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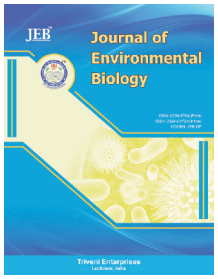

A novel laccase producing *Brevundimonas* sp. MVSP from paper and pulp industry waste water

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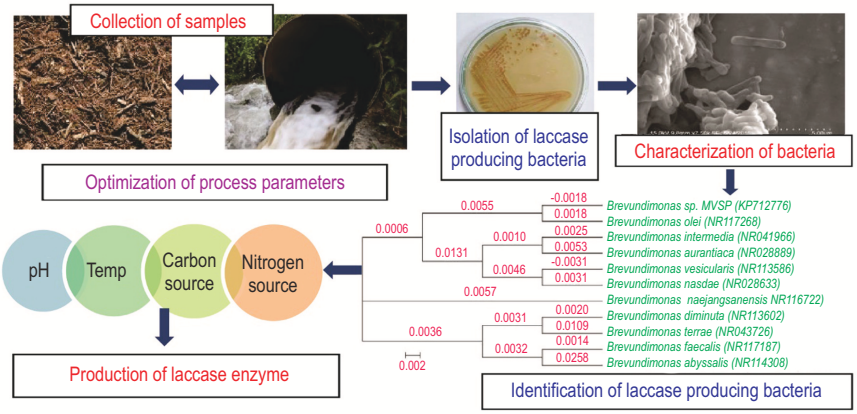
Abstract

Aim : The present study deals with the isolation, screening and characterization of laccase producing bacterial strain from paper and pulp industry waste water. A novel laccase producing bacterial strain *Brevundimonas* sp. MVSP was identified and the optimum conditions required for maximal laccase enzyme activity was determined.

Methodology : Serial dilution method was used for screening laccase producing strain. Out of 58 isolated microorganisms, seven positive strains were screened. Among the seven isolates, "MVSP" showed highest enzyme activity towards laccase production. The isolated microorganism was characterized by biochemical tests and molecular analysis. The 16S rRNA sequence data were analysed and the results were compared with Genbank database.

Results : Various operating parameters such as incubation time, pH, temperature, carbon and nitrogen sources were optimized for intensification of laccase activity. On the basis of laccase activity, *Brevundimonas* genus was found to have higher laccase activity among the other isolates, and was determined using phylogenetic analysis. The sequence received the Gen Bank accession number KP712776. The crude enzyme isolated from *Brevundimonas* sp. MVSP strain showed the maximum laccase activity of 5.24 U ml^{-1} at the optimum conditions.

Interpretation : This is the first report which claims that *Brevundimonas* sp. MVSP produced laccase enzyme using $CuSO_4$ and guaiacol as an inducer. The novel *Brevundimonas* sp. MVSP strain had achieved maximum laccase activity of 5.24 U ml^{-1} at optimized pH (6.5) and temperature (35°C) in 96 hr. This potent organism could be a suitable candidate for the large-scale production of laccase and it may be used to treat various industrial effluents from textile, paper and pulp industry etc.



