



SCREENING AND ASSESSMENT OF LACCASE PRODUCING BACTERIA FROM DIFFERENT ENVIRONMENTAL SAMPLES

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ABSTRACT

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 including wood-rotting fungi where it is often associated with lignin peroxidase, manganese dependent peroxidase, or both. Because of its importance in bioremediation. These include abundant applications in effluent detoxification, enzymatic conversion of chemical intermediates, wine clarification degradation of textile dyes etc. In the present study, two potential microbes were isolated on solid medium containing guaiacol and ABTS for laccase activity out of 10 microbes. Two cultures PHP7 and PKD5 were selected for molecular characterization was carried out using 16S rRNA gene technology of PHP7 revealed as *Bacillus cereus* (KU878970.1). Using laccase from PHP7 and PKD5 isolates, explored at industrial level for decolorization of coloured effluents that significance in environmentally friendly and play critical role as bioremediation at commercial scale.

INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) are multicopper enzymes belonging to the group of blue oxidases which exist widely in nature and are defined in the Enzyme Commission (EC) nomenclature as oxidoreductases which oxidize diphenols and allied substances and use molecular oxygen as an electron acceptor (Thurston, 1994; Kiiskinen et al., 2002; Kiiskinen and Saloheimo, 2004). They are predominantly found in higher plants and fungi (Thurston, 1994; Mayer and Staples, 2002). Literature review reported that laccases are extensively dispersed among the bacteria. For example, laccases from *Azospirillum lipoferum* (Diamantidis, et al., 2000), *Marinomonas mediterranea* (Solano, et al., 1997), *Streptomyces griseus* (Endo, et al., 2003), *E. coli* (Kim, et al., 2001), *Bacillus subtilis* (Muthukumarasamy, et al., 2015) and many more bacteria have been identified and characterized by Shrestha, et al.(2016).

Laccases are also useful for the decomposition of azo dyes by oxidative methods (Michael et al., 2005). In view of its importance in large scale application, the present endeavor is to search for highly efficient laccase producing fungi with secretion of abundant amounts of laccase and to reduce the cost of production.

Many more bacteria have been identified and characterized by Shrestha, et al.(2016). In laccases,

molecular oxygen work as an electron acceptor to catalyze the oxidation of array of substrates (Revanker and Lele, 2006). Due to laccase activity on mixture of substrates, numerous diverse compounds used as indicators for conformation of laccase production. Some indicators used are Guaiacol, Tannic acid, 2, 2-azino-bis (3- ethylbenzthiazoline-6- sulphonic acid (ABTS), Syringaldazine and polymeric dyes like remazol brilliant blue-R (Cristina et al., 2004; Jiejie et al., 2007; Buddolla et al., 2008; Teck et al., 2010; Chun et al., 2010). Laccases producing bacteria have been screened by visualizing laccase activity on a solid state fermentation of especially fungi (Nishida et al., 1988; De Jong et al., 1992) or with liquid cultivation examined with enzyme activity measurements. Laccases are recognized for their wide spread applications including ethanol production, food processing, dye bleaching, paper and pulp processing, and production of value added chemicals from lignin (Giardina et al. 2010; Shrestha et al., 2016). Literature review reported numerous industrial applications of laccases as textile dye bleaching, bioremediation, effluent detoxification and biopolymer modification (Xu, 1999; Lu et al., 2010; Aravind et al., 2016). The enzyme concerned in linking monomers, polymer degradation and also break down of aromatic compounds. It has received a lot of scientific awareness over last couple of decade for their proficiency to oxidize phenolic and non-phenolic lignin linked compounds as well as highly recalcitrant environmental pollutants

treatment (Brenna and Bianchi, 1994; Roy *et al.*, 2005). Rapid industrialization and widespread use of pesticides have become a grave environmental trouble that led to soil pollution and effect agricultural productivity to major extent. Synthetic dye containing effluents causes unresponsive impact to the surrounding environment and also poisonous and hazardous to biological life (Lee *et al.*, 1999). In this context, the objectives of the present work were to isolate and molecular characterized laccase producing bacteria that could be use as bioremediation for industrial dye effluent treatment.

