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Screening and Characterization of High Laccase Producing *Enterobacter sp*. from Devarayanadurga Forest Soil, Tumkur District

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ABSTRACT

Eight laccase producing bacteria were isolated from forest soil samples using an enrichment culture technique. The bacterial isolates were grown on LB/Cu^{2+} agar medium containing 10 mM guaiacol for detection of extracellular laccase enzyme. The bacterial isolates produced reddish brown coloration around the colonies was further purified and plate assayed on the same media for laccase enzyme production. In liquid culture, the highest laccase production (7.12 U/ml) was achieved on the 3rd day of incubation at 37°C and pH 7.0. The most potent bacterial isolate-ISL6 was identified as Enterobacter sp. by morphological and biochemical characterization. The characteristic feature of the enzyme indicates that it is active in alkaline pH and has substrate specificity.

Key words: *Bacteria, Laccase, Forest soil, Guaiacol, Enterobacter sp.*

INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) are copper-containing enzymes that belong to the so-called blue copper oxidases. These enzymes are responsible for the oxidation of a variety of aromatic amines as well as phenolic compounds with the reduction of molecular oxygen to water[1]. In addition, in the presence of small-molecular-weight compounds named redox mediators, laccases are able to oxidize non-phenolic structures[2]. This together with the fact that they only use molecular oxygen as a co-substrate instead of hydrogen peroxides as used by peroxidases makes laccases very attractive for different biotechnological applications. To date, fungal laccases have been extensively studied while the potential of bacterial laccases for the same purpose has not been equally assessed until now[3]. It was reported that of fungal laccases lack stability at high temperature and pH[4], however, bacterial laccases have the ability to overcome these drawbacks of fungal laccases[5], making them compatible with almost all industrial processes[6].

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The first bacterial laccase was reported from *Azospirillum lipoferum*, subsequently, laccases were discovered in a number of bacteria such as *Bacillus subtilis, Bordetella compestris, Caulobacter crescentus, Escherichia coli, Mycobacterium tuberculosis, Pseudomonas syringae, Pseudomonas aeruginosa* and *Yersinia pestis*[7]. In the present study, we report the isolation, screening, and characterization of high laccase producing *Enterobacter sp*. from Devarayanadurga forest soil samples of Tumkur District, based on guaiacol assay[8].

MATERIAL AND METHODS

Sample Collection:

The soil samples were collected from Devarayana Durga forest, (13.375 N latitude, 77.213 E longitudes), Tumkur District. Soil samples were collected in sterilized plastic bags from a depth of 10–15 cm below the earth's surface. All the samples were kept at 4°C until used.

Isolation and screening of extracellular laccase producing bacterial strains

Eight laccase producing bacterial strains were isolated by enrichment technique by using M9 culture medium supplemented with 0.2 mmol/L Cu^{2+} were inoculated with 10 g of soil sample and incubated at 37°C on a rotary shaker (150 rpm) for 2 days. The 5 ml culture was transferred to 100 ml Luria-Bertani (LB) culture medium containing 0.2 mmol/L Cu^{2+} and incubated at 37°C at 150 rpm for 6 days. Stable enrichment cultures were obtained after subculture. To isolate pure cultures, the enriched bacterial cultures were appropriately diluted with the sterile saline solution (0.9% NaCl) before spreading onto LB/Cu^{2+} agar plates supplemented with guaiacol (10 mM) and incubated at 37°C for 3-5 days. The bacterial isolates were reconfirmed for laccase enzyme production on LB/Cu^{2+} agar medium[9] supplemented with guaiacol (10 mM) as substrate and without guaiacol as control[8]. Later the pure, positive cultures of bacterial isolates showing extra- cellular laccase secretion on guaiacol supplemented plates were developed in LB/Cu^{2+} broth and the cultures were maintained in 15% glycerol as a stock culture for further analysis[10]. The enzyme was produced in 250 ml Erlenmeyer flasks, containing 100 ml of LB/Cu^{2+} broth devoid of guaiacol, and micronutrient solution[11]. Finally, the pH was adjusted to 7.0 prior to sterilization (15 lbs, 121°C, and 15 min). The production medium was inoculated with 1% inoculum (2.0 A_{610}) and incubated at 37°C under shaking condition (150 rpm) for six days. After incubation, the culture broth was centrifuged at 10,000 RPM for 10 min at 4°C and the cell free supernatant was used as crude enzyme to measure laccase activity.