Introduction

Laccase production has been a significant attention in scientific community since it has a wide range of applications in many industries including chemical, food, paper and pulp, biofuel production, pharmaceutical and textile industries etc. (Plácido and Capareda, 2015). Laccase (benzenediol : oxygen oxidoreductase) is an excellent multi-copper containing polyphenol oxidase which acts on various substrates from one laccase to another. Laccase is widely distributed in living organisms including bacteria, insects, higher plants and fungi (Alexandre and Zhulin, 2000). Several reports are available for fungal laccase such as *Ascomycetes*, *Basidiomycetes* and *Deuteromycetes* (Brijwani *et al*., 2010). The production of laccase was also found in some prokaryotic species such as *Azospirillum lipoferum* (Givaudan *et al*., 1993), *Bacillus sphaericus* (Claus and Filip 1997), *Escherichia coli* (Uthandi *et al*., 2012), *Marinomonas mediterranea* (Solano *et al*., 1997), *Pseudomonas putidia* (Sharma *et al*., 2007) *Pseudomonas syringae* (Mellano and Cooksey, 1988) and *Streptomyces griseus* (Endo and Ueda, 2003), respectively. Both the bacterial and fungal laccases are similar in structures but they found to differ in their amino acid sequences. Though laccase is found in various sources, fungal laccase is widely isolated and used in many industrial applications (Baldrian, 2006). Most of the fungal and plant laccase are extracellular in nature, whereas bacterial laccase is intracellular (Diamantidis *et al*., 2000). The property of laccase from bacteria and fungi varies, based on their metabolic pathway. For instance, the stability of fungal laccase is low compared to bacterial laccase (Baldrian, 2006). It is interesting to note that the bacterial laccase is highly active and more stable at wider pH, temperature levels and exhibits high salt tolerance (Sharma *et al*., 2007). The major functions of bacterial laccase such as morphogenesis, pigment biosynthesis and copper homeostasis are well established (Strong and Claus, 2011). Compared to fungal laccase, bacterial laccase has many significant advantages. In general, the production of bacterial strain can be achieved at low-cost media which is more suitable for commercial production of laccase. Bacterial laccase is used as the best biocatalyst in many research purposes due to the broad range of substrate utilization, grows at neutral pH and catalysed by various biochemical reactions (Lončar *et al*., 2014). The commercial production of bacterial laccase has not been established but few reports have been published on conventional and statistical methods on laccase production (Singh *et al*., 2009). Therefore, an attempt was made to investigate the production of laccase from a bacterial strain. In this study, the novel laccase producing bacterial strain *Brevundimonas* sp. MVSP was isolated, screened, characterized and various process parameters were optimized to intensify the production of laccase for scale up industrial process.

Materials and Methods

The wastewater samples were collected from pulp and paper industry located in the vicinity of Karur, Tamil Nadu, India. Soil samples were collected from a depth of 10-15 cm below the earth's surface, near the mountaineous area of Namakkal, Tamil Nadu, India. The collected samples were stored in an air-tight polyethylene bag and maintained at 4ºC until further use.

Isolation and screening of laccase-producing bacteria : For isolation and screening samples, 5 g of soil sample, 5 g of the degraded region of tree barks and 5 ml of pulp and paper industry wastewater were collected and mixed. The mixture was taken in an 250 ml Erlenmeyer flask which was suspended in 99 ml of sterile saline solution (0.9% w/v NaCl) (Bains *et al*., 2003). The flasks were then incubated at 37°C for 180 min in an orbital shaker maintained at 120 rpm. Then one ml of the sample was serially diluted and dilutions were spread on M162 minimal medium (Degryse *et al*., 1978) with an addition of 0.1% Guaiacol and 0.1 mM $CuSO_4$, which was used as an inducer and incubated at 32°C for three days. The isolated bacterial colonies were selected and grown on nutrient agar medium containing 0.1% Guaiacol and 0.1 mM $CuSO_4$ incubated for 48 hrs at 32°C. Isolated colonies were streaked several times until the isolates were found to be pure colonies. The positive laccase producing colonies was confirmed by the visual appearance of reddish-brown colour formation (Kuddus *et al*., 2013). The morphological, physiological and biochemical characteristics of laccase producing strains were examined. Isolated bacterial cultures were maintained on nutrient agar and stored at 4°C for further studies.

Biochemical and morphological characterization of isolates: The isolated strains were observed morphologically using high-resolution Scanning Electron Microscope (SEM) (JEOL 100 Vegas 3 TESCAN SEM). Biochemical tests were performed using Hi-Assorted Biochemical test Kit, HI-MEDIA. The genomic DNA of the isolated culture was extracted following the method described by Cheng and Jiang, (2006). The 16S rRNA gene was amplified using the 16S universal primers *i.e*., forward primer 5'AGAGTTTGATCCTGGCTCAG3' and reverse primer 5'ACGGCTACCTTGTTACGAC3' (Weisburg *et al*., 1991). Using polymerase chain reaction (PCR), the amplification was performed by initial denaturation at 94°C for 2 min, followed by 40 cycles at 94°C for 45 sec, then at 55°C for 30 sec, and 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were subjected to cycle sequencing using Big Dye Terminator V.1.1. (Applied Biosystems, Foster City, California) in an ABI PRISM Genetic Analyzer 3730 (Applied Biosystems). Extended products were precipitated and purified by ethanol. Sequences were evaluated with Seq scape analysis software V2.5. The 16S rRNA sequence results were analysed with BLAST program (National Centre for Biotechnology (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and it was used for sequence similarity with the standard program module as default. The 16S rRNA sequence was deposited in GenBank database and accession number was retrieved.

