

Full Length Research Paper

Production of extracellular laccase from the newly isolated *Bacillus* sp. PK4

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Laccase belongs to the family of multicopper oxidase and have wide range of biotechnological applications starting from the food industry to bioremediation. However, the use of laccase at commercial scale is hindered by factors like high enzyme cost, low activity and /or stability under given conditions. This study was carried out with aim of screening for extracellular thermostable laccase producing bacteria. Twenty-two (22) laccase positive strains were isolated from the selected environmental samples while extracellular laccase activity was detected only in six strains namely TM1, TMT1, PK4, PS1, TMS1 and ASP3. The laccase enzyme produced from PK4 was found to be more thermostable with a half-life of 60 min at 80°C. The strain PK4 was identified and designated as *Bacillus* sp. PK4. The laccase production was optimized using one- factor-at-a-time method and maximum enzyme production were observed at the temperature of 37°C, pH 7.5, 10% inoculum size with yeast extract and glucose as the nitrogen and carbon sources, respectively at an agitation rate of 150 rpm. Copper sulphate at 0.1 mM concentration was found to maximize the laccase production among the tested inducers. Among the trace elements, FeSO₄ and ZnSO₄ gave the maximum laccase production for the isolated strain in comparison with the control. The effect of copper-induced time showed that the addition of copper before inoculation effectively increased the laccase production compared to the addition of copper after 2, 4, 6 and 8 h of inoculation. The optimization of the media resulted in 11.8 fold increase in laccase production.

Key words: Laccase, thermostability, *Bacillus* sp., optimization.

INTRODUCTION

Enzymes have been utilized in several industries for many years. Laccase (EC 1.10.3.2) is one of the important enzymes in terms of applicability and versatility in industries (Dabrimanesh et al., 2015). Laccases are the members of multi-copper oxidases and contain

histidine-rich copper binding domains. They can oxidize lignin related compounds and highly recalcitrant environmental pollutants. Moreover, unlike many other oxidoreductases, laccases do not require cofactors such as NAD (P) H and, unlike peroxidases, they do not

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produce toxic peroxide intermediates. These characteristics of laccases position them as potential industrial oxidative enzymes (Shi et al., 2015).

Laccases are widely distributed in nature and have been described in fungi, plants, and insects and more recently in bacteria and archaea, indicating that the laccase redox process is ubiquitous in nature. Laccase plays an important role in several metabolic steps, including those involved in fungal pigmentation, plant lignification, lignin biodegradation, humus turnover and cuticle sclerotization, wherein naturally occurring low molecular weight phenolic compounds and natural fiber polymers are utilized as substrates (Jeon et al., 2012). The first bacterial laccase was reported in *Azospirillum lipoferum* in the year 1993. Thereafter, laccases have been discovered in a number of bacteria including *Bacillus subtilis*, *Bordetella compestris*, *Caulobacter crescentus*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Pseudomonas syringae*, *Pseudomonas aeruginosa* and *Yersinia pestis* (Dhiman and Shrikot, 2015).

Laccases have attracted considerable interest in many fields of industry and environmental processes due to their broad substrate specificity and ability to oxidize high redox potential substrates in the presence of certain low molecular weight compounds called mediators (Diwaniyan et al., 2012). Application of laccase in different industrial fields includes detoxification of industrial effluent, mostly from paper and pulp, textiles and petrochemical industry, used as a tool for medical diagnostics and as a bioremediation agent to clean up herbicide, pesticide and certain explosives in soil and as cosmetics ingredient (Kalia et al., 2014).

Keeping in view the importance of laccase in pollution degradation and to increase the toolbox of bacterial laccase from different subfamily, bioprospecting new bacterial strains displaying laccase activity are the need of time. Therefore, a study on bacterial laccases is important from the perspectives of basic science as well as for the development of novel biotechnological applications.

The objective of the present study was to isolate and screen for thermostable laccase producing strains from the selected samples and to maximize the laccase production of the isolated strain by optimizing the media components.

