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Research Article

OPTIMIZATION OF LACCASE PRODUCTION BY A NEWLY ISOLATED BACTERIUM *PSEUDOMONAS* SP. DW-1A USING 16S rDNA SEQUENCING

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Abstract

Background: *Laccase belongs to the group of enzyme oxidoredutasethatknown to be a potential candidate in different industrial and biotechnological applications throughout the world.* ***Objective:****To isolate and identify a new source of bacteriallaccase and enhanced its production yield using additional parameters like carbon and nitrogen sources, pH, temperature and incubation time.****Methods:*** *The strain was isolated from the decayed bamboo sample using enrichment culture and identified with 16S rDNA sequencing. Further, the strain was screened for laccase activity and*

optimized the laccase production using different process parameters in shake flask condition. ***Results:*** *The bacterial strain was Gram-negative and rod-shaped, identified as Pseudomonas sp. DW-1A (accession number: MH819294). The strain waslaccase positive that confirmed by the formation*

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*of a reddish brown colour zone in a guaiacol plate assay.Xylanandyeast extract was found to be the optimum carbon and nitrogen source for the growth and metabolism of the strain.Pseudomonassp. DW-1 is a pH tolerant strain that can actively produce laccase between pH 4 to pH 10 though highest production (351.45 ± 36.89 U/ml) at pH 10 ($p < 0.001$). The optimum temperature was found to be at 35ºC that exhibited 1318.48 ± 131.74 U/mllaccase productions ($p > 0.05$).The highest laccase production was recorded as 2118.84 ± 90.14 U/ml at 96 h of incubation time ($p < 0.001$).**Conclusion:** Above findings reveal that Pseudomonas sp. DW-1 is an active laccaseproducingbacteriumthat might be suitable for industrial as well as other biological applications.*

Keywords: Bacterial strain; Assam; *Pseudomonas* sp. DW1; Laccase; Process parameters optimization.

Introduction

Laccases (EC 1.10.3.2) are multicopper oxidases that catalyze the oxidation of various aromatic compounds, particularly those with electron-donating groups such as phenols (-OH) and anilines ($-NH_2$) by using molecular oxygen as an electron acceptor (Solomon*et al* 1996).These enzymes are distributed in a wide range of higher plants, insects, and fungi as well as incertain bacteria (Thurston 1994; Dwivedi *et al* 2011). The laccase enzyme was first described from the exudates of the Japanese lacquer tree, *Rhusvernicifera* (Yoshida 1883). Most of these laccases studied so far are of the origin of white-rot fungi, such as *Phlebia radiate*, *Pleurotus ostreatus* and*Trametes versicolor* (Bourbonnais *et al* 1992; Niku-Paavola *et al* 1988;

Palmieri *et al* 2000). However, the laccase enzyme was first produced by the bacterium*Azospirillumlipoferum*(Givaudan*et al* 1993).Later, bacteria from different generic groups such as *Streptomyces lavendulae*, *Bacillus vallismortis*, *Pseudomonas extremorientalis* BU118 and *y-proteobacterium*have been established by many researchers (Gianfreda*et al* 1999; Sharma *et al* 2007; Zhang *et al* 2012; Arunkumar*et al* 2014; Neifer*et al* 2016). Today, bacterial laccases have been reported as a promising candidate in diverse industrial areas like dye decolourization, pulp delignification, waste water treatment, deinking of paper pulp and pulp sewages (Huan *et al* 2011; Godlewska *et al* 2014; Sondhi *et al* 2015; Neifer*et al* 2016; Ozer *et al* 2018). Only a few workers have been focused on the application like pulp bio-bleaching using laccase enzyme from certain bacteria (Arias *et al*2003; Kumar *et al*2005; Virk*et al* 2012; Sondhi*et al*2015; Ozer*et al* 2018).

Owing to its vivid industrial applications, there was keen to develop a new laccase positive bacterial sourcefor their successful utilization.Although bacteria having laccase activity were reported by many types of research from different areas (Kuddus et al 2013; Suljic*et al* 2015; Guo *et al* 2017; Kumar *et al* 2018), this region has not been studied so far. Recently, work on conditions that affect production, statistical optimization of media composition and immobilization of laccase have been carried out by several groups (Niladevi*et al* 2009: Verma *et al* 2016; Kumar *et al* 2018). The ever-increasing demand for thelaccaserequires the production process to be economical and less time consuming (Kumar *et al* 2018). Addition of inexpensive sources like carbon and nitrogen for enhancing the laccase production could be effective in case of making the entire process economically sounds (Sivakumar 2010). Abiotic factors like pH, temperature

and incubation time has been seen to influence laccase production in many literatures (Kudduset *al* 2013; Sondhiet *al* 2015: Kumar *et al* 2018). However, similar work has been reported on the optimization of laccases production in submerged fermentation by a novel source *Streptomyces psammoticus* using the coffee pulp as substrate (Niladevi and Prema 2008). This study was undertaken to study the optimization of laccases production in submerged fermentation by newly identified bacterium using ABTS as substrate. The results have been reported in the present manuscript.

Materials and methods

Chemical and reagent:

Guaiacol and ABTS (2, 2′-Azino-bis, 3-ethylbenzthiazoline-6-sulfonic acid) were purchased from Sigma- Aldrich, USA. Other reagents were procured from HiMedia, Laboratories, Mumbai, India and were of analytical grade.

Collection of sample:

Decayed bamboo samples were collected from the experimental farm of CSIR-NEIST, Jorhat, Assam, India. As soon as collected the samples were transferred to the laboratory and stored at 4˚C in the culture room.

Isolation and screening of laccase producing bacterial strain: Isolation and screening werecarried out by using the method described by Kudduset *al* (2013). For this, 10 g of collected sample was dispensed in 250 ml of Erlenmeyer flask containing 100 ml of nutrient broth supplemented with 0.2 mM CuSO4 and 2 mMguiacoland incubated at shaking condition (150 rpm). Bacterial colonies were isolated by standard serial dilution plate technique using nutrient agar medium containing 0.2mM CuSO4 and incubated at 30˚C for 48 h.