Sequence analysis : The 16S rRNA sequence was compared with other neighbour joining sequence and the multiple sequence alignment was performed by clustal W program and a

phylogenetic tree were constructed using the software package MEGA (Molecular Evolutionary Genetics Analysis) version analysis tool V 5.1 1 as described by Tamura *et al.* (2011).

Laccase activity assay and protein concentration : The selected positive bacterial strains were subjected to centrifugation (10,000×g) for 20 min at 4°C and the supernatant was used for enzyme assay. The presence of laccase activity in the supernatant was determined spectrophotometrically at 530 nm using 10 mM guaiacol as a substrate in a reaction mixture containing 100 mM acetate buffer (pH 5) (ε465=48,000 M^{-1} cm^{-1}). The change in the absorbance due to the oxidation of guaiacol was monitored at 37°C for 10 min of incubation. The enzyme activity was measured on Elico double beam SL 210 UV-Visible spectrophotometer (Elico Ltd, India). Laccase activity was expressed as International Units (IU), where one IU is defined as the amount of laccase required to oxidize one µmol of substrate per min. The protein concentration of laccase was determined using UV-Visible spectrophotometer at 280 nm by Lowry's method using Bovine serum albumin as standard (Lowry *et al*., 1951).

Effect of incubation time : The effect of incubation time on laccase activity was determined by recording the absorbance of enzyme catalysed reaction using copper sulphate and guaiacol as an inducer in the nutrient medium. Inoculation of one ml of 24 hrs old culture of isolated bacterial strain was kept at 35°C at 120 rpm for different incubation time ranging from 0-120 hrs. The samples were withdrawn at regular time intervals (0, 24, 48, 72, 96 and 120 hrs) and the growth was measured by taking optical density at 600 nm. The crude culture was centrifuged at 10,000 rpm for 20 min at 4°C.The presence of laccase activity in the supernatant was measured at 530 nm. The time at which the laccase showed maximum activity was noted as optimum incubation time of enzyme.

Effect of pH : The influence of pH on crude laccase activity was determined at optimum incubation time using guaiacol as a substrate in all activity assays. The reaction was subjected to different pH ranging from (5-7.5) with a pH interval of 0.5. The reaction mixture was centrifuged at 10,000 rpm for 20 min at 4°C, and the supernatant was used to determine the laccase activity and the pH at which the maximum laccase activity was taken as optimum pH.The enzyme activity was measured at 530 nm.

Effect of temperature : The influence of temperature on crude laccase activity was determined at optimized incubation time and pH, using guaiacol as a substrate. The reaction was subjected to different temperatures ranging from 20-45°C at an interval of 5°C, respectively. The temperature, at which the crude enzyme showed maximum activity, was recorded as the optimum temperature of the enzyme. The reaction mixture was centrifuged at 10,000 rpm for 20 min at 4°C and the fresh supernatant used to determine the laccase activity. The enzyme activity was measured at 530 nm.

Effect of carbon sources : To analyse the effect of seven different carbon sources such as fructose, glucose, maltose, mannitol, starch and sucrose was amended at the concentration of 0.5% in the culture medium. A 100 ml of nutrient medium was incorporated individually with 0.5% of the above-mentioned carbon sources and it was prepared with optimized pH in 250 ml Erlenmeyer flasks. The flasks were sterilized, cooled and inoculated 24 hrs old *Brevundimonas* sp. MVSP at 35°C for an optimized time interval at 120 rpm. The culture medium from six different carbon sources were centrifuged at 10,000 rpm for 20 min at 4°C, and the supernatant was used to measure the enzyme activity at 530 nm.