MATERIALS AND METHODS

Isolation of laccase secreting bacteria

The laccase producing bacteria were isolated in 12 different samples, namely, the treated and untreated effluents from textile industries of Ganapathypalayam and Muruganpalayam (Tirupur District, Tamil Nadu, India) and untreated effluent of two paper industries one located at Karamadai (Coimbatore District, Tamil Nadu, India) and another at Kothamangalam (Erode District, Tamil Nadu, India), soil from textile and paper industry effluent discharged

site, sludge from the textile industry, soil contaminated with automobile waste from Pollachi (Coimbatore District, Tamil Nadu, India) and composted coir pith. The isolation was based on serial dilution technique. In this, 1.0 ml of each of the sample was added to a tube containing 9 ml of sterile water (10^{-1}) and mixed vigorously for 30 s. Dilution was repeated till 10^{-7} and 0.1 ml of suspension was spread from each dilution tube on nutrient agar with 0.1% guaiacol and 0.005% cycloheximide, and incubated at 37°C for three days. Cycloheximide was added to inhibit the fungal growth. The colonies showing reddish brown color were selected. The positive colonies from all the samples were subcultured for three times to get the pure colony. The isolated bacterium was streaked on the same medium, namely nutrient agar with 0.1% guaiacol to confirm the positive activity.

Growth characterization and extracellular laccase production

The isolated bacterial strains were grown in Luria-Bertani broth supplemented with 1 mM copper sulphate for laccase production and growth. The broths were incubated at 37°C with the rotation of 100 rpm for 24 h. The culture was harvested after 24 h and centrifuged at 8000 rpm for 20 min. The supernatant was used as a crude enzyme extract for the assay of laccase activity using 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as substrate.

Assessment of optimal temperature and thermostability of bacterial laccase

The temperature optima and thermo-stability of laccase produced by the isolated strain were assessed in acetone-precipitated sample. Acetone was added to the crude enzyme extract in the ratio of 1:1, vortexed for 1 to 2 min and incubated overnight at -20°C. The sample was centrifuged at 8000 rpm for 30 min and kept at room temperature for 1 - 2 h for the complete removal of the acetone. The pellet was dissolved in 100 mM potassium phosphate buffer of pH 7.0

The optimal temperature for the laccase activity was determined over the range of 20 to 70°C using ABTS as substrate. The thermostability of the laccase produced by the isolated strain was studied by incubating the acetone-precipitated samples at three different temperatures 50, 60 and 70°C and aliquots were withdrawn every 30 min up to 3 h. The sample was slowly cooled down to room temperature and residual laccase activity was measured. All the experiments were carried out in triplicates. The results were expressed either as mean or mean standard deviation.

Identification of thermostable laccase producing bacteria

The maximum thermostable laccase producing bacteria was identified according to the morphological, physiological and biochemical characteristics by following the Bergey's Manual of Systematic Bacteriology and by 16S rDNA phylogenetic method.

Optimization of laccase production by one factor at a time method

Basal media used for the production of laccase

The basal media used for optimizing the enzyme production had following composition in g/L: K_2HPO_4 - 0.7, KH_2PO_4 - 1.4, $MgSO_4$ - 0.01, NaCl - 0.5, tryptone - 0.5, $CaCl_2$ - 0.01 and pH 7.0. The basal medium was inoculated with overnight grown culture with $OD_{600\text{ nm}}$ of 0.5 to 0.7 and incubated at 37°C at 100 rpm for 42 h.

Extraction of extracellular and intracellular enzyme

The culture medium was centrifuged at 10000 x g for 15 min at 4°C after fermentation at different experimental conditions described below and the supernatant was used as the crude enzyme extract. The cells obtained as pellet were washed twice with 100 mM phosphate buffer, pH 7.0. It was preserved in ice and resuspended in 0.5 ml of phosphate buffer. The protein extraction was carried out by sonication for 5 min at 40% amplitude and 0.7 s / cycle with the sonicator. Then it was centrifuged at 13,000 rpm for 20 min at 4°C to pull down the cell fragment and the supernatant was taken carefully in the cool tube. The supernatant obtained was used as the intracellular enzyme.