MATERIAL AND METHODS

A. Collection and screening of samples

The samples was collected from four different industrial chemical dye contaminated sites, Khadi udyog, Industrial discharge from various parts of Kanpur and Bijnor District. Collected samples contain the mixture of polluted water and soil with soil sediments. The samples were collected in sterile plastic bags, were sealed and brought to the lab aseptically for further studies sealed and brought to the lab aseptically for further studies.

B. Enrichment and Isolation of bacteria

The 5 g of soil sample was inoculated individually in 500 mL Erlenmeyer flask containing 100 mL Bushnell Haans medium (BHM) (3.72 g/L) addition with wheat stalk powder (5 g/L) as the sole carbon source for enrichment purpose. The medium was incubated at 37°C at 120 rpm on rotary shaker for 7 days. Laccase producing microbes were isolated on LB agar plate incubated at 37°C for 24 h. Distinct bacterial colonies were isolated and frequently subculture until pure cultures was obtained. All the pure cultures were store on LB agar slant at 4 °C.

C. Qualitative screening for laccase activity

Primary screening of the isolates for laccase production was prepared on different dye containing agar plates. Toluidine Blue, Methylene Blue, Congo Red, Malachite Green dye selected for screening potent microbes (Chun *et al.*, 2010). LB agar supplemented with different dye at specific concentration (25 mg/L) with substrate as a 4.0mM guaiacol and 1.0 mM ABTS (2, 2'- Azino-bis-[3-ethylbenzthiazoline6-sulphonicacid]). All cultures were incubated on LB media containing dye. Laccase secretion was monitored by appearing clear zone surrounding the colonies. Laccase activity was specified by the diameter of haloes on agar plate.

D. 16s rDNA identification and laccase gene amplification of potential isolates

The isolated colonies were further taken for genomic DNA extraction for amplification of 16S rDNA gene (Ahmed *et al.*, 2007; Patel *et al.*, 2017) by suspending in TE buffer in a micro-centrifuge tube. These cells were heated for 10 minutes at 94°C and were centrifuged at 6,000 rpm for 5 minutes. The upper layer was used as template DNA for the amplification of 16S rDNA gene and partial laccase gene amplification were carried out by ABI Veriti PCR Machine (Applied Biosystems, USA)

using improved PCR Program (Sambrook *et al.*, 2009; Safary *et al.* 2016; Patel *et al.*, 2017).

E. Production of laccase enzymes

Optimization and Biomass production at pH and temperature level The optimum pH value for Laccase production varies depending on the substrates employed, even though numerous literature have been reported a bell shaped profile as normal distribution curve for laccase activity. Four 100 mL Erlenmeyer flask containing 50 mL Laccase Production Medium were prepared. Then, some flasks were marked with different pH like 5, 6, 7, and 8 while some flask were place at different temperature RT, 30, 40 and 50°C. Different pH was set by pH meter using 1M NaOH and 1M HCl according to the marking on the flask. 25 mg/L toluidine blue dye was added, flasks were inoculated by 1 mL of microbial culture of 24 h having OD of 0.6. and then flasks were incubated for 9-10 days at 125 rpm. (Sivakumar *et al.*, 2010; Bhuvaneshwari *et al.*, 2015).

F. Dye degradation at different pH and temperature

The toluidine blue dye, were prepared individually with the concentration of 25 mg/L in sterilized distilled water as blank and for test the medium was prepared in LB broth along with 1 mL of culture. The culture filtrate with laccase activity was used as a source of enzymes to test its efficiency degradation of effluents and dyes. The percentage of degradation achieved was calculated with reference to the control samples that were not treated with enzyme for 8 days at an interval of 48 h at 450 nm. (Chun, *et al.*, 2010).