Effect of nitrogen sources : The culture medium was amended with the concentration 0.5% of different nitrogen sources such as peptone, yeast extract, tryptone, ammonium sulphate, sodium citrate and calcium nitrate were taken separately in a 250 ml Erlenmeyer flask. It was then sterilized, cooled and inoculated with one ml of 24 hrs old *Brevundimonas* sp. MVSP culture and was incubated at 35°C at 120 rpm. The culture medium was centrifuged at 10,000 rpm for 20 min at 4°C, and the supernatant from six different nitrogen sources were used to measure the enzyme activity at 530 nm.

Results and Discussion

The preliminary screening of soil samples, tree barks and pulp and paper industry wastewater for bacterial strains showed the presence of fifty eight different bacterial strains. Out of fifty eight isolated microorganisms, seven positive strains were

Table 1 : Morphological and biochemical characteristics of *Brevundimonas* sp. MVSP

Characteristics	*Brevundimonas* sp. MVSP
Colony colour	Brownish
Cell morphology	Slender
Gram's staining	-
Spore staining	-
Motility	Motile
Indole	+
Methyl-Red	-
Voges-Proskauer	-
Urease	-
H_2S production	+
Catalase test	-
Oxidase test	+
Nitrate reduction test	-
Dextrose	-
Fructose	-
Galactose	-
Lactose	-
Mannitol	-
Sucrose	+

The sign (-) indicates negative results and (+) indicates positive results

screened on the nutrient media containing 0.1% guaiacol and 0.1 mM $CuSO_4$, a noble substrate, for the detection of laccase secretion. Among the seven isolates, it was observed that one strain which was visually identified as reddish-brown colour colonies (Fig. 1 A) named as *Brevundimonas* sp. MVSP, showed higher laccase activity. It was evident from the literature that most of the bacterial (Mongkolthanaruk *et al*., 2012) and fungal laccase strains (Prathibha *et al*., 2015) showed reddish brown formation, which denotes the laccase positive strains. This strain was supposed to be highly potent among the seven isolates, and hence *Brevundimonas* sp. MVSP was used for further studies. The morphological characteristics of isolated strain were further subjected under scanning electron microscope (Fig. 1 B). The biochemical and physiological characteristics were identified using Hi Assorted™ Biochemical test kit, which was compared with the Bergey's Manual of Systematic Bacteriology. The morphological, biochemical and physiological characteristics of strain, MVSP was similar to the genus *Brevundimonas,* and