Identification of the bacterial strain:

Morphological observation:

Image of pure colonies of bacteria isolate was taken by scanning electron microscope using Leo 1430 rP operated at 5 kV on gold-coated samples. The image of electron micrograph was used to determine its shape and size. Gram staining was also performed and the result was interpreted according to the colour retained by the cell wall of the bacterium.

For biochemical characterization, the pure strain was inoculated in a carbohydrate test kit (Himedia TM KB009TM Hi Carbo Kit).

Identification using 16S rDNA sequencing:

The pure culture was centrifuged at 6000 rpm for 10 min and the cell pellets were used for DNA extraction using Genei^{TM} Bacterial DNA Isolation kit. The extracted DNA was quantified using Nanodrop 2000c spectrophotometer and the purity was ascertained on the basis of $A_{260/280}$ ratio. PCR (Polymerase chain reaction) was performed using universal 16S rDNA primers 27 F- forward primer (5'-AGAGTTTGATCMTGGCTGAG-3') and 1492- reverse primer (5'-TACGGYTACCTTGTTACGACT-3') as per Frank *et al* (2008). The PCR products were visualized using 1% agarose gel electrophoresis with EtBr (Ethidium Bromide) staining. The amplified product (1500 bp) was gel purified, sequenced and analyzed using standard online tools of NCBI (National Centre for Biotechnology Information) and submitted to Gen Bank.

Laccase assay:

The laccase enzyme activity was determined by oxidation of ABTS using the modified method described by Bourbonnais and Paice 1992. The crude enzyme was collected as supernatant by centrifugation at 10,000 rpm for 10 min at 4ºC. The enzyme reaction was prepared in a citrate-phosphate buffer (50 mM, pH 7) using ABTS (0.3 mM) and 100 µl of crude enzyme. The

laccaseactivity was measured using UV-VIS spectrophotometer (Lambda 35, Perkin Elmer, USA) by monitoring A_{420} (ε_{420} = 3.6 x$10^4 M^{-1}$ cm^{-1}) and the enzyme was expressed in Units/ml (Unit= µmol/min).One unit of laccase activity was defined as the amount oflaccase oxidized 1 µmol of ABTS per min. All assays were performed in triplicate.

Optimization of laccase production by OFAT approach:

The OFAT approach was used for screening of various media components influencing the growth of the bacterial strain and laccaseproduction(Kudduset *al*2013; Peter and Vandana 2013; Sondhi*et al* 2015).Bushnell Hass (BH)broth was preferred for media optimization study as it is free of any carbon sources and containsminor trace elements suitable for OFAT study design.

Effect of carbon and nitrogen source:

Different carbon sources likeglucose**,** lactose, dextrose, maltose, malt extract, starch, molasses, oats, sucrose and xylan along with two agricultural wastes; rice bran and wheat bran were added at 1% (w/v) concentration to flasks containing BH broth in triplicates. Similarly, different nitrogen sources like yeast extract, ammonium chloride, ammonium sulphate, glycine, peptone, tryptone, potassium nitrate, sodium nitrate and urea were added at 0.25% (w/v) concentration. All the flasks were inoculated with 0.5 ml of pure culture and kept at 30 ˚C for 48 h under shaking condition (150 rpm). Simultaneously two flasks were also inoculated devoid of carbon and nitrogen source to act as a control.

Effect of different concentrations of optimum carbon and nitrogen source:

The culture medium containing optimized carbon source at different concentrations 0-5% (w/v) was evaluated for its effect on laccase production.

Similarly, the culture broth containing optimized nitrogen source at different concentrations 0-1% (w/v) was also evaluated for its effect on laccase production.

Effect of pH, temperature and incubation time and agitation:

The effect of temperature on laccaseproduction was determined by incubating the inoculated medium at different temperatures (25-40˚C) with the above-optimizedcondition.Similarly, the influence of pH on laccase production was studied by varying pH range of the culture mediumfrompH(3-12).The laccase production was also studied by growing the culture with optimized parameters by incubating at different time intervals (18-120)h.All the experiments were conducted under shaking condition (150 rpm) and the results are mean of three independent experiments.

Statistical analysis:

All the tests were conducted in triplicate and data are presented as the mean values of triplicate tests. Statistical analysis like ANOVA & Multiple comparisons was performed by Tukey-HSD was performed using IBM SPSS Statistics, Version 20.0. Armonk, NY: IBM Corp USA and GraphPad Prism 7, Graph Pad Software, Inc.

Results

Isolation and screening of laccase producing bacterial strain:

The bacterialstrain was screened and isolated by enrichment culture using nutrient broth supplemented with 0.2 mM $CuSO_4$ and 2 mMguaiacol as an inducer (Fig. 1).A positive result was further confirmed as evident from the formation of green colour in the test tube containing the culture broth and ABTS as a substrate. ABTS is the substrate for laccase enzyme in the absence of hydrogen peroxide, turns into dark green colour in oxidized form

and thus used to detect laccase activity (Gonzalez *et al* 2006; Lonergan *et al* 1997; Dwivedi *et al* 2011).

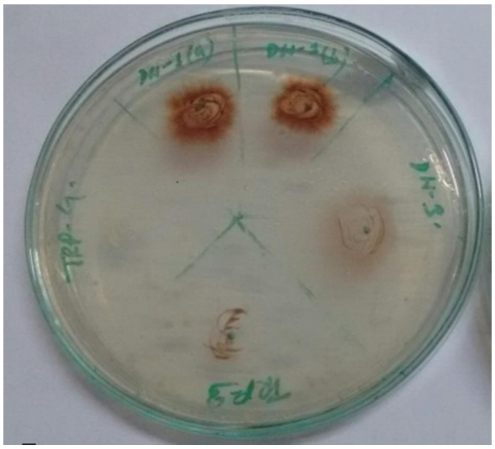

Fig. 1: Isolation and screening of laccase positive of bacterial strain on guaiacol plate assay

Identification of the bacterial strain:

Morphological observation:

The morphological observation indicated it as a non-motile, Gram negative& rod-shaped bacterium as seen under 1000 x magnification and scanning electron micrograph (SEM) as shown in Fig. 2a & 2b.