Laccase activity assay:

Laccase activity in the supernatant was determined with two different substrates viz., Guaiacol[8], and hydroquinone[12]. Laccase activity in the supernatant of bacterial culture was determined in 30 mM Tris buffer (pH 8.0), containing 10 mM guaiacol and 10 mM hydroquinone as substrates and incubated at 37°C for 10 min. One unit of laccase was defined as the amount of the enzyme required to transform 1µmol substrate per min under standard assay conditions. All the experiments on laccase activity were performed in triplicates, with an average standard deviation of laccase activity less than 5%. Laccase activity in supernatant of bacterial culture was determined in Tris-HCl buffer (pH 8.0), the reaction was started by adding the stock solutions of guaiacol and hydroquinone into buffer to make a final concentration of 10 mm, and then absorbance was measured at 470 nm for guaiacol (e= $6740M^{-1}Cm^{-1}$) and 248 nm for hydroquinone (e= $17252M^{-1}Cm^{-1}$) respectively in an UV-Visible spectrophotometer. The laccase activity was calculated as per the following formula:

$$E.A = A \times V/t \times e \times v$$

Where;
A=Absorbance at λ_{max}
V= Volume of the reaction mixture (in ml)
v= Volume of the enzyme extract (in ml)
t=Incubation time (in min)
e=Extinction coefficient of the substrate

Morphological and Biochemical characterization of high laccase producing bacterial isolate:

High laccase producing bacterial isolate (ISL-6) was examined for morphological and biochemical characteristics with reference to Bergey's Manual of Systematic Bacteriology. Gram staining, catalase, and cytochrome-C oxidase tests were performed according to standard protocol. Gram's characteristics and cell morphology of the isolated strain (ISL-6) were determined by light microscopy and further, the morphology of bacterial cells grown overnight in culture tubes were examined with a scanning electron microscope (SEM) (S-4800, Hitachi Corp., Japan). For SEM, the suspended cells were fixed in a 2.5% glutaraldehyde and 0.1 M phosphate buffer solution (0.1 M, pH 7.2) for 2 hr, and then dehydrated with a graded ethanol series from 30 to 100% for 20 min each. After dehydration, the samples were critical-point dried and then sputter-coated with gold under vacuum for SEM examination[13]. High laccase producing bacterial isolate (ISL-6) tested for carbon sources utilization, the pure culture was inoculated respectively into peptone-water culture medium containing 1% substrates (disaccharides such as Glucose, Maltose, Lactose, Sucrose, and Mannitol) and all the tubes were incubated at 37°C for 24 to 48 hr. Control tubes contained uninoculated medium and were incubated at the same conditions. Further, the isolate (ISL-6) is tested for few biochemical characteristics by inoculating the isolate on to selective media such as casein agar, urea agar, nutrient gelatin, tributyrin and starch agar medium, to identify protease, urease, gelatinase, lipase and amylase production, respectively. We used a nitrate broth medium to determine if our bacterial isolate (ISL-6) is capable of reducing nitrate (NO^{3-}) to nitrite (NO^{2-}) or other nitrogenous compounds via the action of the enzyme nitratase (also called nitrate reductase). After incubation, these tubes are first inspected for the presence of gas in the Durham tube and further testing includes the addition of sulfanilic acid (nitrate I) and dimethyl-α napthylamine (nitrate II). If nitrite is present in the media, then it will react with nitrate I and nitrate II to form a red colored compound. If instead, the tube turns red after the addition of a pinch of Zinc dust, this indicates a negative result. MR/VP test was used to determine which fermentation pathway is used to utilize glucose. We used the Voges-Proskauer test to detect the presence of acetoin, a precursor of 2, 3-butanediol. In order to test this pathway, an aliquot of the MR/VP culture is removed and α-naphthol and KOH were added. The tubes were shaken vigorously and set aside for about 1 hr until the results can be interpreted. The bacterial isolate (ISL-6) was also tested for indole production in nutrient broth medium. It tests the ability of the strain to produce indole. We used Simmon citrate agar plates. It is used to determine if our strain can use citrate as its sole carbon source and energy. The pure culture was inoculated into Simmons citrate agar and incubated at 37°C for 24 hr. All the above test and other biochemical tests were carried out in triplicate[9,14]. The results were tabulated.

Effect of buffer strength on laccase activity

Effect of buffer strength on laccase activity of bacterial isolate (ISL-6) was studied by assaying the culture supernatant in Tris-HCl buffer and Sodium acetate buffer of varying buffer ionic strengths (10 mM, 20 mM, 30 mM, 50mM, 60 mM, 100 mM) with 10 mM guaiacol and hydroquinone. The test tube containing reaction mixture (laccase substrate) of each buffer strength level was incubated for 10 minutes for evaluation of laccase activity at 470 nm and 248 nm under assay conditions. Similarly, the effect of buffer pH on laccase activity was carried for different pH ranged.

