *P. sanguineus* exhibited higher activity at pH 9 (Garcia *et al*., 2007). Moreover, laccase from *M. albomyces* showed the optimal pH range of 6-7.5 (Kiiskinen *et al*., 2004) and 8.5 for *S. psammoticus* (Niladevi and Prema, 2007). Also, *B. subtilis* MTCC 241 immobilized laccase showed optimum pH of 9 and pH 7 for free laccase which was similar to previously discussed results. The thermo-stability of *Shiraia* sp. SUPER-h168 was found to exhibit the maximum activity at 50°C (Yang *et al*., 2013) using guaiacol as a substrate whereas we have found the maximum activity at 35°C for both free and immobilized laccase. Similarly, Qi *et al*. (2014) reported that laccases Lacc C and Lacc B from *T. versicolor* were stable at 55 and 60°C.

To best of our knowledge, this was the first report on the degradation of synthetic dyes (Orange 3R, Yellow GR and T-Blue) using culture filtrate, free and immobilized laccase from *B. subtilis* MTCC 2414. The polyazo dye solophenyl red 3BL was degraded up to 79.66% by *Fomes fomentarius* laccase in the presence of acetosyringone at 14 h 30 min incubation (Neifar *et al*., 2011) and 47% acid violet 7, 81% Indigo caramine and 70% Acid green 27 by *Panus rudis* laccase (Zhang *et al*., 2006). Although, Pereira *et al*. (2009) showed that *B. subtilis* CotA laccase was able to decolorize

Sudan Orange G by 98% within 7 h of incubation but present study showed the degradation efficiency up to 81.72% Yellow GR at 120 h incubation, the reason for longer incubation period was due to substrate inhibition or prolonged enzyme reduction during degradation process (Osma *et al*., 2007).

In the present study the immobilized laccase showed more efficiency (81.72% of Yellow GR) than free laccase (74.69% of T-Blue) which is due to higher biomass concentration per volume unit and have higher operational stability of immobilized beads leads to process intensification and acceleration of degradation rates (Krastanov *et al*., 2013). Also, Mogharabi *et al.* (2012) reported that laccase immobilized by gelatin alginate exhibited maximum removal of dye of about 80% amino black and eosin, which was similar to this study. Although, Kuddus *et al*. (2013) showed *P. putida* MTCC 7525 laccase degraded Brilliant blue, Congo red and Crystal violet up to 74-93% at 24 h incubation which crossponds to 74.69% of T-Blue degradation by free laccase at 120 h incubation. Moreover, Kalme *et al*. (2009) suggested that, presence of amino group represents the starching of pyrrol ring like structure at a peak of 1340 cm^{-1} through FTIR, indicated that the some of the metabolites produced after decolourization. Therefore, the development of processes based on laccase seems to be an attractive biocatalyst due to their potential in degrading synthetic dyes of diverse chemical structure, currently employed in the industry (Hou *et al.,* 2004; Couto and Sanroman, 2005).

Laccase produced from *B. subtilis* MTCC 2414 exhibited maximum enzyme activity and stability at 40°C and pH 7. The synthetic dyes were degraded up to 81.72% when treated with immobilized laccase after 120 h incubation. Therefore, *B. subtilis* MTCC 2414 laccase proved to be efficient agent for degrading synthetic dyes that are currently used in textile industries. Further pilot scale studies are required with this biocatalytic process for actual industrial applications and detailed study is needed to explore the mechanism involved.

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