RESULT AND DISCUSSION

Recently, research on pollution research has offered a simple and cost efficient ways of handling synthetic dye containing effluents. Dye decolourization using bioenzymes like laccases has been suggested as an eco-friendly and Bioremediation alternative for treatment of industrial synthetic dyes (Robinson *et al.*, 2001; Gnanasalami and Gnanadoss, 2013). However, yield of laccases produced for industrial and commercial purposes are insufficient to meet the market demand. Screening of potent laccase producing microbes that are competent and secrete elevated yields of laccase are required. In the present study, two Potential microbes (PHP7 and PKD5) have been isolated from different region of Kanpur and Bijnor Districts that could be used as bioremediation for synthetic dye degradation.

Enrichment and Isolation of bacteria

5 gm each of all the 4 samples collected from different collection sites were inoculated in to Bushnell Haans medium (BHM) supplemented with wheat stalk powder separately as the solitary carbon source for fortification of desired microbes. These enriched cultures were used to isolate microbes having high laccase activity. After enrichment, microbes were isolated using spread plate technique (Abdulkarim, *et al.*, 2005). Colonies showing distinct morphological features were selected for further

qualitative screening. Bacteria were the major source isolated from the different samples, followed by fungi and actinomycetes. For easy cultivation and maintenance, the bacteria were selected for further examination. Ten different colonies were selected on the basis of its morphological characteristics i.e. PHP7, PHP6, PHP8, PSG3, PSG9, PKD2, PKD5, PPS4, PPS1 and PPS10. The isolated bacteria were further purified and streaked on LB agar slant and preserved at 4°C in refrigerator.

Biomass at different pH by isolates PHP7 and PKD5

Determination of biomass indicated the growth rate of microbes. Higher the biomass, higher is the growth rate of isolates. Efficient biomass was determined at different pH at room temperature on shaking condition. The biomass produced by PHP7 was 0.053 mg/5 mL, while

the biomass produced by PKD5 was 0.058 mg/5 mL (Table 1). PKD5 and PHP7 growth rate were higher at pH 8, pH 7 respectively at 8 days of incubation.

Dye degradation at different pH by isolates PHP7 and PKD5

PHP7 isolate showed highest degradation of dye of 64.28% after incubation of 6 days at pH7 while from PKD5 isolate shows highest degradation of dye i.e. 61.90% after incubation of 8 days at pH8 (Table 2).

Laccase enzyme production at different pH by isolate PHP7 and PKD5

Isolate PHP7 showed highest Laccase activity of 0.489 U/L at pH 7 while PKD5 showed 0.404 U/L Laccase activity at pH 8 at 8th day of incubation, respectively in Table 3.

Table 1: Biomass of PHP7 and PKD5 at different pH

Sr. No.	Time	pH5	pH6	pH7	pH8	pH5	pH6	pH7	pH8
1	Day2	0.006	0.012	0.023	0.016	0.004	0.014	0.023	0.024
2	Day4	0.014	0.021	0.036	0.024	0.009	0.021	0.033	0.043
3	Day6	0.019	0.029	0.049	0.032	0.016	0.027	0.049	0.056

Table 2: Dye degradation of PHP7 and PKD5 at different pH.

S. No.	Day	Biomass of PHP7 at different pH				Biomass of PKD5 at different pH			
		pH5	pH6	pH7	pH8	pH5	pH6	pH7	pH8
1.	Day 2	2.38	7.14	21.42	2.38	2.38	7.14	11.9	23.8
2.	Day 4	14.28	19.04	42.85	21.42	0.39	16.66	35.71	42.85
3.	Day 6	21.42	33.33	64.28	33.33	0.37	42.85	47.61	59.52
4.	Day 8	21.42	35.71	64.28	42.85	0.36	40.14	53.02	61.9

Table 3: Laccase enzyme activity of PHP7 U/L and PKD5 U/L at different pH.

S. No.	pH	Laccase enzyme activity of PHP7 U/L	Laccase enzyme activity of PKD5 U/L
1.	5	0.112	0.086
2.	6	0.290	0.272
3.	7	0.485	0.385
4.	8	0.374	0.401
5.	9	0.240	0.329

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