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Research Article

Isolation and characterization of Laccase producing bacteria from contaminated sites

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Abstract

In the present investigation, indigenous bacterial strains were isolated capable of laccase production. Total 58 different bacterial isolates were isolated from effluent and soil sediment samples collected from chemically contaminated sites near the vicinity of Surat. These isolates were screened for laccase production under submerged condition in Luria Bertani broth supplemented with 1% fructose and 0.01% $CuSO_4$ and laccase activity was measured using ABTS assay. Out of these, bacterial isolate namely SB1 was selected on the basis of maximum laccase activity of 0.694 U/l. The effect of pH, incubation temperature and copper supplementation on laccase production by both isolates were studied. Dye decolorization potential of laccases produced by both isolates were studied on Reactive red 152 dye in the presence of mediator ABTS. The isolated bacterial strain was identified as *Enterobacter spp.* on the basis of cultural and biochemical conditions.

INTRODUCTION

Lignocelluloses, the most abundant organic substances available on the globe, are becoming more valuable biomass resources as they can be easily converted to variety of energy containing products as well as can be used as substitute to fossil fuel resources (Fu *et al.,* 2013). Lining degradation is one of the critical factor in many of the industrial processes like pulp industry, bleaching industry and paper making industry which uses wood as substrate. Conventional methods used for delignification of wood involves harsh chemicals and conditions that causes serious environmental pollution by releasing chlorinated water and sulfur containing gases (Eugino *et al.,*2008; Lara *et al.,* 2003). Among all, most well-known lignin degrading enzymes have been classified from fungi, typically form those belonging to white-rot basidiomycetes. The enzymes produced by such microorganisms mainly include lignin peroxidase (LiP), manganese peroxidase (MnP) and laccases (Gassara *et al.,* 2010).

Laccases (EC 1.10.3.2, benzenediol oxygen oxydoreductase) are also called as "BLUE ENZYME" because they are multinuclear copper containing enzymes (Ryan *et al.,* 2003). They can catalyze the oxidation of various organic and inorganic compounds, including diphenols, polyphenols, diamines, substituted phenols, and aromatic amines with reduction of molecular oxygen to water (Kiiskinen *et al.,* 2004). Laccase also oxidizes other substrates such as aromatic amines, syringaldazine, 2, 2-azinobis (3-ethylbenzothiazoline-6-sulphonate) (ABTS), 1-hydroxybenzotriazole (HBT), 2, 2, 6, 6-tetramethylpiperidineoxy (TEMPO), violuric acid to form free radicals (Zille *et al.,* 2003). Laccases are widely distributed among higher plants, fungus (Mayer and Richard, 2000), and bacteria (Claus, 1994). Despite of wide occurrence in plants, laccases have not been used or characterize so far

because their detection and purification is difficult (Ranocha *et al.,* 1999). Fungus like ascomycetes, basidiomycetes, deuteromycetes, and other cellulolytic fungi are well known laccase producers and majority of laccase have been characterized from them only (Sharma *et al.,* 2007). Recently some bacterial laccases have been characterized from *Azospirillum lipoferum*, *Streptomyces lavendulae, Streptomyces cyaneus*, and *Bacillus subtilis*. Some bacterial laccases can be highly active and much more stable at high temperatures, at high pH as well as at high chloride concentrations as well (Bugg *et al.,* 2011; Dwivedi *et al.,* 2011; Reiss *et al.,* 2011; Peter *et al.,* 2014; Demissie and Kumar, 2014; Naz *et al.,* 2015).The aim of the present study was to isolate and characterize indigenous bacterial isolates, capable of laccase production, form industrial effluents and to establish optimal physiological parameters for laccase production and to investigate dye decolorization potential of laccases thus produced.

MATERIALS AND METHODS

Chemicals

All the chemicals used in this study were of analytical grade and procured from Hi-media laboratories, Mumbai, India.

Sample Collection and Enrichment

Effluent and soil sediment samples were collected form chemically contaminated sites and enriched by inoculating 1 ml of sample in 50 ml sterile nutrient broth. The flasks were incubated at 30 °C for 48 hr. at 120 rpm. After enrichment samples were screened for bacterial isolates.

Isolation and Screening of Laccase producing bacteria

For isolation of bacterial stains, loop full of enriched sample was streaked on nutrient agar plates and pseudomonas isolation agar plates and incubated at 37°C for 24-48 hr. After incubation plates were observed for morphologically different bacterial isolates.