Effect of buffer pH on laccase activity

Effect of medium pH on laccase activity was studied by assaying the culture supernatant at different pH ranged from pH 5.0-10.0 with 10 mM Guaiacol and 10 mM hydroquinone as substrates respectively. The test tube containing the reaction mixture of each pH was incubated at 37°C for 10 min for estimation of laccase activity at 470 nm and 248 nm respectively.

RESULTS AND DISCUSSION

The result showed that total of eight bacterial isolates shows laccase activity on guaiacol plate assay, they developed brown coloration around the line of streak on LB/Cu^{2+} agar medium (Fig.1). One of the potent bacterial isolate (ISL-6) having high level of laccase activity was selected for further studies (Table.1). Several reports are available on laccase production by the microorganisms isolated from different habitats. Forest soil samples itself is a source of microorganisms which can be used for isolation of potential microorganism for industrial applications. It was around 96 bacterial isolates were isolated from pulp and paper mill effluent, soil samples, bagasse, and degraded tree barks and found that 16 *Bacillus* strains had capability to produce laccase in the presence of guaiacol[8]. The marine culture of Alteromonas MMB-1 was isolated from Mediterranean Sea using Marine Agar 2216 plates to produce neutral laccase[15]. Laccase producing bacterial isolates has been isolated by using enrichment technique with the addition of copper ion as inducer[16]. It was used lignin-based laccase-mediator compounds (LCMOs) were used such as guaiacol as substrate to detect extracellular laccase producing isolates[17]. The conventional laccase assay method uses *viz.*, guaiacol,2,6-dimethoxyphenol, syringaldazine, ABTS [2, 2-azino-bis (3ethylbenzothiazoline-6-sulfonic acid)] to detect oxidized coloured products of substrate. However, it has been reported that plate assay test for screening based on colour indicator compounds *viz.* guaiacol and tannic acid are efficient substrates to obtain novel laccase producers[18] but some of the microbes that showed positive reactions on colour indicator but did not produce laccase in liquid cultures but may produced other ligninolytic enzymes, such as lignin peroxidases or manganese-dependent peroxidases[19], because these enzymes are also capable of decolourizing the polymeric dyes[20]. Hence, the quantitative detection of laccase activity is very much important for the isolated microorganisms. In the present investigation, a total of eight strains showed brown coloration around the line of streak on LB/Cu^{2+} agar medium containing guaiacol (Fig.1). Thereafter, all the eight positive strains were screened quantitatively with guaiacol under assay condition. Among all, bacterial isolate (ISL 6) showed maximum laccase activity (7.12 U/ml) with guaiacol after 3rd day of incubation at 37°C (Table.1; Fig.2).

Morphological and Biochemical characteristics:

The morphological and cultural characteristics of the bacterial isolate (ISL-6) was found to be Gram's negative, and the SEM image illustrated that they are 0.6-1.0 × 0.7-1.8 µm long rods (Fig.3,4). The cells occurred in aggregation, and the strain can grow both aerobically and anaerobically suggests that the strain is facultative anaerobe. Colonies appear to be opaque, circular white to slightly pink in color, slightly moist to mucoid, with center slightly raised, margin flattened, edges minutely lobed and 3-6 mm in diameter (Table.2; Fig.5). It was non-spore former, capsulated, and shows motility. Further, the bacterial isolate (ISL 6) was tested for biochemical characterization as per Bergey's manual of Systematic Bacteriology[9,14,21]. The bacterial isolate (ISL-6) showed the positive reaction for nitrates reduction and citrate utilization. The isolate (ISL-6) showed the negative reaction for gelatinase, casein protease, indole, urease and amylase production (Table.3). The fermentation test results showed that gas was produced among all the carbohydrates tested by inverted Durham's tube containing broth of dextrose, maltose, lactose, sucrose, and mannitol. However, acid production was not observed in dextrose, maltose, and sucrose fermentation broths but it was observed in lactose and mannitol fermentation broth (Table.4). These properties are similar to those of genus *Enterobacter* according to Bergey's Manual of Systematic Bacteriology. On the basis of the above results, the bacterial isolate (ISL6) was identified as *Enterobacter sp*. Similar analysis is conducted for the identification of *Enterobacter sp*[22].