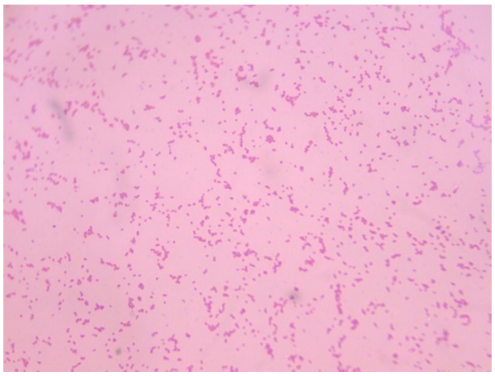

Fig. 2a: Gram staining of the bacterial strain under 1000 x magnification

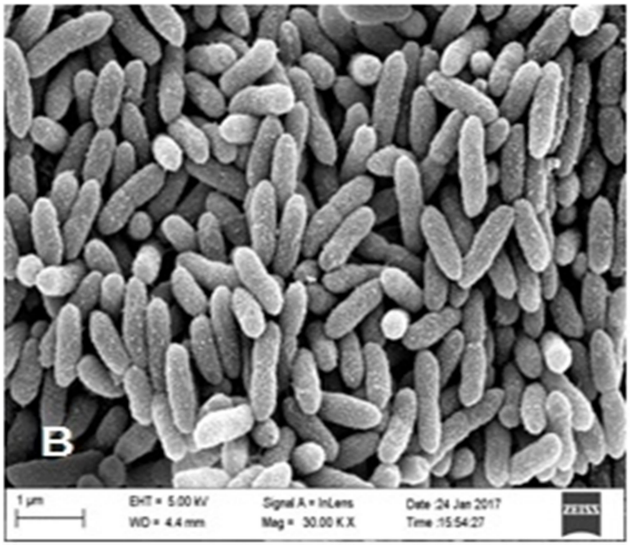

Fig. 2b: SEM image of isolated strain showing morphological characteristics.

For biochemical characterization, the bacterial strain DW1 was grown in the carbohydrate test kit and the results of the biochemical test of the isolated strain were presented in Table 1.

Identification using 16S rDNA sequencing:

The evolutionary history was inferred using the Neighbour-Joining method. The optimal tree with the sum of branch length = 0.22327092 is shown in Fig. 2c. The percentage of replicate trees where the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the base substitutions number per site. The analysis included 21 nucleotide sequences. All the positions with less than 95% site

Table 1: Biochemical test using Himedia TM KB009 TM HiCarbo Kit (KB009A/ KB009B1 /KB009C) Part A, Part B each having 12 carbohydrates utilization tests and Part C containing 11 sugars and 1 control.The temperature of incubation: 35 ± 2˚C. Duration of incubation: 18 - 24 h.

HiCarbo™ Kit- Part A (KB009A)		**Result**
1	Lactose	+
2	Xylose	-
3	Maltose	+
4	Fructose	+
5	Dextrose	+
6	Galactose	-
7	Raffinose	-
8	Trehalose	-
9	Melibiose	-
10	Sucrose	+
11	L-Arabinose	-
12	Mannose	-
HiCarbo™ Kit- Part B (KB009B1)		**Result**
1	Inulin	-
2	Sodium gluconate	-
3	Glycerol	+
4	Salicin	-
5	Dulcitol	-
6	Sorbitol	-
7	Inositol	-
8	Mannitol	-
9	Adonitol	-
10	Arabitol	-
11	Erythritol	+
12	alpha-Methyl-D-glucoside	-
HiCarbo™ Kit- Part C (KB009C)		**Result**
1	Rhamnose	-
2	Cellobiose	-
3	Melezitose	-
4	alpha-Methyl-D-Mannoside	-
5	Xylitol	-
6	ONPG	-
7	Esculin	-
8	D-Arabinose	-
9	Citrate	+
10	Malonate	-
11	Sorbose	-
12	Control	+

coverage was eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. A total number of 1247 positions werein the final dataset. The sequence was submitted to GenBank with accession number MH819294 as a partial 16s rRNA nucleotide sequence. Molecular identification by Sanger's sequencing and nucleotide analysis of 16S-rDNA region engaged the following criteria for identification; (a) when the resultant sequence yielded a similarity score of 99% with a reference sequence belonging to a classified species, the unknown isolate was assigned to this species; (b) when the score was between 95% and 99%, the unknown isolate was assigned to the corresponding genus; and our strain showed 99% with many strains of different species and thus, designated as *Pseudomonas* sp.DW-1.

Optimization of laccase production by OVAT approach:

Carbon and nitrogen sources are the basic requirements for survival and metabolism of bacteria under laboratory conditions along with various environmental factors like temperature, pH and incubation time. The production of laccase by *Pseudomonas* sp. DW-1was studied by OVAT approaches under submerged fermentation adopting the above parameters and results were discussed accordingly.

Effect of carbon and nitrogen source:

In our study, xylan was found to be the best carbon source for the strain DW-1 and exhibiting highest laccase production 157.06±12.73U/ml,which is highly significant ($p< 0.001$). The other carbon sources that contributed to the laccase production are shown in Fig. 3a.Among all the nitrogen sources used, yeast extract was found to be the best nitrogen that influenced thegrowth and enzyme production by *Pseudomonas* sp. DW-1 and recorded as