Isolates were primarily screened for laccase production by plating them on Luria Bertani (LB) agar supplemented with Guaiacol (0.01%). Development of brown color zone, surrounding the bacterial growth, was indicating the production of Laccase. Colonies with brown color zone was selected and subjected for secondary screening. For secondary screening, a loop full of bacterial suspension was inoculated in 50 ml of sterile LB broth supplemented with 1% fructose and 0.01% $CuSO_4$ (Travers *et al.,* 2008) and incubated at 30°C at 125 rpm. Laccase activity was checked daily by method described below.

Selected bacterial isolates were streaked on nutrient agar and sent to Advanced Diagnostic Laboratory, Param doctor house, Lal Darvaja, Near Surat Railway Station, Surat, Gujarat for biochemical characterization. Biochemical tests were carried out on Phoenix Instrument, version: 6.01 A and EpiCenter, version: V6.20A.

Laccase Activity Assay (ABTS assay)

Laccase activity was measured using ABTS {2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)} as a substrate for Laccase. The reactive mixture consists of 1.5 ml sodium acetate buffer (1 mM, pH 5.0), 1.5 ml ABTS (0.5 mM) and 1.5 ml cell free supernatant. The absorbance was noted down at 420 nm using UV/Visible (Shimadzu-UV-3600 Plus). One unit of enzyme activity was defined as 1 micro mole of ABTS oxidized per minute (e = 3.6 x 104 M-1 cm-1) (Bourbonnais and Paice, 1990).

Laccase production under Submerged Condition

Laccase production was carried out under submerged condition in Laccase production medium (Tryptone 10 gl-1, Yeast Extract 5 gl-1, Sodium Chloride 10 gl-1, Fructose 0.1 gl-1 and Copper Sulphate 0.001 gl-1). Isolates form secondary screening were inoculated in the medium and incubated at 30°C at 120 rpm. Laccase activity was measured.

Optimization of process parameters

Effect of pH and Incubation Temperature on Laccase activity

Effect of initial pH of the media on Laccase enzyme activity was checked. For this initial pH of the Laccase production medium (LPM) was adjusted to 6, 7, 8 and 9. 1.5 ml of bacterial suspension was inoculated in LPM and incubated at 30°C at 120 rpm. Samples were and checked for laccase activity. The optimal temperature of laccase production differs greatly from one bacterium to another. 1.5 ml of bacterial suspension was inoculated in LPM and incubated at various temperatures like room temperature (30°C), 37°C and 45°C at 120 rpm. Samples were removed and checked for laccase activity.

Effect of Copper supplementation on Laccase activity The Laccase activity is influenced by the presence and absence of Cu^{2+} (Zourai *et al.,* 2006). Here, for optimization of copper supplementation LPM was prepared supplemented with different amount of $CuSO_4$ like 0.005, 0.01, 0.02 and 0.03%. 1.5 ml of bacterial suspension was inoculated in flasks containing different

amount of $CuSO_4$ and incubated at 37°C at 120 rpm. Samples were removed and checked for laccase activity.

Decolorization of Synthetic Dyes

For finding out dye decolorization potential of laccase enzyme a slightly modified protocol of Molina *et al.,* (2009) was adopted. The reaction mixture contained 50 mM phosphate buffer (pH 8), crude enzyme from broth and Reactive red 152 dye of various concentrations (100, 200, 300, 400 and 500 ppm) along with mediator ABTS. The decolorization was monitored spectrophotometrically at 490 nm using UV-VIS Spectrophotometer (Shimadzu-UV-3600 Plus). Crude enzyme was obtained from cell free extracts of selected isolates. Percentage dye decolorization was calculated according to formula: $D = 100(Aini - Aobs)/Aini$, where D is decolorization (%), Aini, initial absorbance and Aobs, observed absorbance.

RESULTS AND DISCUSSION

Sample collection and Enrichment

Six different effluent and soil sediment samples were collected from three different contaminated sites namely Ankleshwar GIDC, Amla Khadi and Koyali Khadi. Enrichment was done to promote the growth of bacterial populations in the samples.