Enzyme production and assay:

The Guaiacol assay was used to monitor laccase production by *Enterobacter sp*. in broth culture. The substrate Guaiacol (10 mM) oxidation rates were observed linear at all laccase concentrations. A rapid and linear increase pattern was observed at 470 nm till complete exhaustion of the substrate (Guaiacol). The most influenced parameters on the laccase assay were evaluated as different buffer strength and pH. The hydrogen ion concentration (pH) of a solution can affect laccase structure in the same way the buffer strength thereby may affecting the ability of laccase activity. Fungal laccases were reported generally active at low pH ranged of pH 3.0-5.0[23]. Minor changes in the optimal pH range were observed for the related to the protonation and or de-protonation state of the phenolic substrates[24]. In all cases, laccase activity was substantially higher in 30 mM and 50 mM Tris-HCl buffer for guaiacol and 20 mM and 30 mM Tris-HCl buffer for hydroquinone, respectively (Table.6; Fig.6). The laccase activity was maximum 7.12 U/ml at optimum pH 8.0 of 30 mM Tris-HCl buffer for 10 mM Guaiacol. Whereas, it was observed 5.90 U/ml during the 3rd day of incubation at 37°C at optimum pH 8.0 in 10 mM Tris-Hcl buffer containing 10 mM hydroquinone (Table.7; Fig.7). Compared to its activity with guaiacol at optimum pH 8.0 was 82% less active with hydroquinone at pH 8.0 this showed a broader substrate specificity in bacterial laccase. The strong pH influence observed on the laccase activity of both substrates correlated to equilibrium between two contrasting observable facts. Firstly as pH increases, the redox potential difference also increases and as a result oxidation rate between substrate and laccase type 1 (T1) Cu, which assigned for the substrate oxidation. Second, binding of the hydroxide anion (OH^-) to the T2/T3 Cu, where the reduction of oxygen to water takes place and as a result it leads to inhibition of laccase activity at high pH due to increased hydroxide anion (OH^-)[25].

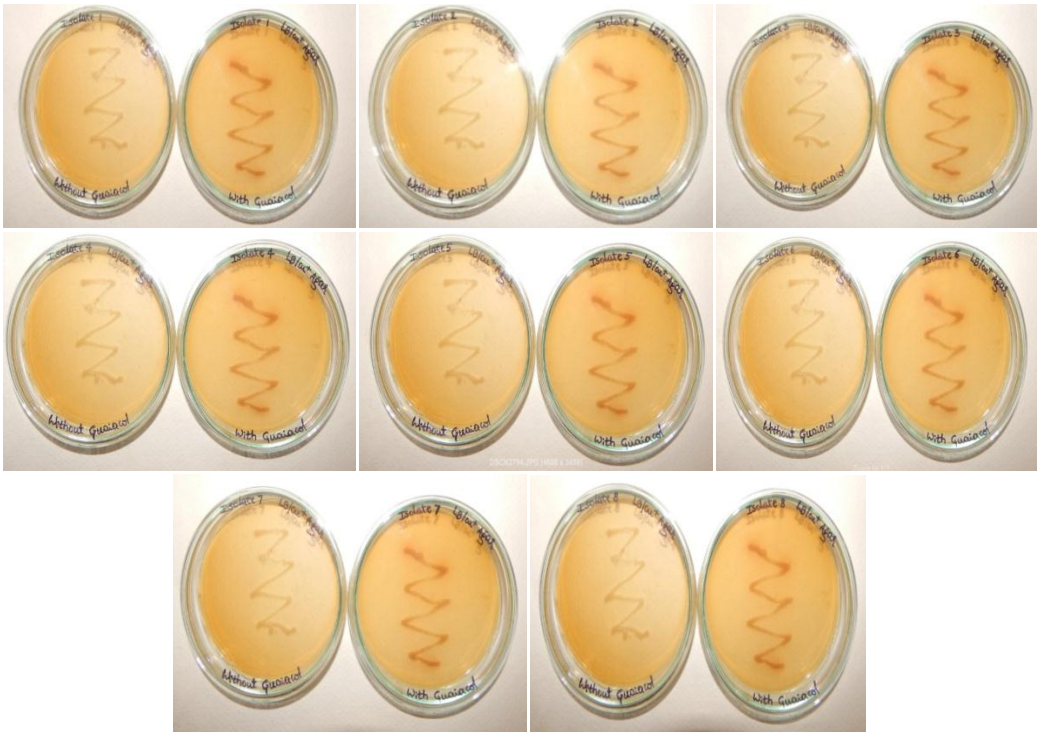

Fig. 1: Screening of Bacterial isolates (ISL1-8) on LB/Cu^{2+} agar medium Supplemented with guaiacol (10 mM)