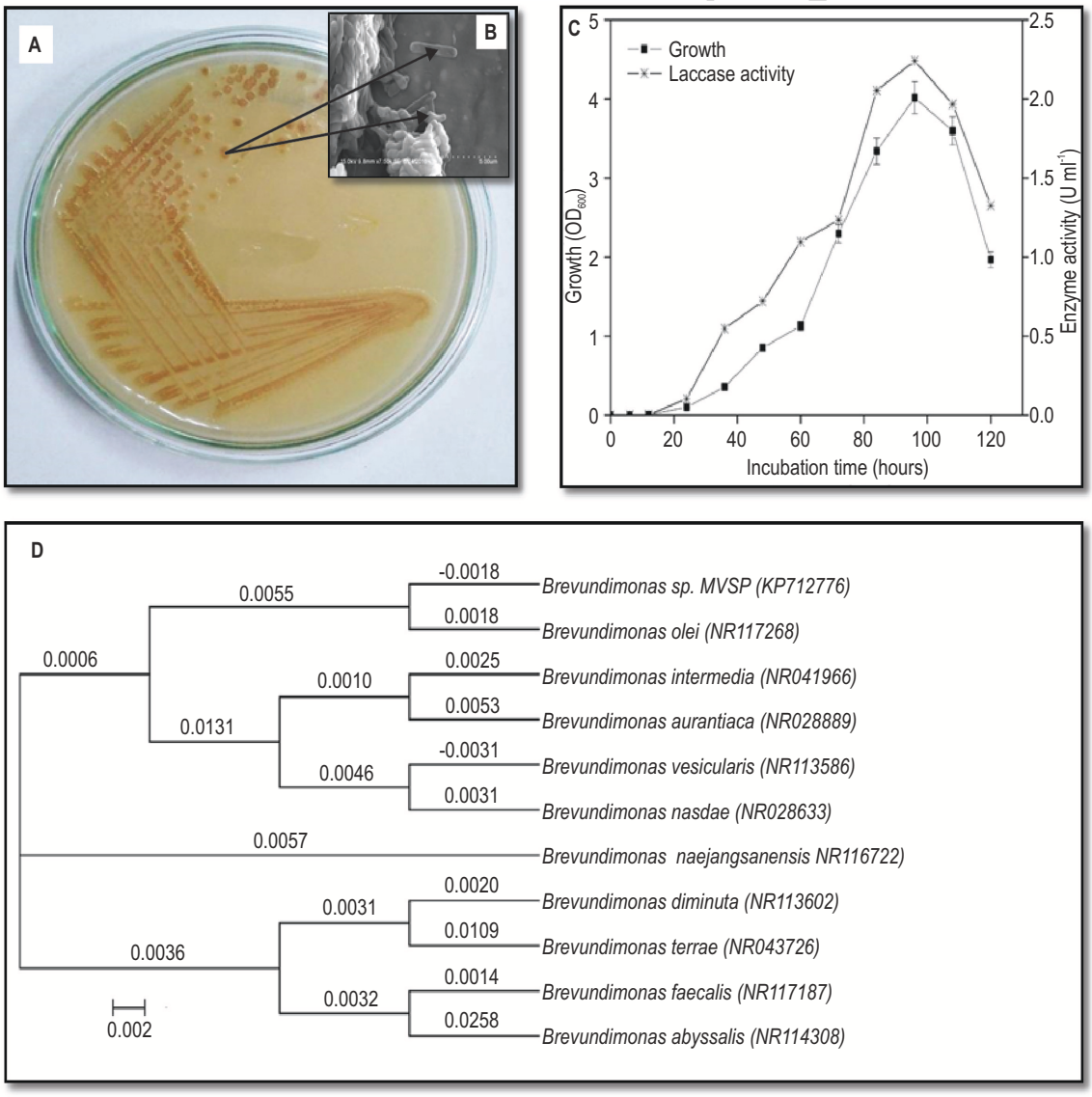


Fig. 1 (A) : Screening of novel laccase producing *Brevundimonas* sp. MVSP strains in nutrient agar medium, the inset (B) shows the scanning electron microscopic view of rod-shaped isolated MVSP strain, (C) Growth curve and laccase enzyme activity of *Brevundimonas* sp. MVSP (OD at 600 nm) (D) Phylogenetic analysis of novel *Brevundimonas* sp. MVSP and other related *Brevundimonas* sp. based on 16S rRNA gene sequence. Bootstrap consensus tree was drawn by multiple sequence alignment with the neighbour-joining method using software MEGA5