Growth kinetics and laccase production

The growth kinetics and enzyme secretion were followed every 6 h for 78 h. The culture was inoculated in basal medium and incubated at 37°C with shaking at 100 rpm. The sample was withdrawn every 6 h, bacterial biomass and both extracellular and intracellular laccase activity were measured using 2,6 dimethoxyphenol (DMP) as substrate. The growth of bacterial culture was estimated in terms of biomass by measuring the optical density at 600 nm

Optimization of different physicochemical parameters for laccase production

In one factor one time method, laccase production was optimized using selected variables such as pH (3 to 10 with the increment of 1 pH), inoculum size (5, 10, 15 and 20%), temperature (27, 37 and 47°C), carbon at 1% level (glucose, galactose, fructose, lactose, maltose, sucrose, mannose, starch, sodium citrate and sodium acetate) and nitrogen sources at 0.5% level (peptone, casein, yeast extract, gelatin, urea, ammonium sulphate, ammonium phosphate, ammonium nitrate, potassium nitrate, ammonium carbonate and sodium nitrate), agitation (100, 150 and 200 rpm), inducers at 0.1mM concentration (guaiacol, ABTS, 2,5 xylydine, veratryl alcohol, catechol and copper sulphate), trace elements (KCl, BaCl₂, MnSO₄, ZnSO₄ and FeSO₄ - 0.2 g/L) and copper addition time (0 to 8 h). The culture was incubated at 37°C for 42 h at 100 rpm except for the experiments with different temperature and agitation. The growth and laccase activity (using 2,6 DMP as substrate) were determined under each set of experimental conditions. All the experiments were carried out in triplicates and the results were expressed as mean ± standard deviation

Laccase assay

The enzyme assay was done using 2 mM ABTS or 2 mM 2,6 DMP as substrate. 50 to 100 µl of the sample was added to 10 mM phosphate buffer (pH 7) containing the substrate 2,6 DMP or 10 mM sodium acetate buffer (pH 5) containing ABTS at a concentration of 2 mM, and oxidation was measured by the increase in the absorbance at 470 nm ($\epsilon = 14800 \text{ M}^{-1}\text{cm}$) and 405 nm ($\epsilon = 35000 \text{ M}^{-1} \text{ cm}^{-1}$) respectively. One unit of laccase was defined as the amount of the enzyme required to transform 1 µmol substrate per min under standard assay conditions.

RESULTS AND DISCUSSION

Isolation and screening for extracellular laccase producing bacteria

A total of 22 laccase producing strains were isolated from

the selected 12 different environmental samples. The extracellular laccase activity was detected in the six strains namely TM1, TMT1, PK4, PS1, TMS1 and ASP3. The strain TM1 produced the maximum laccase (0.51 U/ml) which was followed by PK4 (0.49 U/ml), ASP3 (0.32 U/ml), TMS1 (0.29 U/ml), TMT1 (0.29 U/ml) and PS1 (0.25 U/ml).

Effect of temperature on the extracellular laccase activity

The temperature optima for the extracellular laccase produced by the strains namely TM1, TMT1, PK4, PS1, TMS1 and ASP3 was determined over the temperature range of 20 to 70°C. The temperature activity profiles of the isolated strains are shown in Figure 1. From Figure 1, it can be inferred that the optimum temperature for the laccase activity of TM1, TMT1, PS1, TMS1 and ASP3 was 40°C whereas for PK4 it was 50°C. The laccase activity of the selected strains was found to increase with an increase in temperature up to the optimal temperature and further increase of temperature, above the optimal temperature reduced the enzyme activity for all the selected strains. The temperature optimum for extracellular laccase from *Bacillus* sp. ADR (Telke et al., 2011) and *Pseudomonas putida* (Kuddus et al., 2013) was stated to be 40°C. Similar to the previously analyzed bacterial laccases, the extracellular laccase of the isolated strains had an optimum temperature in the range of 40 to 50°C.

Assessment of thermostability of extracellular laccase

One of the key factors determining the suitability of the enzyme laccase in industrial applications is its thermostability. Hence, the thermostability of the laccase produced by the isolated strains was assessed by incubating the acetone-precipitated samples at three different temperatures, namely 50, 60 and 70°C for 3 h. The results are presented in Figure 2a to d.

For the strain PK4, there was no loss of laccase activity at 50°C, 98 and 77% of the activity remained at 60 and 70°C respectively after 3 h of incubation. TM1 retained 86, 42 and 6% activity up to 3 h at 50, 60 and 70°C respectively. 90, 63 and 12% of the initial laccase activity were retained after 3 h of incubation at 50, 60 and 70°C respectively for the strain TMT1. The isolated strains PS1, TMS1 and ASP3 retained 66, 74 and 82% of initial activity respectively at 50°C. These strains lost more than 50% activity at 60°C at the end of 2 h of incubation (Figure 2a, b and c).

