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ISOLATION AND SCREENING OF INDIGENOUS LACCASE PRODUCING FREE LIVING DIAZOBACTERIA FROM DYE WASTEWATER

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Abstract

Textile industries are one of the high generators of synthetic dye effluent. Many of these dyes are recalcitrant in nature and accumulate in nature. Some of these are mutagenic and carcinogenic and responsible for many deleterious effects on human health. Most of the conventional oxidation technologies have their own limitations. The ultimate solution of this is the search of biological systems such as enzymatic oxidations with advantages of specificity, biodegradable and reactions carried out in mild conditions. Among different decolorizing enzymes, laccase is getting much attention in detoxification and bioremediation of these synthetic pollutants. The important obstacle to commercialize the bacterial laccases was lack of sufficient stock and cost to achieve cheaper production and alteration by chemical means to obtain more robust and active enzyme. They also carry limitations of high cost of isolation and purification, non reusability, the instability of their structures and their sensitivity to harsh process conditions. But these limitations can be overcome by the use of immobilized enzymes. The role of free living diazotrophs is well known in soil sustainability and increasing fertility by atmospheric nitrogen fixation. Their role in degradation of environmental pollutants is also documented. In current studies the efforts are made to isolate and screen for indigenous laccase producing free living diazobacteria. The 57 laccase producing free living diazobacteria were enriched, isolated using Burks Nitrogen Free media as selective media. The laccase producing ability of isolate was confirmed in the range of 0.003 – 0.031 U/L by Guiacol assay. The potent isolate (0.031 U/l) was identified as *Klebsiella* sp. with reference to Bergey's manual of Determinative Bacteriology. The extracellular laccase enzyme was partially purified by neutral salt precipitation and dialysis. The extracellular enzyme was immobilized by entrapment method using calcium alginate beads. The immobilized potent diazobacteria showed 62% decolorization compared to 37% decolorization shown by free cells. The studies are in progress for application of enzyme *in situ* decolorization.

Key words: Bioremediation, Decolorization, Free living diazobacteria, Enzyme Immobilization, Laccase.

I. Introduction

Textile industries are one of the high generators of synthetic dye effluent. Currently, around 10,000 dyes are used in textile industries with approximately 7-10 tons annual consumption. These dyes may be classified based on their chromophore group viz. azo, anthroquinone, indigo, triphenylmethyl and phthalocyanine. Many of these dyes are recalcitrant in nature and accumulate in nature. Some of these are mutagenic and carcinogenic and responsible for many deleterious effects on human health. Hence, it becomes essential to treat before discharge **(Afreen S. *et al.*, 2016).** Most of the conventional oxidation technologies have the limitations of non specificity, side reactions, and use of environmental hazardous chemicals. The ultimate solution of this is the search of biological systems such as enzymatic oxidations with advantages of specificity, biodegradable and reactions carried out in mild conditions. The enzymatic treatment of synthetic water is recently getting importance. Among different decolorizing

enzymes, laccase is getting much attention in detoxification and bioremediation of these synthetic pollutants (**Rehan A. *et al.,* 2016).** Laccase (Benzene diol: oxygen oxidoreductase, EC 1.10.3.2) is the most extensively studied group of enzymes among oxidases. They belong to the family of blue multicopper oxidases, catalyzing the one electron oxidation of four reducing substrate molecules simultaneously reducing molecular oxygen to water. These enzymes are known to oxidize variety of phenolic compounds and aromatic amines **(Dittmer Neal T. *et al.,* 2004 and Kumar R. *et al.,* 2016).** Their commercial applications are in paper and pulp industries, biobleaching, biosensing and bevarages refining. They are also used in removal of pollutants viz. alkenes, chlorophenols, dyes, herbicides, polycyclic aromatic hydrocarbons and benzopyrene **(Grigorios Diamantidis *et al.,* 2000 and Gochev V.K. and Krastanov 2007).** Enzymes perform different features that make their use advantageous over conventional chemical catalysts. They also carry limitations of high cost of isolation and purification, non reusability, the instability of their structures and their sensitivity to harsh process conditions. But these limitations can be overcome by the use of immobilized enzymes (**Singh Gursharan *et al.,* 2010).** The alginate entrapment of enzyme is more advantageous and cheaper in comparison to other methods.

The most extensive application of laccase is in denim finishing, pulp delignification, textile dye bleaching, wastewater detoxification and transformation of antibiotics and steroids **(Fatemeh Sheikhi *et al.,* 2012).** The important obstacle to commercialize the bacterial laccases was lack of sufficient stock and cost to achieve cheaper production and alteration by chemical means to obtain more robust and active enzyme **(Allos Mina M. and Asmaa A. Hussein 2015).** The practical applications of the laccase in biotechnology point out the need of isolating microorganisms with laccase activity and having robust performance.