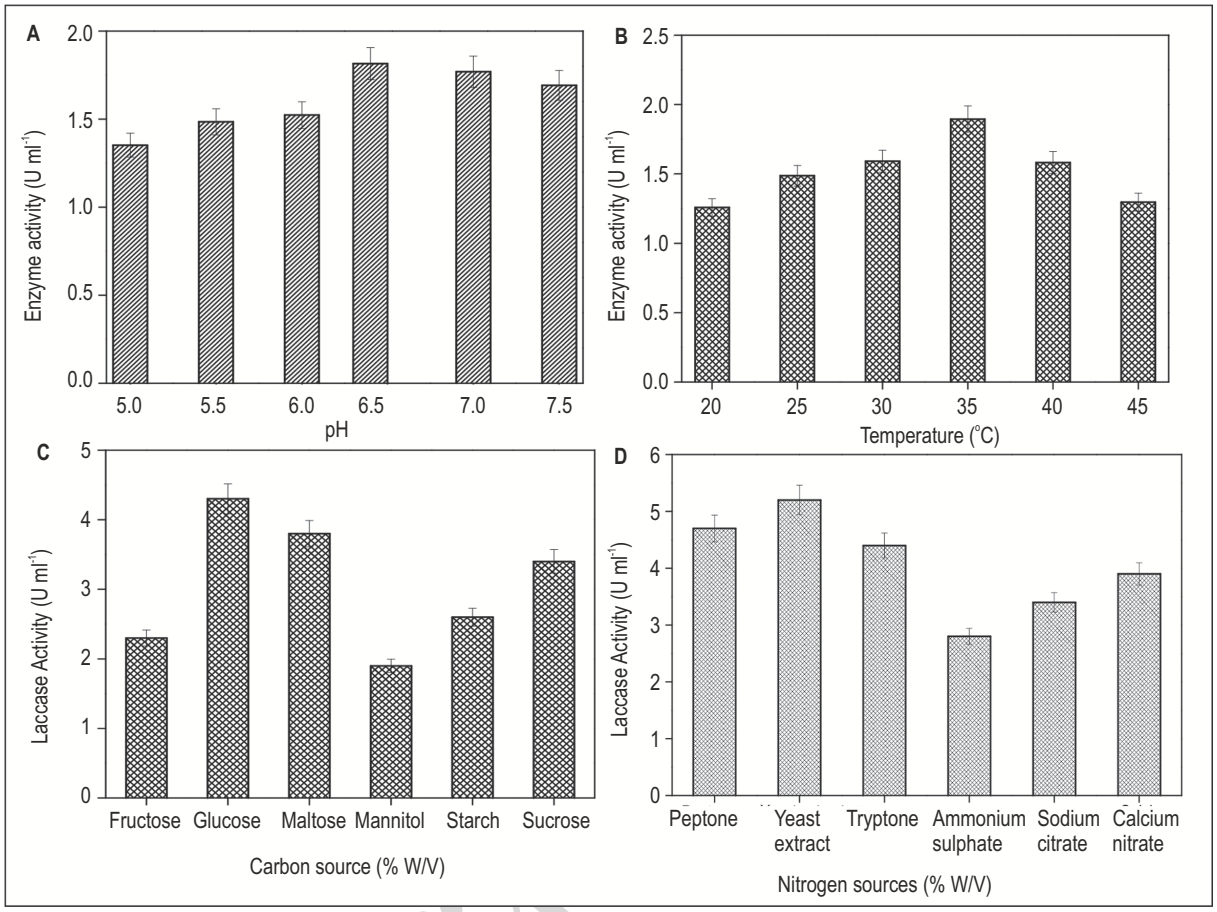


Fig. 2 (A) : Effect of pH [Conditions : Temperature: 35°C and Time : 120 hrs at 120 rpm], (B) Temperature [Conditions: pH: 6.5,Time: 120 hrs at 120 rpm], (C) Carbon sources [Conditions: pH: 6.5, Temperature: 35°C, Time: 120 hrs at 120 rpm] (D) Nitrogen source on laccase enzyme activity [Conditions: pH: 6.5, Temperature : 35°C, Carbon source : glucose and Time : 120 hrs at 120 rpm]

hence the isolate was confirmed by performing 16S rRNA sequence analysis. The biochemical and morphological test results is summarized in the Table 1.

The PCR amplification and the 16s rRNA sequencing were performed. The retrieved 16S rRNA gene sequencing was analysed using BLAST from National Centre for Biotechnology (http://blast.ncbi.nlm.nih.gov/Blast.cgi), which showed similarity to *Brevundimonas* sp. The phylogenetic tree analysis was closely related to *Brevundimonas* sp and the sequence was assigned the Gen Bank accession number KP712776. The phylogenetic tree of *Brevundimonas* sp. MVSP (Fig.1D) and 16S rRNA gene sequences of the strain was analysed using MEGA 5.1 software. The neighbour-joining phylogenetic tree was constructed using Kimura 2-parameter model.

Seven isolated strains were selected and screened for laccase enzyme production. Among the seven isolates, *Brevundimonas* sp. MVSP was found to be a potent strain based on higher laccase activity which produced 2.24 U ml^{-1} at 96th hrs using guaiacol, which acts as the best carbon source and inducer. The strain showed a progressive increase in enzyme activity from 24 to 96 hrs and further increase in incubation time up to 120 hrs, the laccase activity decreased due to the decreasing phase of the bacterial strain (Fig.1C).