The PK4 laccase was found to retain 77% of the initial activity after 3 h of exposure at 70°C. By contrast, the strain ASP3 lost 98% activity and the strains PS1 and TMS1 lost 100% of initial activity under the same

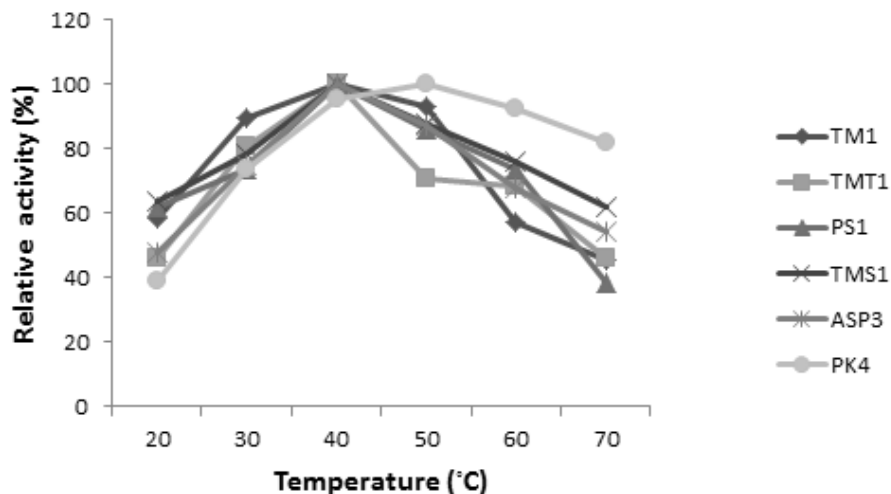


Figure 1. Temperature activity profile of laccase from the isolated strains (Values are the mean of triplicates).