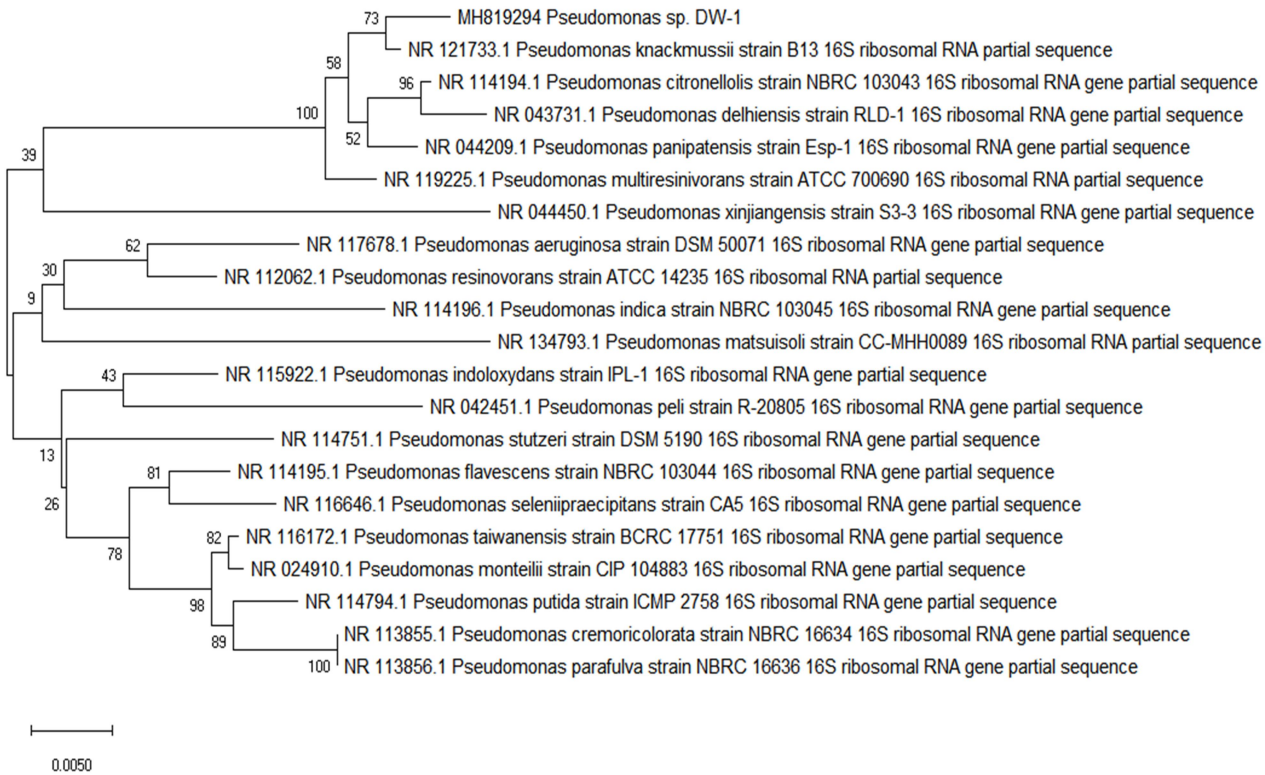

Fig. 2c: Evolutionary relationships of different *Pseudomonas* sp. DW-1A with our strain MH819294

171.55±13.40U/ml (p< 0.001). The other nitrogen sources e.g., peptone, urea, ammonium sulphate, ammonium chloride and glycine have also a significant effect on laccase production as seen in Fig. 3 b.

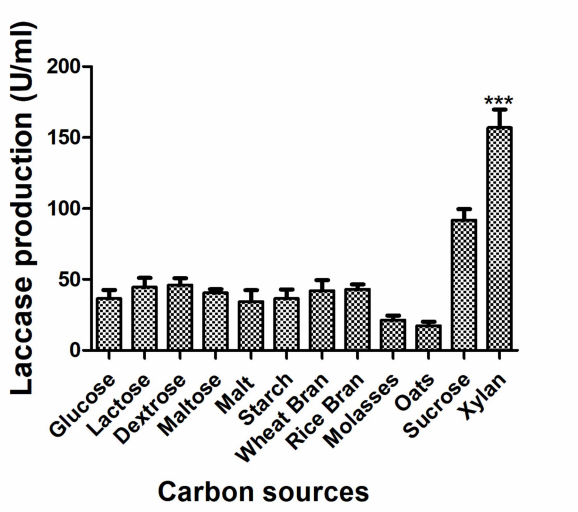

Fig.3a: Effect of different carbon sources on laccase production

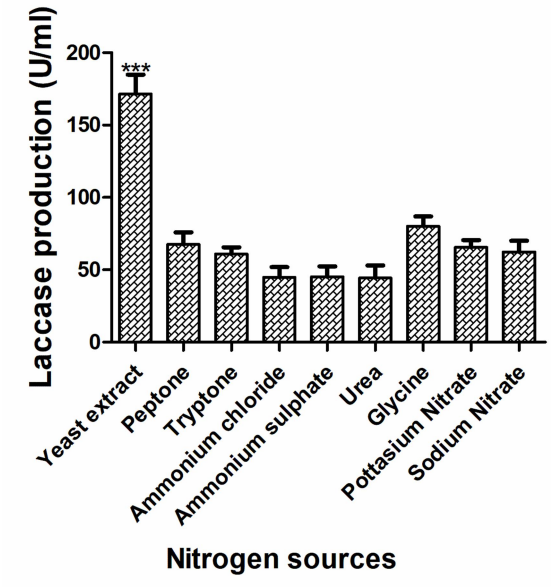

Fig.3b: Effect of different nitrogen sources on laccase production

Effect of different concentration of selected carbon and nitrogen source:

While the concentration of the optimum carbon source was varied, the maximum laccase production was recorded as 215.94±13.76 U/ml at 2% xylan($p< 0.001$) (Fig. 4a). Likewise, the concentration of optimum nitrogen source was varied and the highestlaccaseproduction was quantified as 296.01±17.20 U/ml at 0.5% yeast extract ($p< 0.001$) (Fig. 4b).