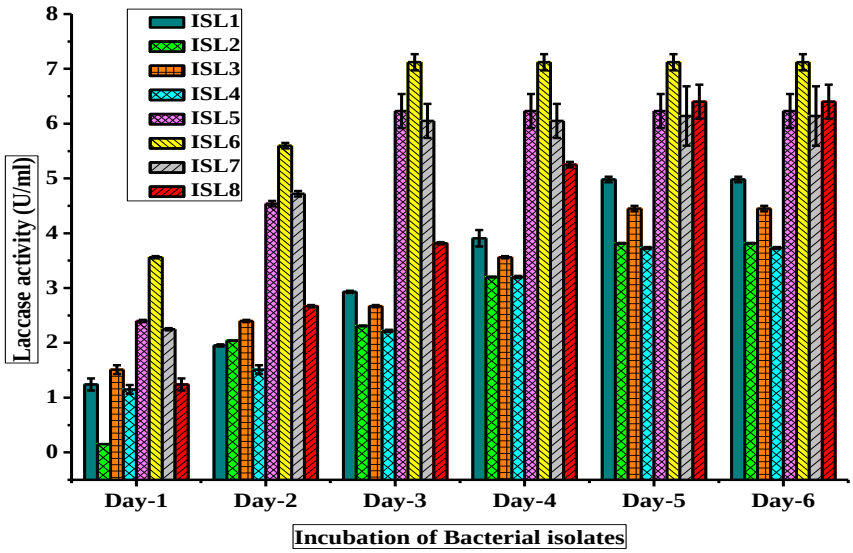

Fig. 2: Quantitative screening of Bacterial isolates for extracellular laccase production using guaiacol as substrate at 37°C at pH 7.0 for the different period (values are the mean ± SD of triplicates)

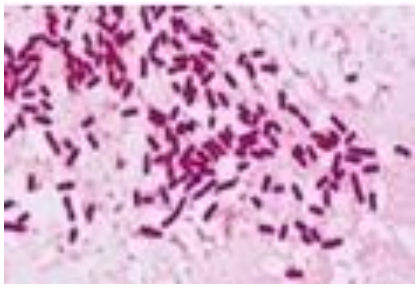

Fig. 3: Gram's staining of Bacterial Isolate (ISL-6)

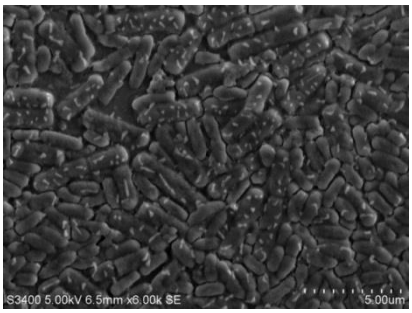

Fig. 4: SEM image of Bacterial Isolate (ISL-6)

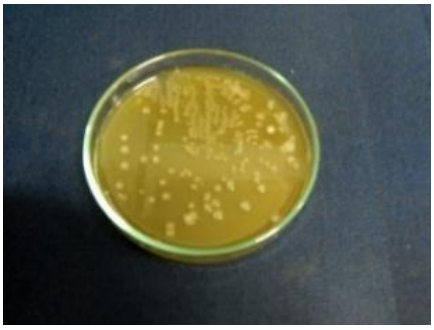

Fig. 5: Bacterial Isolate (ISL-6) on LB/Cu^{2+} Agar medium

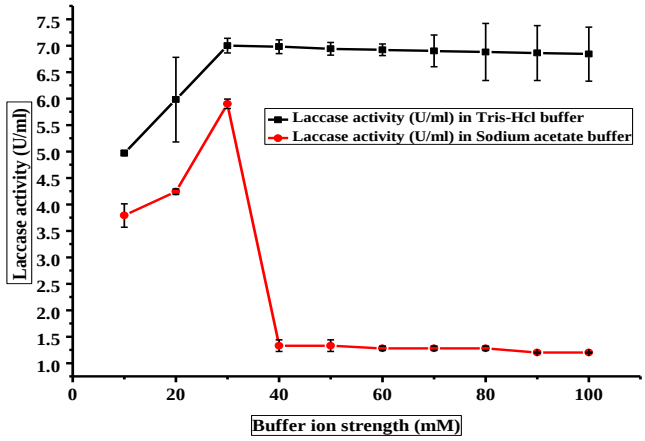

Fig. 6: Effect of buffer ion strength on laccase assay by *Enterobacter sp.* grown on LB/Cu^{2+} broth at initial pH 7.0 under shake flask culture at 37°C ± 0.5 on 3rd day of incubation (values are the mean ± SD of triplicates)