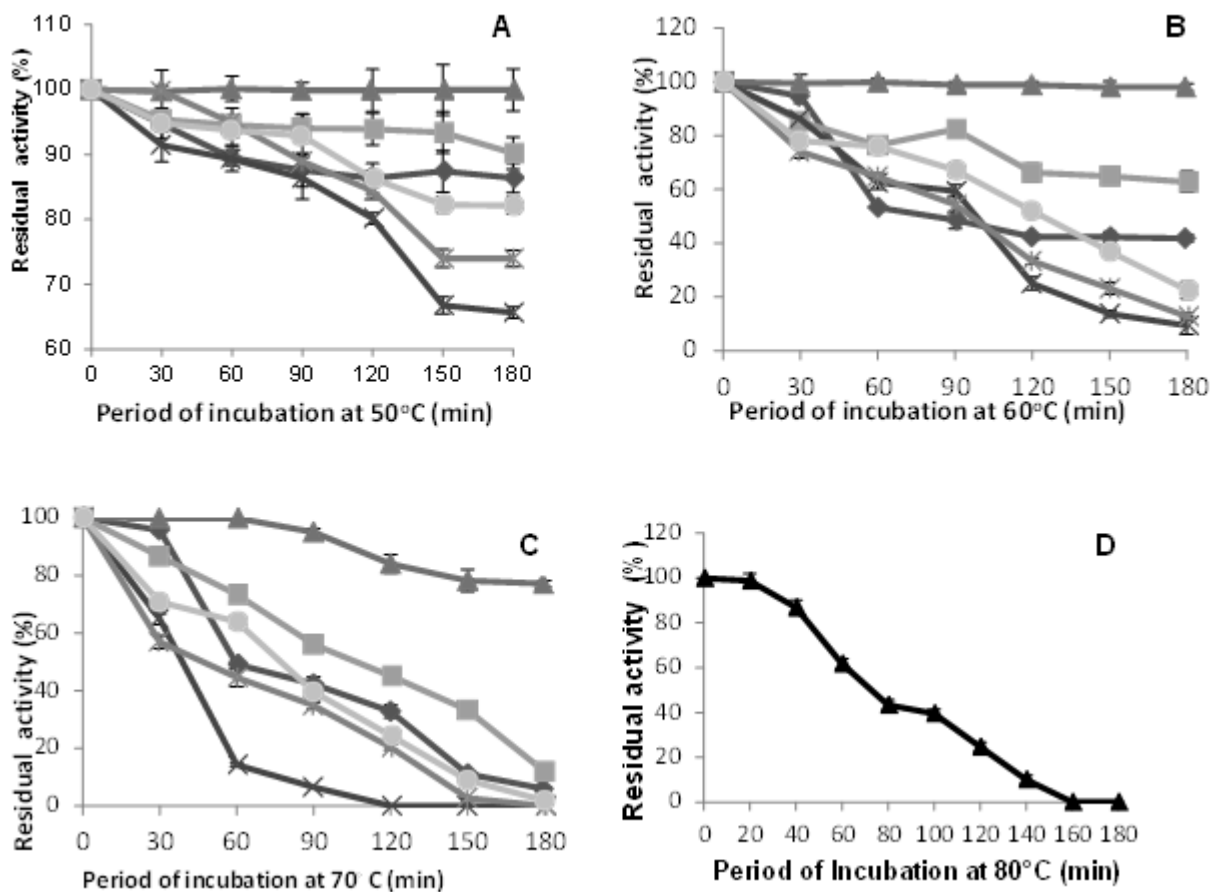


Figure 2. Laccase activity of the isolated strains at different temperatures A) at 50°C, B) at 60°C, C) at 70°C, and D) laccase activity of PK4 at 80°C (Values are the mean \pm SD of triplicates

◆ TM1 ■ TMT1 ▲ PK4 ✕ PS1 * TMS1 ● ASP3).

conditions. Except for PK4 extracellular laccase, the laccase activity of all other tested strains was lost after 3 h incubation at 70°C. Therefore, only PK4 laccase thermostability was assessed at 80°C for a period of 3 h (Figure 2d) and it was found that PK4 laccase retained 62% activity after 60 min of incubation at 80°C. Most of the thermostable laccases reported are from bacterial source whereas fungal enzymes lost their activity at temperatures above 60°C (Baldrian, 2006). Ihssen et al. (2015) showed that laccase from bacterial origin was more heat stable than the commercially available fungal laccase from *Tinea versicolor*.

According to Hilden et al. (2009) thermostability is the ability to resist irreversible inactivation at high temperature and to keep the activity at 60°C for a prolonged period. Singh et al. (2000) stated that thermostable enzymes are generally defined as those with an optimum temperature above that of the maximum for the growth of an organism or with exceptional stability above 50°C over an extended period of time. A thermostable laccase from *Streptomyces lavendulae* REN-7 retained its original activity up to 20 min at 70°C (Suzuki et al., 2003). Endo et al. (2002) described in their study that *Streptomyces griseus* laccase retained 40% activity after 60 min of incubation at 70°C and quoted this as thermostable laccase. Lu et al. (2013) affirmed that laccase from *Streptomyces* sp. C1 retained 30% activity after 120 min incubation at 70°C and more than 60% at 50°C and regarded this as moderately thermostable laccase.

The purified laccase from the thermophiles *Mycelipophthora thermophila* and *Scytalidium thermophilum* did not withstand 1 h period of incubation at 80°C (Xu et al., 1996) whereas the laccase from *Chaetomium thermophilum* was stable only for 8 min incubation at 80°C (Chefetz et al., 1998). Among the tested strains, PK4 laccase was found to retain 62% enzyme activity at 80°C after 60 min of incubation. Hence, it was selected for identification and further studies.

Identification of the selected strain

The morphological, physiological and biochemical characteristics indicated that the selected strain PK4 probably belong to the genus *Bacillus* according to the Bergey's manual of systematic classification (Table 1). The 16S rDNA sequence of strain PK4 (GenBank Accession No. KF651984) was compared with the 16S rDNA sequences present in the rDNA database of NCBI using BLAST to further identify the species of the organism. The homologous search (data not shown) indicated that the strain shared 98% identity with the *Bacillus safensis* and *Bacillus pumilus*. The neighbor-joining phylogenetic tree was constructed using the type strains of *Bacillus* sp. and presented in Figure 3a. The percentage of replicate trees in which the associated taxa

Table 1. Morphological, physiological and biochemical characteristics of the strain PK4.