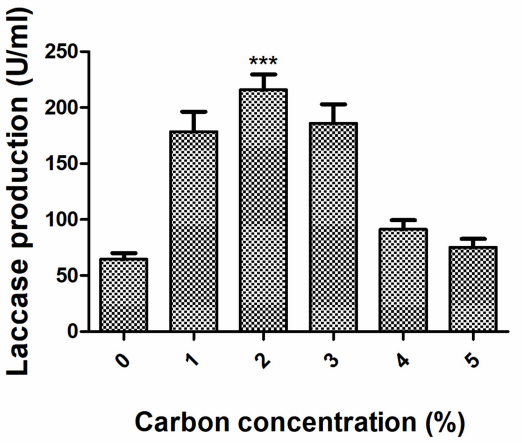

Fig. 4a: Effect of different concentration of selected carbon source (Xylan) on laccase production

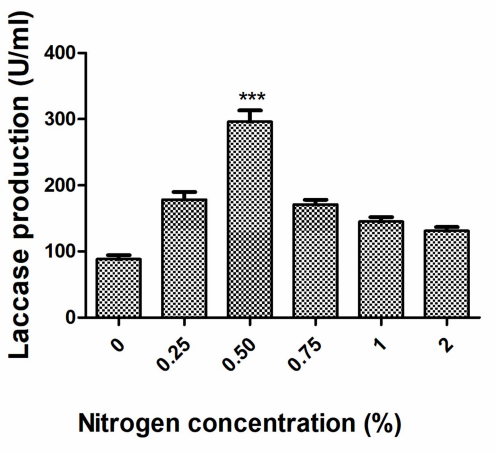

Fig. 4b: Effect of different concentration of selected nitrogen source (Yeast Extract) on laccase production

Effect of pH on laccase production:

Thebacterial strain *Pseudomonas* sp. DW-1 was grown on production medium varying with different pHs range (3-12) to evaluate the influence of pH on laccase production. The strain DW-1 producelaccase within pH (7-12) and maximum yield of enzyme production were observed from pH (8-10) as shown in Fig. 5. The optimum laccase production was seen at pH 10 exhibiting 351.45±36.89 U/ml ($p < 0.001$). However, the yield of laccase production by the strain was found to be less from pH (3-6).

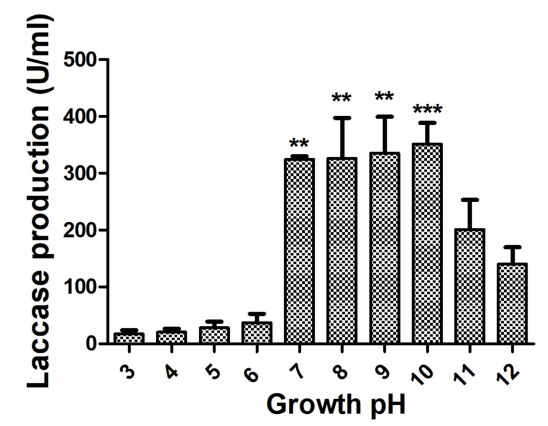

Fig. 5: Influence of pH on laccase production from the bacterial strain

Effect of temperature on laccase production:

In this study,the temperature at 25˚C leads to slightly decreases in the rate of enzyme production as compared to that of other temperatures (30-45˚C). The highest laccase production 1318.48 ±131.74 U/ml was recorded at optimum temperature 35ºC and 1188.95± 99.23 at 40˚C which are highly significant (p <0.001). The maximum amount of laccaseproduction was also obtained by

Pseudomonas sp. DW-1 at 30 and 45˚C as shown in Fig. 6.

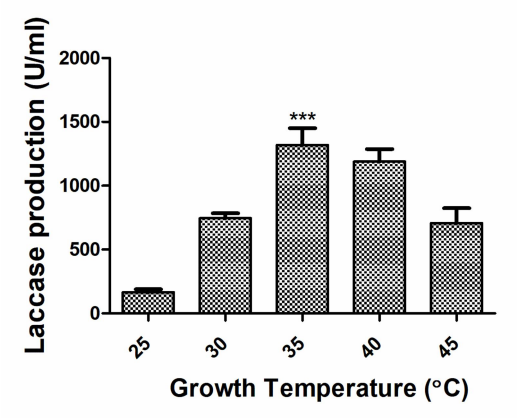

Fig. 6: Effect of temperature on laccaseproduced by *Pseudomonas* sp. DW-1

Incubation time forlaccase production:

The incubation time was studied to optimize the laccase production at optimized parameters like pH and temperature. It plays an important role in the enzyme secretion as evident from the results of our study that showing laccase production by *Pseudomonas* sp. DW-1 enhanced at all the incubation period of time (18-120 h). The maximum laccase production was observed at1109.60 ±53.74 at 72 h and found to be statistically optimum at 96 h of incubationand recorded as 2118.84 ± 90.14 U/ml ($p < 0.001$). A decreasing trend of enzyme production was observed from 96 h to 120 h exhibiting 761.59 ± 27.45 U/ml as shown in Fig. 7. This may be due to the change in the culture conditions such as diminishing of oxygen, nutrients and accumulating of toxic metabolites that inhibit the bacterial growth and metabolism.

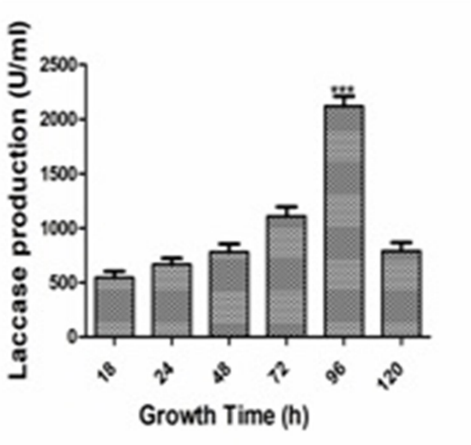

Fig. 7: Different incubation time (h) showing laccase production by the strain

Discussion

The newly identified bacterium*Pseudomonas* sp. DW-1was rod-shaped and Gram-negative in morphology known from the observation under a scanning electron microscope and Gram staining.Other isolates such as *Pseudomonas* sp. K9 has been reported as rod-shaped and Gram-negative like our strain (Huan *et al* 2011). The strain DW-1 showed the ability to produce laccase activity using 2 mM $CuSO_4$as inducer in a guaiacol plate assay. Guaiacol has been preferred for laccase screening in many bacteria like *Bacillus tequlensis*, *Pseudomonas putida*and also including *Pseudomonas extremorientalis* BU118, as it can be rapidly utilized by a wide range of bacterial strains (Kudduset *al*2013; Sondhiet *al*2015; Neifaret *al*2016). The isolated strain was also found to oxidize ABTS in accordance with other studies involving laccase screening by ABTS as preferred substrate (Collet *al* 1993; Kudduset *al* 2013; Sondhiet *al* 2015).The ability of an enzyme to oxidize ABTS is a confirmation of true laccase activity as it is mostly used