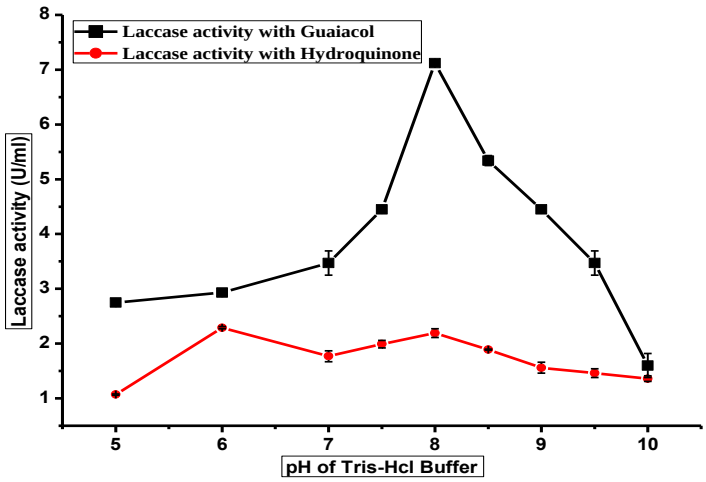

Fig. 7: Effect of pH on substrate guaiacol and hydroquinone on laccase assay by *Enterobacter sp.* has grown on LB/Cu^{2+} broth at initial pH 7.0 under shake flask culture at 37°C ± 0.5 on the 3rd day of incubation (values are the mean ± SD of triplicates)

Table 1: Quantitative screening of laccase activity from bacterial isolates (The values are presented as the mean ± SD of triplicate tests)

Incubation of Bacterial isolates	Laccase activity of ISL-1 (U/ml)	Laccase activity of ISL-2 (U/ml)	Laccase activity of ISL-3 (U/ml)	Laccase activity of ISL-4 (U/ml)	Laccase activity of ISL-5 (U/ml)	Laccase activity of ISL-6 (U/ml)	Laccase activity of ISL-7 (U/ml)	Laccase activity of ISL-8 (U/ml)
Day 1	1.24±0.11	0.15±0.03	1.51±0.08	1.15±0.08	2.4±0.02	3.56±0.02	2.25±0.02	1.24±0.11
Day 2	1.95±0.02	2.04±0.02	2.4±0.08	1.51±0.08	4.54±0.05	5.6±0.05	4.71±0.05	2.67±0.02
Day 3	2.93±0.02	2.31±0.02	2.67±0.02	2.22±0.02	6.23±0.31	7.12±0.15	6.05±0.31	3.82±0.02
Day 4	3.91±0.15	3.2±0.02	3.56±0.02	3.2±0.02	6.23±0.31	7.12±0.15	6.05±0.31	5.25±0.05
Day 5	4.98±0.15	3.82±0.02	4.45±0.02	3.73±0.02	6.23±0.31	7.12±0.15	6.14±0.54	6.4±0.31
Day 6	4.98±0.15	3.82±0.02	4.45±0.02	3.73±0.02	6.23±0.31	7.12±0.15	6.14±0.54	6.4±0.31

Table 2: Morphological characterization of the bacterial isolate (ISL 6)

Characteristics	***Enterobacter sp.***
Colony diameter	3mm-6mm in diameter
Colony texture	mucoid
Colony colour	Slight pink
Cell morphology	Gram –ve rods
Cell motility	+ve

Table 3: Biochemical characterization of the bacterial isolate (ISL 6)

Sl. No.	**Biochemical Test**	**Test Result***
1	Gelatin hydrolysis	+
2	Urease	-
3	Lipase	-
4	Oxidase	-
5	Catalase	+
6	Casein protease	-
7	Amylase	-
8	Nitrate reduction	+
9	Indole production	+
10	Methyl red	+
11	Voges-Proskauer	+
12	Citrate Utilization	+

*Results are expressed as means of three independent experiments.

Table 4: Sugar fermentation profile of the bacterial isolate (ISL 6)

Sl. No.	**Carbohydrates**	**Acid Production**	**Gas Production**	**Test Result***
1	Glucose	-ve	+ve	Negative
2	Maltose	-ve	+ve	Negative
3	Lactose	+ve	+ve	Positive
4	Sucrose	-ve	+ve	Negative
5	Mannitol	+ve	+ve	Positive

*Results are expressed as means of three independent experiments.