The newly isolated strain and its corresponding laccase activity were observed at different pH. The maximum laccase activity of 1.815 U ml^{-1} was achieved from *Brevundimonas* sp. MVSP at pH 6.5 (Fig. 2A). Similar results were observed using various substrate such as Syringaldazine (SGZ) and 2,6-dimethoxyphenol (2,6-DMP) for laccases production at pH 6.5 (Lončar *et al*., 2014). Lu *et al*. (2013a) observed that *B. licheniformis* showed the optimum pH of 6.2 and 6.6 for higher laccase production, when oxidized with different substrates like SGZ and 2,6-DMP respectively, and it showed similar activity as

compared to *Brevundimonas* sp. MVSP. *Bacillus tequilensis* SN4 also showed the optimum pH as 8.0, 5.5, 6.5 and 8.0 for activity with distinct substrates such as 2,6-DMP, 2,2'-azinobis [3-ethylbenzthiazoline-6-sulfonate] (ABTS), SGZ and guaiacol, respectively. This study revealed that most of the bacterial laccase was found to be highly stable at acidic to alkaline pH. It was observed from the study that the laccase enzyme was highly stable at the pH range from 6.5 to 7. The optimum pH for increased activity will differ according to the substrate because every substrate causes different reactions for laccase (Shraddha *et al*., 2011).

The influence of temperature plays a significant role in the stability of enzyme activity. The laccase activity of isolated bacterial strain was measured at different temperatures (25, 30, 35, 40, 45 and 50°C) and it can be seen from Fig. 2B that an optimum laccase activity of 1.895 U ml^{-1} was achieved at 35°C. The temperature is an important parameter which highly favours the rate of biochemical reactions, either by inducing or repressing enzyme yield (Strnadova *et al*., 1991). In the present study, when the experimental temperatures varied from 25-35°C, the laccase activity increased but on further increase in temperature, the laccase activity declined due to the repression of the enzyme producing gene sequences. Similar to the study, Wang *et al*. (2010) also have reported that the maximum laccase activity using *Bacillus subtilis* WD23 was found to be at 25°C. In another study where *Streptomyces* sp. C1 was used, the maximum laccase activity was achieved at 40°C, and it was found that the enzyme was highly resistant towards high temperature (Lu *et al*., 2013b).

Different carbon source such as fructose, glucose, maltose, mannitol, starch and sucrose whose dosage was at a fixed concentration of 0.5% in growth medium was added to determine the intensification of laccase activity. The results showed that the isolated *Brevundimonas* sp. MVSP produced higher enzyme activity using glucose (4.32 U ml^{-1}) in the culture medium compared to other carbon sources as shown in the (Fig. 2C). Jang *et al*. (2002) and Ding *et al*. (2012) reported that glucose is an excellent carbon source for laccase production which supports the current study. In the present study, it was observed that the complete utilization of glucose was achieved at a minimum incubation time by the isolated strain compared to other carbon sources.

The nitrogen sources are essential for amino acid synthesis which makes up the proteins and other value added substances in microorganisms. In the study, six different nitrogen sources (peptone, yeast extract, tryptone, ammonium sulphate, sodium citrate and calcium nitrate) were used at fixed concentration of 0.5% to determine the effect of nitrogen sources on laccase production. The reaction was carried out at optimized incubation time (96 hrs), pH (6.5), temperature (35°C) and carbon source (glucose). Among the six various nitrogen sources, Yeast extract added to the confined medium showed higher laccase enzyme production as seen in Fig. 2D. It was evident that the organic nitrogen sources are highly useful to enhance the laccase activity. Niladevi *et al*. (2007) also stated that the organic nitrogen sources showed higher laccase production than inorganic nitrogen sources using *Streptomyces psammoticus*. Apart from yeast extract, L-asparagine also produced increased laccase activity in *Botryosphaeria rhodina* (Dekker *et al*., 2007). Earlier reports have mentioned that the use of nitrogen sources on laccase production was doubtful (Dobson, 1997), however the present study suggest that use of yeast extract (nitrogen source) is a vital nutrient to enhance the laccase activity.

The results of this study suggests that *Brevundimonas* sp. MVSP strain can be further used for scale up industrial applications.

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