and laccase specific substrate (Williamson 1994; Sondhi*et al* 2015). Moreover, SGZ (syringaldazine) has also been reported as a substrate for confirmation of laccase since it is a specific substrate oxidized by laccase (Givaudan *et al* 1993; Wang *et al* 2010).

The nutritional parameters such as carbon and nitrogen sources are the basic requirement for survival and metabolism of bacteria along with various environmental factors like pH, temperature and incubation time under laboratory conditions. The production of laccase by *Pseudomonas* sp. DW-1was studied by OVAT approaches under submerged fermentation using the above parameters and the results were discussed accordingly. In our study, xylan was found to be the best carbon source for the strain DW-1 and exhibiting highest laccase production 157.06 ± 12.73U/ml, which is highly significant ($p< 0.001$). Unlike our result, glucose showed the highest laccase production (1317 U/mg) by *Pseudomonas aeruginosa*SR3 and selected to be the optimum carbon source (Hussein *et al*2017). A possible explanation for this may be due to the gradual depletion of micro and macronutrients in the growth medium utilized by different species (Nyanhongo *et al* 2002).

The nature and concentration of the nitrogen in the culture medium are important for growing and metabolism of the organisms (Kudduset *al*2013). However, yeast extract was observed to be the best nitrogen source that influenced the rate of laccase production by *Pseudomonas* sp. DW-1 (171.55± 13.40U/ml) ($p < 0.001$). In another study, peptone was the optimum source of nitrogen that supported the enzyme production (1525 U/mg) by *Pseudomonas aeruginosa*SR3 as compared with other nitrogen sources (Hussein *et al*2017). In *Pseudomonas aeruginosa* of the same genus, peptone was found as best nitrogen source with very less laccaseproduction as 0.0382 U/ml (Peter and Vandana 2013).

Pseudomonas sp. DW-1 was grown on production medium with different pH range (3-12) to evaluate the effect of pH on laccase production. The strain showed a significant effectof pH on laccase productionfrom pH range (7-12) except for pH (3-6) and found to be optimum at pH 10 exhibiting 351.45 ± 36.89 U/ml ($p< 0.001$). Thus, *Pseudomonas* sp. DW-1 is a pH tolerant bacterial strain that can grow and metabolize at high pH. In a similar study, laccase production by *Pseudomonas putida* was found to produce 23.14 U/ml and 34.45 U/ml at pH 8 and 9 respectively which was lower in comparison to our strain (Kuddu s*et al*2013). However, Peter and co-worker reported very low enzyme productionby another bacterial strain *Pseudomonas aeruginosa* (0.03 & 0.04 U/ml) at pH 7 and pH 12 (Peter and Vandana 2013). The optimum pH for laccase production by *Pseudomonas putida*LUA15.1 was also found to be pH 8 and exhibited maximum extracellular laccase of 34.30 U/ml (Muthukumarasamy *et al* 2015). Another strain, *Pseudomonas aeruginosa* SR3 was studied with different pH values (5-10) and highest laccase specific activity (1780) U/mg was obtained at pH 7.5 (Hussein *et al* 2017). All these findings demonstrate wide pH variation exists among different species belonging to the same genus. Other bacterial strains such as *Aquisali bacillus elongates* and *Streptomyces bikiniensis*CSC12 produce optimum extracellular laccase 4.4 U/ml and 34.30 U/ml at pH 8 (Rezaei *et al*2017; Devi et al. 2016). Another laccase producing bacterium *γ-proteobacterium* JB has also been reported to exhibit laccase at optimum pH 7 unlike our study (Singh *et al* 2008). Thus, there exist wide pH ranges for different bacteria at which they can produce maximum laccases at definite pH and said to be optimum. Generally, the influence of pH on laccase production is accredited to its role in the

solubility of the nutritional medium, its effect on the substrate ionization as well as its availability for the microbial strains (Bull *et al* 1976).

It is known that temperature also affects enzyme production in almost all bacterial species with the exception of a few thermophilic ones found rarely in normal habitats. In this study, the highest laccase production (1318.48 ± 131.74 U/ml) was recorded at 35ºC and 1188.95± 99.23 at 40˚C which are highly significant ($p < 0.001$). In consonance with our results, *Bacillus* sp. WT can also grow at a temperature range from 20˚C to 45˚C, with optimum growth and laccase production at 35˚C (Siroosi*et al*2016). The optimum temperature for laccase production by *Pseudomonas aeruginosa* SR3 was 30˚C with the specific enzyme activity of 1684 U/mg (Hussein *et al*2017). However, another strain *Pseudomonas*putida LAC-5 produced optimum laccase as 8.845 U/ml at 40˚C (Kuddus *et al*2013). Bacteria like *Aquisali bacillus elongate* and *Stenotrophomonasmaltophilia* also produce high extracellular laccase at 40ºC that is quite near to our findings (Galai *et al* 2009; Rezaei*et al* 2017).