Table 5: Effect of buffers ion strengths on laccase activity of *Enterobacter sp.* (The values are presented as the mean ± SD of triplicate tests)

Buffer ion strength (mM)	**Laccase activity (U/ml) in Tris-Hcl Buffer**	**Laccase activity (U/ml) in Sodium acetate Buffer**
10 mM	4.97±0.05	3.79±0.22
20 mM	5.98±0.80	4.24±0.05
30 mM	7.12±0.13	4.75±0.02
40 mM	7.0±0.14	5.90±0.09
50 mM	6.98±0.13	1.33±0.11
60 mM	6.94±0.12	1.33±0.11
70 mM	6.92±0.11	1.28±0.04
80 mM	6.90±0.30	1.28±0.04
90 mM	6.88±0.54	1.28±0.04
100 mM	6.86±0.51	1.20±0.01

Table 6: Effect of pH on substrate Guaiacol and Hydroquinone on laccase activity of *Enterobacter sp.* (The values are presented as the mean ± SD of triplicate tests)

pH of Tris HCl Buffer	Laccase activity (U/ml) for Guaiacol	Laccase activity (U/ml) for Hydroquinone
5.0	2.75±0.02	1.07±0.01
6.0	2.93±0.02	2.29±0.02
7.0	3.47±0.02	1.77±0.10
7.5	4.45±0.05	1.99±0.07
8.0	7.12±0.01	2.19±0.08
8.5	5.34±0.09	1.89±0.01
9.0	4.45±0.05	1.56±0.10
9.5	3.47±0.22	1.46±0.08
10.0	1.60±0.02	1.36±0.05

CONCLUSION

In view of the results obtained, it can be concluded that high laccase producer can be discovered from forest soil samples by very simple plate test screening method. Guaiacol is a responsive substrate for screening the laccase producing bacterial isolates. In our study, one of the form was showing high laccase activity (ISL6) was identified as *Enterobacter sp*. according to morphological and biochemical characterization. This strain was more sensitive towards guaiacol than hydroquinone as a substrate. This is in agreement with the results reported for a purified laccase from the Ascomycete *Xylaria polymorpha*[26] and for laccase from the Basidiomycete *Trametes sp*[27]. In our study, the lower laccase activity towards hydroquinone (substrate) is due to laccase inactivation by reaction products[28] and highest laccase activity was obtained with guaiacol than hydroquinone. In this study catalysis of guaiacol and hydroquinone by an *Enterobacter sp*. were assayed. This shows laccases vary in their substrate specificities, organism to organism and several substrates should be tested to assess laccase activity. The results obtained in the present study indicated *Enterobacter sp.* is a potential strain for laccase production. Further, it is valuable to identify up to species level accurately by molecular identification using a 16S rRNA sequence-based technique for various biotechnological applications.

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REFERENCES

1. Thurston, C. F., The structure and function of fungal laccases. *Microbiology*, **140(1):** 19-26 (1994).
2. Desai, S., Nityanand, C., Microbial laccases and their applications: a review. *Asian J Biotechnol*, **3(2):** 98-124 (2011).
3. Thakur, I. S., Screening and identification of microbial strains for removal of colour and adsorbable organic halogens in pulp and paper mill effluent. *Process Biochemistry*, **39 (11):** 1693-1699 (2004).