The role of free living diazotrophs is well known in soil sustainability and increasing fertility by atmospheric nitrogen fixation. Their role in degradation of environmental pollutants is also documented. In current studies the efforts are made to isolate and screen for indigenous laccase producing free living diazobacteria from dye wastewater.

II. Methodology

The congo red dye (model azo dye) and other chemicals were purchased from Loba Pvt. Ltd., Mumbai and the media were purchased from Hi media Pvt. Ltd., Mumbai.

i. Sample- The dye effluent sample was collected from local dyeing units and small scale dye industries in and around Ulhasnagar.

ii. Dye acclimatization and enrichment of collected wastewater

The collected dye effluent sample was acclimatized with 50 mcg/ml of azo dye with 20 % increasing dye concentration at interval of 5 days for 15 days. The dye acclimatization was carried out to adopt the organisms for dye toxicity. The dye acclimatized effluent sample was enriched for 3 cycles at interval of 5 days using Burks Nitrogen Free (BNF) liquid media containing 50 mcg/ml of congo red dye.

iii. Isolation and screening of laccase producing free living diazobacteria

The enriched broth was used for isolation of diazotrophs on solid BNF media. The screening for laccase producing diazobacteria was carried out using Luria Bertani (LB) agar media supplemented with 0.01% Guaicol. The development of brown color zone surrounding the bacterial colony on plate indicates laccase producer **(Desai Sagar A. 2017).** The isolates showing brown color zone around the colony were selected for further reconfirmation by colorimetric Guaicol assay.

iv. Determination of Laccase activity by Guaicol assay

The enzyme activity of selected isolates was reconfirmed and compared by Guaicol assay.

The oxidation of Guaicol by laccase enzyme results in formation of reddish brown color, which is read at 450 nm. Briefly, the 2 mM Guaicol in 10 mM sodium acetate buffer (pH 5.0) was used as substrate. The reaction mixture consisting of 1 ml Guaicol, 3 ml acetate buffer (pH 5.0) and 1 ml enzyme, was incubated at 30 ^{o}C for 15 min. and absorbance was read at 450 nm. In blank the enzyme was replaced with distilled water. The enzyme activity was calculated using formula (**Desai Savitha S. *et al*., 2011).**

Enzyme activity (U/ml)=(A x V) / (t x e x v)
Where, A is absorbance at 450 nm, V is volume of reaction mixture in milliliters, t is incubation time in minutes, e is extinction coefficient (12,100 m^{-1} cm^{-1}) and v is enzyme volume in milliliters.

v. Identification of potent laccase producer

Potent laccase producing bacterial isolate was identified on the basis of morphology, cultural and biochemical utilization with reference to Bergey's Manual of Determinative Bacteriology (2009) .

vi. Partial purification of crude laccase enzyme produced by potent isolate

The potent laccase producing bacteria was selected for further crude enzyme production and its partial purification. The partial purification of enzyme was carried out by neutral salt precipitation method using saturated solution of ammonium sulphate solution. This step was followed by centrifugation at 2000 rpm for 60 minutes. The precipitate pallet was solubilized in minimum quantity of phosphate buffer (pH 6.5). Further enzyme purification was done by dialysis against 100mM sodium phosphate buffer **(Verma Ambika *et al.,* 2016).** The protein content was determined by lowry method**(1951).**

vii. Immobilization of laccase enzyme using alginate entrapment method

To prepare alginate beads, 4% sodium alginate solution mixed with 10 ml partially purified enzyme and then dropped uniformly using hypodermal syringe in chilled 2% $CaCl_2$ solution to form beads. The beads were preserved in $CaCl_2$ solution overnight at 4^oC for hardening. On next day, beads were washed with sterile distilled water to remove excess $CaCl_2$. The 10 gms of beads were added in dye effluent sample. During studies the control flask was also run in parallel loaded with sterile beads and another control flask was loaded with free cells of isolate.. The sample was withdrawn after 24 hrs. to read %ge Decolorization by spectrophotometer assay.

%ge Decolorization = A_c - A_t x 100 / A_c

Where, A_c is absorbance of control flask and A_t is absorbance of test flask.