Isolation and Screening of Laccase producing bacteria

For isolation, enriched samples were streaked on nutrient agar plates and pseudomonas isolation agar (PIA) plates. The plates were incubated at 37°C for 24-48 hr. Total 58 different bacterial colonies were isolated and primarily screened for laccase production by plating them on Luria Bertani (LB) agar supplemented with Guaiacol (0.01%). Out of these 58 isolates, 19 showed brownish zone surrounding the colonies indicating guiaicol oxidation. Colonies with brown color zone was selected and subjected for secondary screening. Selected 19 isolates were subjected to secondary screening by laccase production assay in liquid medium. Out of 19, bacterial isolate SB1 was selected on the basis of maximum laccase activity of 0.361 U/L. On the basis of cultural and biochemical conditions, selected isolate was identified as *Enterobacter spp.* (Data not shown).

Laccase production under Submerged Condition

Selected bacterial isolatewas tested for their potential to produce laccase under submerged condition. Maximum laccase activity observed was 0.490 U/L as shown in figure1.

Optimization of process parameters

Optimization of the production media and physicochemical parameters are considered as key factors to maximize the yield of laccase. Optimization by one parameter at a time is still considered as good method for optimization.

Effect of pH and Incubation Temperature on Laccase activity

The results of pH optimization suggested that isolated stain SB1 showed highest Laccase activity of 0.694 U/L at pH 9 as showed in the figure2. The results of pH optimization showed that pH in alkaline range was the ideal condition for laccase production by both selected isolates. The initial media pH shifted towards the higher pH as the enzyme production started. The ability of both isolates to grow and produce laccases in media of high pH values makes this enzyme more suitable for industrial applications.

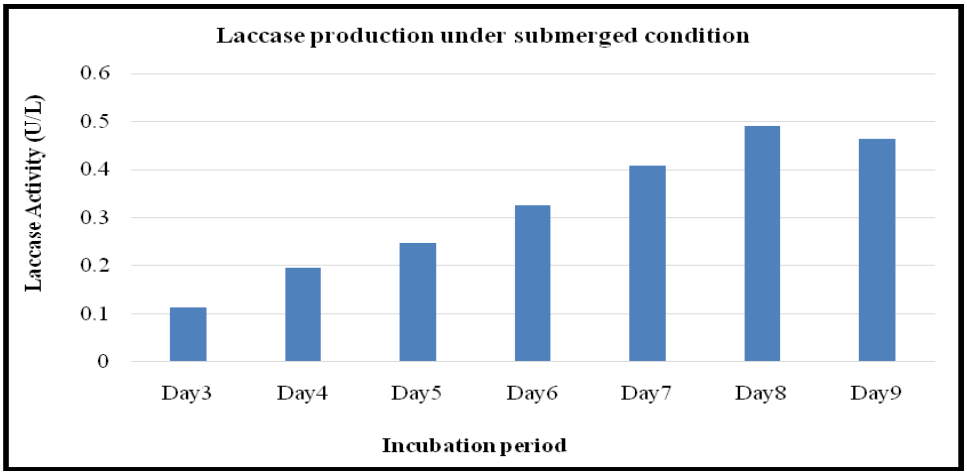

Figure 1. Laccase production by bacterial isolates under submerged condition.

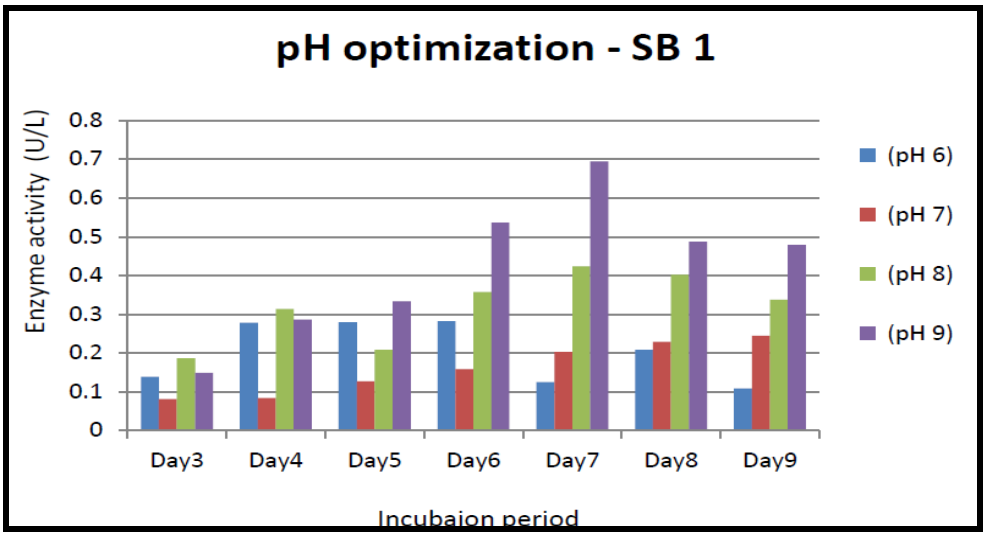

Figure 2. pH optimization for laccase production by isolate SB1.