4. Mukhopadhyay, A., Dasgupta, A. K., Chakrabarti, K., Thermostability, pH stability and dye degrading activity of a bacterial laccase are enhanced in the presence of Cu2O nanoparticles. *Bioresource technology*, **127:** 25-36 (2013).
5. Ye, M., Li, G., Liang, W. Q., Liu, Y. H., Molecular cloning and characterization of a novel metagenome-derived multicopper oxidase with alkaline laccase activity and highly soluble expression. *Applied microbiology and biotechnology*, **87(3):** 1023-103 (2010).
6. Arias, M. E., Arenas, M., Rodríguez, J., Soliveri, J., Ball, A. S., Hernández, M., Kraft pulp biobleaching and mediated oxidation of a nonphenolic substrate by laccase from Streptomyces cyaneus CECT 3335. *Applied and Environmental Microbiology*, **69(4):** 1953-1958 (2003).
7. Dhiman, K., Shirkot, P., Bioprospecting and molecular characterization of laccase producing bacteria from paper mills of Himachal Pradesh. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, **85(4):** 1095-1103 (2015).
8. Sheikhi, F., Ardakani, M. R., Enayatizamir, N., Rodriguez-Couto, S., The determination of assay for laccase of Bacillus subtilis WPI with two classes of chemical compounds as substrates. *Indian journal of microbiology*, **52(4):** 701-707(2012).
9. Wang, C.-l., Zhao, M., Li, D.-b., Cui, D.-z., Lu, L., Wei, X.-d., Isolation and characterization of a novel Bacillus subtilis WD23 exhibiting laccase activity from forest soil. *African Journal of Biotechnology*, **9(34):** (2010).
10. Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Short protocols in molecular biology, (1992).
11. Bains, J., Capalash, N., Sharma, P., Laccase from a non-melanogenic, alkalotolerant γ-proteobacterium JB isolated from industrial wastewater drained soil. *Biotechnology letters*, **25(14):** 1155-1159 (2003).
12. Ögel, Z., Yüzügüllü, Y., Mete, S., Bakir, U., Kaptan, Y., Sutay, D., Demir, A., Production, properties and application to biocatalysis of a novel extracellular alkaline phenol oxidase from the thermophilic fungus Scytalidium thermophilum. *Applied microbiology and biotechnology*, **71(6):** 853-862 (2006).
13. Alphenaar, P., Groeneveld, N., Van Aelst, A., Scanning electron microscopical method for internalstructure analysis of anaerobic granular sludge. *Micron*, **25(2):** 129-133 *(*1994).
14. Palleroni, N., Genus, I., Pseudomonas. *Bergey's manual of systematic bacteriology*, **2:** 323-379 (2005).
15. Solano, F., Garcia, E., Perez, D., Sanchez-Amat, A., Isolation and characterization of strain MMB-1 (CECT 4803), a novel melanogenic marine bacterium. *Applied and environmental microbiology*, **63(9):** 3499-3506 (1997).
16. Singh, G., Sharma, P., Capalash, N., Performance of an alkalophilic and halotolerant laccase from γ-proteobacterium JB in the presence of industrial pollutants. *The Journal of general and applied microbiology*, **55(4):** 283-289 (2009).
17. Wang, Y., Liu, Q., Yan, L., Gao, Y., Wang, Y., Wang, W., A novel lignin degradation bacterial consortium for efficient pulping. *Bioresource technology*, **139:** 113-119 (2013).
18. Kiiskinen, L. L., Rättö, M., Kruus, K., Screening for novel laccase-producing microbes. *Journal of applied microbiology*, **97(3):** 640-646 (2004).
19. De Jong, E., De Vries, F. P., Field, J. A., van der Zwan, R. P., de Bont, J. A., Isolation and screening of basidiomycetes with high peroxidative activity. *Mycological Research*, **96(12):** 1098-1104 (1992).
20. Couto, S. R., Rivela, I., Sanroman, A., In vivo decolourization of the polymeric dye poly R-478 by corncob cultures of Phanerochaete chrysosporium. *Acta biotechnologica*, **20(1):** 31-38 (2000).

21. Aneja, K. R., *Experiments in microbiology, plant pathology, tissue culture and mushroom production technology*. 3rd Edition, New Age International Limited, New Delhi, 276-282 (2001).
22. Feng, C., Li, J., Qin, D., Chen, L., Zhao, F., Chen, S., Hu, H., Yu, C.-P., Characterization of exoelectrogenic bacteria Enterobacter strains isolated from a microbial fuel cell exposed to copper shock load. *PloS one*, **9(11):** e113379 (2014).
23. Slomczynski, D., Nakas, J., Tanenbaum, S., Production and Characterization of Laccase from Botrytis cinerea 61-34. *Applied and Environmental Microbiology*, **61(3):** 907-912 (1995).
24. Rosado, T., Bernardo, P., Koci, K., Coelho, A. V., Robalo, M. P., Martins, L. O., Methyl syringate: an efficient phenolic mediator for bacterial and fungal laccases. *Bioresource technology*, **124:** 371-378 (2012).
25. Xu, F., Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases. *Journal of Biological Chemistry*, **272(2):** 924-928 (1997).
26. Liers, C., Ullrich, R., Pecyna, M., Schlosser, D., Hofrichter, M., Production, purification and partial enzymatic and molecular characterization of a laccase from the wood-rotting ascomycete Xylaria polymorpha. *Enzyme and Microbial Technology*, **41(6-7):** 785-793 (2007).
27. Li, A., Zhu, Y., Xu, L., Zhu, W., Tian, X., Comparative study on the determination of assay for laccase of Trametes sp. *African Journal of Biochemistry Research*, **2(8):** 181-183 (2008).
28. Robles, A., Lucas, R., de Cienfuegos, G. A., Gálvez, A., Phenol-oxidase (laccase) activity in strains of the hyphomycete Chalara paradoxa isolated from olive mill wastewater disposal ponds. *Enzyme and Microbial Technology*, **26(7):** 484-490 (2000).