III. Results and Discussion

In current study, Congo red was used as model azo dye for induction of extracellular laccase enzyme. The microorganisms were acclimatized with increasing concentration of dye to make them adoptive for harsh environment of toxic dye. Further enrichment and isolation was carried out using BNF media for selective enrichment and isolation of free living diazobacteria from effluent. Total 57 isolates were obtained on enrichment and isolation. The diazotrophs were designated as DA1, DA2, DA3 and so on. On screening, 14 isolates were able to oxidize Guaicol and appeared as reddish brown growth on solid media. Arora Daljit Singh *et al.,* (2010) also performed screening of laccase producing isolates using guaicol during their studies on lignolytic fungal laccase and their application. Buddolla Vishwanath *et al.,* (2008) also reported use of gaicol in solidified media for qualitative determination of laccase activity by isolates., during their study on screening and assessment of laccase producing fungi from different environmental niches. By Guaicol oxidation assay, the enzyme activity for isolates was found in the range of 0.003 – 0.031 U/L (Table-1). The potent bacterial isolate was selected based on higher enzyme activity of (0.031 U/L). The potent laccase producing bacteria isolate was identified as *Klebsiella* sp. on the basis of morphology, cultural and biochemical utilization with reference to Bergey's manual.

Table 1 – Quantitative Laccase determination

Sr. No.	Diazotroph isolate	Laccase Enzyme production (U/L)
1	DA4	0.017
2	DA9	0.005
3	DA17	0.021
4	DA19	0.011
5	DA21	0.009
6	DA26	0.031
7	DA30	0.023
8	DA33	0.026
9	DA39	0.009

10	DA41	0.025
11	DA46	0.004
12	DA47	0.019
13	DA51	0.024
14	DA52	0.029

The extracellular crude laccase enzyme was partially purified by using neutral salt precipitation and dialysis method. The partially purified laccase was immobilized using alginate entrapment method and showed higher enzyme activity in terms of *ex situ* decolorization of effluent under optimized conditions in laboratory.

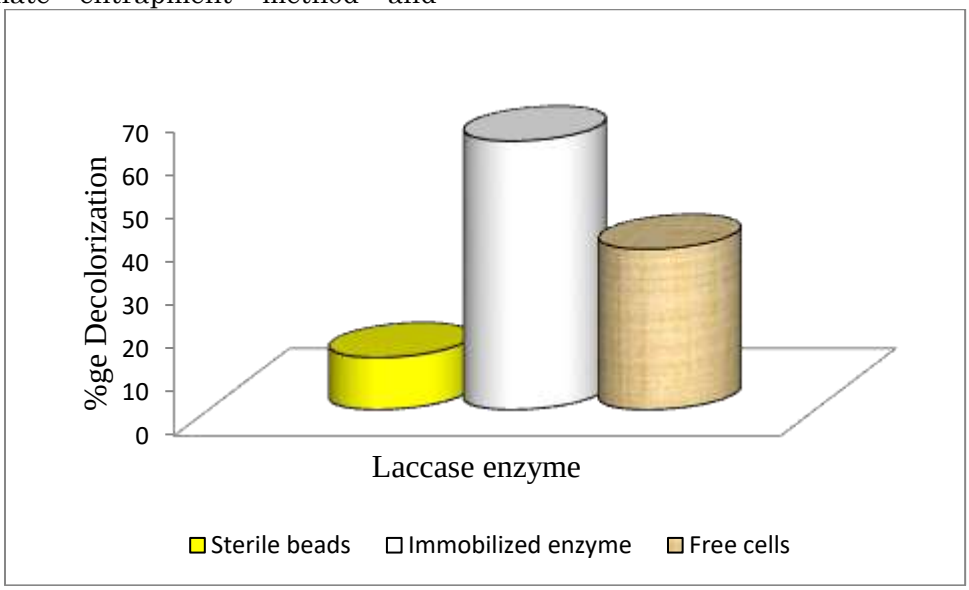

Figure 1- Dye effluent decolorization by laccase enzyme.

The decolorization of dye effluent was seen 62% and 37% for immobilized enzyme and free cells of isolate respectively (Figure 1). The free cells were acting as source of free extra cellular crude enzyme. The sterile beads also showed some decolorization could be because of dye adsorption by alginate beads. The immobilized enzyme was more active and efficient could be because of lack of dye toxicity against cells and alginate beads had given micro environment for direct reaction between enzyme and substrate

IV. Conclusion

In the current study, dye effluent sample was used as source for isolation of laccase producing bacteria. Among 57 free living diazobacteria, 14 isolates were able to produce laccase as confirmed by guaicol assay. The potent laccase producing bacteria (0.031 U/l) was identified as *Klebsiella* sp. The immobilized enzyme was more effective in comparison to free cells (unbound enzyme). Different laccase applications have been successfully carried out in vitro. However, lack of sufficient enzyme stocks and the cost of redox mediators is the major limitation. To overcome this limitation, efforts are made to achieve cheap overproduction of laccase. To obtain stable enzymes with low cost, the combination of following techniques will be helpful- the adsorption of laccase on a suitable inert carrier, increasing life span of the laccase activity and reutilization of the substrate / laccase product. The studies are in progress for application of enzyme *in situ* decolorization.

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