The optimal temperature of laccase production differs greatly from one bacterium to another. The bacterial isolate SB1 showed the maximum laccase activity of 0.351 U/L at incubation temperature of 30°C as showed in the figure3. Similar results were also obtained by Mishra *et al.,* (2017) as they observed maximum laccase production at 30°C temperature by *Streptomyces lavendulae.*

Effect of Copper supplementation on Laccase activity

The results copper supplementation showed that, maximum laccase activity of 0.348 U/L was obtained at 0.01% of Copper sulfate concentration as showed in figure 4. Copper has been previously reported to be a strong laccase inducer in the fungal and bacterial laccases. Similar results were also obtained by the studies carried out by Telke *et al.,* (2012) where *Pseudomonas sp.* LBC1 laccase showed increased activity by the addition of $CuSO_4$.

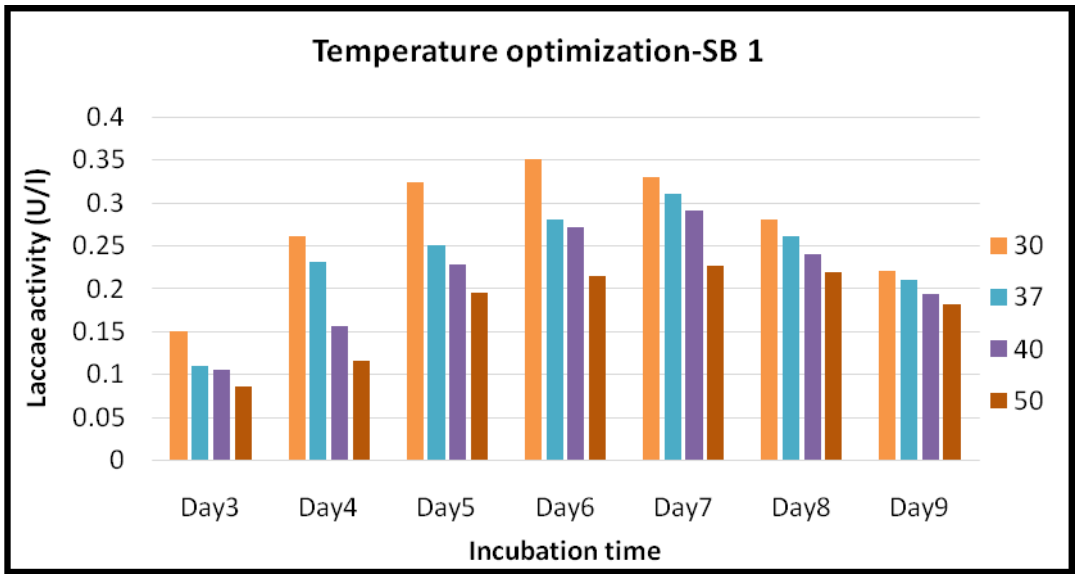

Figure 3. Temperature optimization for laccase production by isolated bacteria.