The incubation time was studied to optimize the laccase production since it had an adverse effect on enzyme secretion. Laccase production by *Pseudomonas* sp. DW-1 was enhanced at all the incubation period of time (18-120) h. The maximum laccase production was observed to be (1109.60 ± 53.74) at 72 h and found to be statistically optimum (2118.84 ± 90.14 U/ml) at 96 h of incubation ($p < 0.001$). A decreasing trend of enzyme production was observed from 96 h to 120 h exhibiting 761.59 ± 27.45 U/ml as shown in Fig. 7. This may be due to the change in the culture conditions such as diminishing of oxygen, nutrients and accumulating of toxic metabolites that inhibit the bacterial growth and its metabolism. Researchers also reported

that laccase produced by *Pseudomonas fluorescence* increased with time till 72 h and then decreased due to depletion of micro and macronutrients in the growth medium (Kiran and Vandana 2014). In relevant to our findings, laccase production by *Pseudomonas aeruginosa* SR3 commenced during the first 18 h where the specific activity was enhanced after 24 h, but reach to optimum at 27 h of incubation and recorded as 1785 U/mg (Hussein *et al* 2017). Another bacterium *Pseudomonas*putida LAC-5 produces an enzyme (4.25 U/ml) at optimum time 108 h which is quite near to 96 h resulted in our study (Kuddus *et al*2013). Some other bacteria like *Aquisali bacillus elongates* shows optimum laccase within 24-72 h and *Stenotrophomonasmaltophilia* reaches the highest peak after 48 h incubation, dissimilar to our study (Galai*et al*2009; Rezaei *et al* 2017). However, a strain like *Streptomyces bikiniensis*CSC12 takes much longer time and produces laccase maximally on the 14th day of incubation (Devi *et al*2016).

Conclusion

The genus *Pseudomonas* is one of the best-studied bacterial groups and includes numerous studies of environmental interest including pulp and paper manufacturing and other biological applications. This is the report of a newly isolated, Gram-negative bacterium of genus *Pseudomonas*, as a potent laccase produced. The strain was identified as *Pseudomonas* sp. DW-1 (accession number: MH819294) and it was a highly alkaline strain that could grow and produce laccase at pH 10, primarily utilized xylan and yeast extract as optimum carbon and nitrogen source. Moreover, the strain was found to be an active laccase producer that exhibited a maximum yield of laccase as 2118.84 ± 90.14 U/ml. The results have been found to be significant and

encouraging so that the laccase may be further explored for applying in different industrial and biological purposes.

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References

Arias ME, Arenas M, Rodríguez J, Soliveri J, Ball AS and Hernández M (2003). Kraft pulp biobleaching and mediated oxidation of a nonphenolicsubstrate by laccase from *Streptomyces cyaneus* CECT 3335. *Appl Environ Microbiol*, 69:1953-58.

Arunkumar T, Anand DA, Narendrakumar G, (2014). Production and partial purification of laccase from *Pseudomonas aeruginosa* ADN04. *J Pure ApplMicrobiol***,** 8:727-31.

Bull AT and Bushnell ME (1976). Environmental control of fungal growth. In: The filamentous fungi (ed.Smith JE and Berry DR) 2:1-29 Edward Arnold London.

Bourbonnais R and Paice MG (1992).Demethylation and delignification of kraft pulp by *Trametesversicolor*laccase in the presence of 2, 2–azinobis (3-ethylbenzthiazoline-6-sulfonate). *Appl Microbiol Biotechnol*, 36: 823–827.

Coll PM, Fernández-Abalos JM, Villanueva JR, Santamaría R, Pérez P (1993).Purification and characterization of a phenoloxidase (laccase) from the lignin-degrading basidiomycete PM1 (CECT 2971). *Appl Environ Microbiol*, 59(8):2607–261.

Devi P, Kandasamy S, Chendrayan K. and Uthandi S. (2016).Laccase producing *Streptomyces bikiniensis* CSC12 isolated from compost. *J Microbio Biotechnol Food Sci*, 6:794–798.

Dwivedi UN, Singh P, Pande VP and Kumar A (2011).Structure–function relationship among bacterial, fungal and plant laccases. *J Mol Catal B Enzym*, 68:117-28.

Frank JA, Reich CI, Sharma S, Weisbaum JS, Wilson BA, Olsen, GJ (2008). Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Appl Environ Microbiol*, 74(8):2461-2470.

Galai S, Limam F and Marzouki MN (2009). A new *Stenotrophomonasmaltophilia* strain producing laccase use in decolorization of synthetics dyes. *Appl Biochem Biotechnol*, 158:416–431.

Gianfreda L, Xu F, Bollag JM (1999). A useful group of oxidoreductive enzymes. *Bioremed J*, 3(1):21–25.

Givaudan A, Effosse A, Faure D, Potier P, Bouillant, ML, Bally R (1993). Polyphenol oxidase in Azospirillumlipoferum isolated from rice rhizosphere: Evidence for laccase activity in non-motile strains of *Azospirillum lipoferum*. *FEMS Microbiol Lett*, 108:205-10.

Godlewska EZ, Przystas W, Sota EG (2014). Decolourisation of different dyes by two *Pseudomonas* strains under various growth conditions. *Water Air Soil Pollut*, 225:1846.

Gonzalez P, Zaror C (2000). Effect of process modifications on AOX emissions from kraft pulp bleaching, using *Chilean* pine and eucalyptus. *J Clean Prod*, 8:233-241.

Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD(2007). Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Sci,* 315:80-807.

Huan M, Tai LL, Fang YC, Jin SJ, Gao Y, Hong Q, Peng LS (2011).Biodegradation of malachite green by strain *Pseudomonas* sp. K9 and cloning of the tmr2 gene associated with an ISPpu12. *World J MicrobiolBiotechnol*, 27:1323–1329.

Hussein SI, Haider NH, Aziz GM, HusseinZA(2017).Determination the optimum conditions of laccase production by local isolate of *pseudomonas aeruginosa* using lab scale fermenter *I J S N*, 8(2):274-283.

Kumar M, SinghG, Kumar AS, Singh BJ, Sharma P, (2018). Artificial Neuro-Fuzzy Inference System (ANFIS) based validation of laccase production using RSM model. *Biocat Agricul Biotechnol,* https://doi.org/10.1016/j.bcab.2018.03.016.