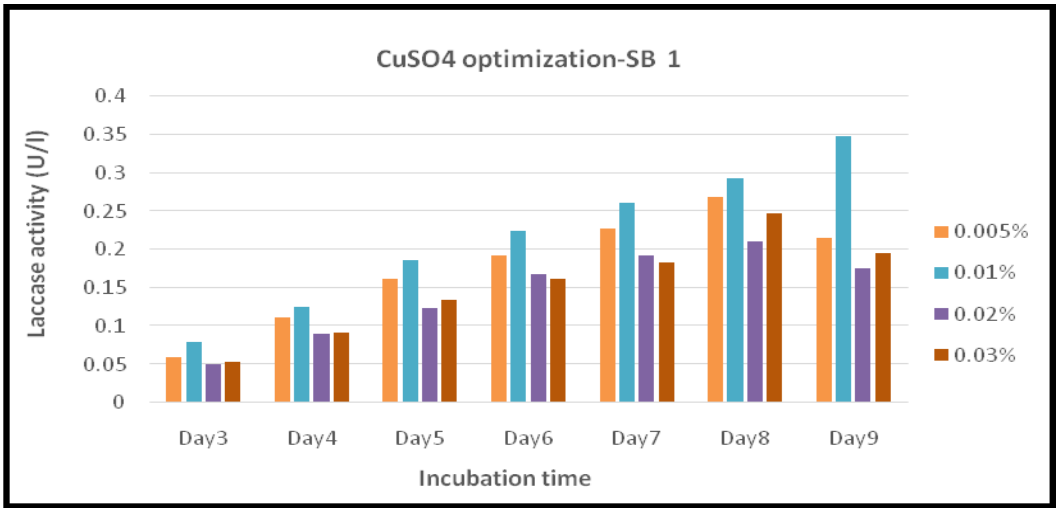

Figure 4. Effect of copper supplementation on laccase production by isolate SB1.

Decolorization of Synthetic Dyes

Different concentrations of Reactive red 152 dye were used for the assessment dye degrading potential of laccase enzyme produced by two selected isolates. For this, cell free supernatant was used as enzyme source and decolorization experiments were carried out in the presence of mediators like ABTS. Laccase from SB1 isolate showed highest dye decolorization of 41 % after incubation of 24 hours. (Figure5).

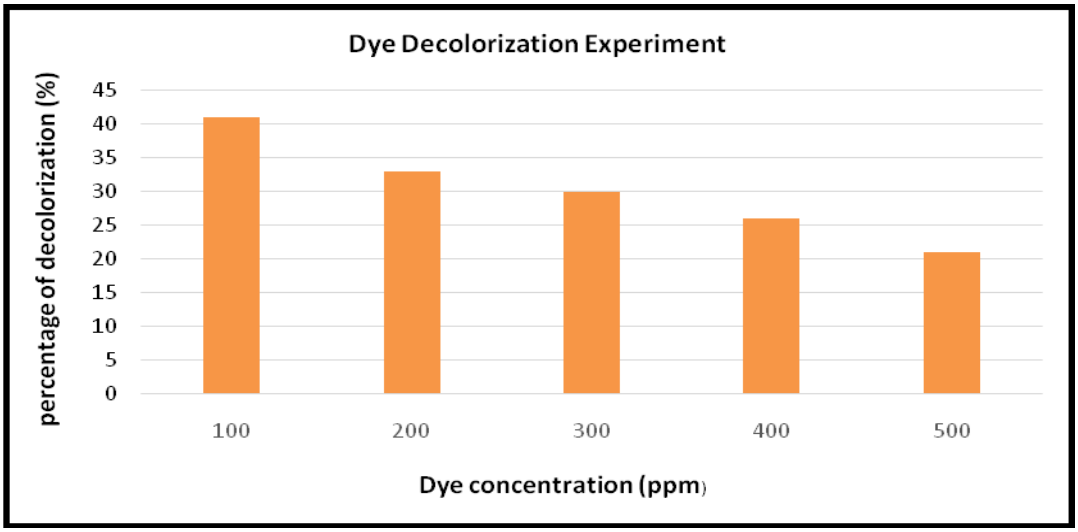

Figure 5. Dye decolorization by both bacterial isolates.

Similar kind of results were also observed by Demissie and Kumar, 2014. They observed 96% decolorization of Congo red dye when treated with laccase produced by *Streptomyces spp.* isolated form soil. This results are also supported by the findings of various other researchers (Baughman and Perenich 1988; Roy *et al.,* 2005).

Thus, bacterial laccases have attracted a great deal of attraction due to their importance in biotechnological applications and economic benefits. Such enzymes may also be useful for many other industrial applications. Here, we reported the isolation of bacteria capable of laccases production form industrially contaminated sites with promising bioremediation potential in dye effluent treatment.

Form the present study it could be concluded, the bacterial isolate capable of laccase production was identified as *Enterobacter spp.* Enzyme production was optimal in presence of 0.01% copper sulfate at pH 9 and 30° C temperature. Also, enzyme produced here has capability of decolorizing Reactive Red 152 azo dye by 41% of decolorization in presence of ABTS mediator. Dye decolorization by crude laccase enzyme shows its economical applicability in textile effluent treatment. Isolated bacteria can be explored at industrial level for decolorization of dye containing wastewater.

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