Kiran PJ and Vandana P (2014). Congo red dye decolorization by partially purified laccases from *Pseudomonas aeruginosa*. *Int J Curr Microbiol Appl Sci*, 3(9):105-115.

Kuddus M, Joseph B and Ramteke PW (2013) Production of laccase from newly isolated *Pseudomonas putida* and its application in bioremediation of synthetic dyes and industrial effluents. *BiocatalAgriculBiotechnol*, 2(4): 333-338.

Lonergan G, Mew E, Schliephake K, Baker WL (1997). Phenolic substrates for fluorometric detect of laccase activity. *FEMS Microbiol Lett*, 153:485–490.

Guo H, Lin C, Wang S, Jiang D, Zheng B, Liu Y and Qin W (2017). Characterization of a novel laccase-producing *Bacillus* sp. A4 and its application in *Miscanthus* degradation, *BioRes* 12(3):4776-4794.

Muthukumarasamy NP, Jackson B, Joseph RA, Sevanan M (2015). Production of extracellular laccase from *Bacillus subtilis* MTCC 2414 using agro residues as a potential substrate *Biochem Res Int*.

Neifar M, Chouchane H, Ahjoubi M., JaouaniA, Cherif A (2016).*Pseudomonas extremorientalis* BU118: a new salt-tolerant laccase-secreting bacterium with biotechnological potential in textile azo dye decolourization. *3 Biotech*, 6(1):107.

Niku-Paavola ML, Karhunen E, SalolaP,Raunio V (1988).Ligninolytic enzymes of the white-rot fungus *Phlebiaradiata*. *Biochem J*, 254:877-83.

Niladevi KN and Prema P,(2008).Effect of inducers and process parameters on laccase production by *Streptomyces psammoticus* and its application in dye decolourization, *BioresourTechnol*, 99:4583-4589.

Niladevi KN, Sukumaran RK, Jacob N, Anisha GS, Prema P, (2009). Optimization of laccase production from a novel strain *Streptomyces psammoticus* using response surface methodology. *Microbiol Res*, 164:105-113.

Nyanhongo GS, Gomes J, Gübitz G, Zvauya R, Read JS, Steiner W (2002).Production of laccase by a newly isolated strain of *Trametesmodesta*. *Bioresour Technol*, 84(3):259-263.

Ozer A, Uzuner U, Guler HI, Ay Sal F, Belduz AO, Deniz I, Canakci S,(2018). Improved pulp bleaching potential of *Bacillus subtilis* WB800 through over expression of three lignolytic enzymes from various bacteria. *Biotechnol Appl Biochem*, 65(4):560-571.

Palmieri G, Giardina P, Bianco C, FontanellaB,Sannia G (2000).Copper induction of laccaseisoenzymes in the ligninolytic fungus *Pleurotusostreatus*.*Appl Environ Microbiol*, 66:920-24.

Peter J and Vandana P (2013). Congo red dye decolourization by partially purified laccases from *Pseudomonas aeruginosa.Int J Cur Microbiol*, 3:105-115.

Rezaei S, ShahverdiAR, Faramarzi MA (2017).Isolation, one-step affinity purification, and characterization of a polyextremotolerantlaccase from the halophilic bacterium *Aquisalibacilluselongatus* and its application in the delignification of sugar beet pulp. *BioresourTechnol,* 230:67-75.

Sharma P, Goe lR,Capalash N, (2007). Bacterial laccases. *World J Microbiol Biotechnol,* 23:823-832.

Singh G, Ahuja N, Batish M, Capalash N, Sharma P (2008).Biobleaching of wheat straw-rich soda pulp with alkalophiliclaccase from *γ-proteobacterium* JB: Optimization of process parameters using response surface methodology. *BioresourTechnol*, 99:7472-9.

Siroosi M, AmoozegarMA,Khajeh K (2016). Purification and characterization of an alkaline chloride-tolerant laccase from a halotolerant bacterium, *Bacillus* sp. strain WT. *J MolCatal B:Enzym*, 134:89–97.

Sivakumar R (2010). Isolation, screening and optimization of production medium for thermostablelaccase production from *Ganoderma*sp.*Int J EngiSciTechnol,* 2(12):7133-7141.

Solomon EI, Sundaram UM, Machonkin TE, (1996).Multicopper oxidases and oxygenases. *Chem Rev*, 96: 2563–2605.

Sondhi S, Sharma P, George N, Chauhan PS, Puri N, Gupta N (2015).An extracellular thermo-alkali-stable laccase from *Bacillus tequilensis* SN4, with a potential to biobleach softwood pulp. *3 Biotech*, 5(2):175-185.

Suljic S, Mortzfeld FB, Gunne M, Urlacher VB, Pietruszka J (2015). Enhanced biocatalytic performance of bacterial laccase from *Streptomyces sviceus*: Application in the michael addition sequence towards 3-Arylated 4-Oxochromanes. *Chem Cat Chem*, DOI: 10.1002/cctc.201500142.

Thurston CF (1994).The structure and function of fungal laccases. *Microbiology*, 140 (1):19-26.

Verma A, Dhiman K, Shirkot P, (2016). Hyper-production of laccase by *Pseudomonas putida* LUA15.1 through mutagenesis. *J Microbiol Exp*, 3:(1)00080.

Wang C, Zhao* M, Li D, Cui D, Lu L and Wei Xing (2010). Isolation and characterization of a novel *Bacillus subtilis* WD23 exhibiting laccase activity from forest soil. *African J Biotechnol*, 9(34):5496-5502.

Williamson PR (1994). Biochemical and molecular characterization of the diphenol oxidase of *Cryptococcus neoformans*: identification as a laccase. *J Bacteriol,* 176(3):656–664.

Yoshida H (1883).Chemistry of laquer (urushi). *J ChemSoc*, 43:472-86.

Zhang C, Diao H, Lu F, Bie X, Wang Y, Lu Z (2012). Degradation of triphenylmethane dyes using a temperature and pH stable spore laccase from a novel strain of *Bacillus vallismortis*. *Bioresour Technol,* 126:80-86.

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