| Description | Inference |
|-------------------------------------|--|
| Morphological characteristics | |
| Colony colour | White |
| Pigment | Yellow |
| Shape | Rod |
| Gram's staining | + |
| Motility | + |
| Physiological characteristics | |
| Spore formation | + |
| Sodium chloride | Growth at 1 to 5% |
| Growth at 4°C | - |
| Growth at 55°C | - |
| Biochemical tests | |
| Gelatin liquefaction | - |
| Voges proskauer's | + |
| Oxidase test | + |
| Starch hydrolysis | + |
| Casein hydrolysis | - |
| Citrate utilization | + |
| Indole production | - |
| Catalase test | + |
| Urease test | - |
| Malonate | - |
| ONPG | + |
| Nitrate reduction | + |
| Catalase | + |
| Arginine | + |
| Carbohydrate fermentation tests | |
| Sucrose | + |
| Mannitol | + |
| Glucose | + |
| Maltose | + |
| Raffinose | + |
| Melibiose | + |
| Inositol | + |
| Methyl- α -D-glucopyranoside | + |
| Arabinose | - |
| Trehalose | + |
| Antibiotic sensitivity tests | |
| Sensitive | Streptomycin, kanamycin, erythromycin, ampicillin, tetracyclin, gentamycin co-trimoxazole, rifampicin, |
| Resistant | Penicillin, teicoplanin |

+ Positive; - Negative.

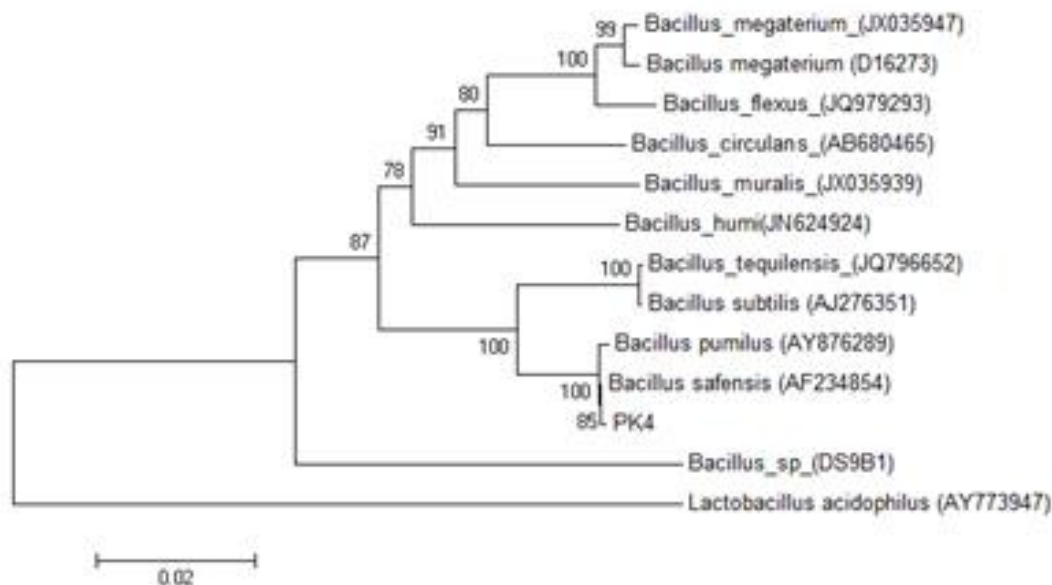


Figure 3a. Neighbor-joining tree showing the phylogenetic position of PK4.

clustered together in the bootstrap test (500 replicates) is shown next to the branches.

The BLAST search based on 16S rDNA gene sequence and phylogenetic tree (neighbor-joining method) using type strains revealed that the isolated strain PK4 shared high similarity with *Bacillus pumilus* and *Bacillus safensis*. According to Stackebrandt and Goebel (1994), strains belonging to the same genus which exhibits less than 97% 16S rDNA gene sequence similarity should be considered as members of the different species. Bozoglu et al. (2013) reported that bacterial isolate from hot springs had exhibited 99% resemblance to *Brevibacillus* according to 16S rDNA gene sequence analysis and concluded that 16S rDNA gene sequence analysis was inadequate in discriminating the closely related species.

In the present study, the isolate had exhibited 98% similarity with the two different species namely *B. pumilus* and *B. safensis*. Hence, 16S rDNA gene sequence analysis and phylogenetic method were not sufficient to identify the isolated strain PK4 at the species level. The present data should be supported by more advanced genomic analysis methods for the identification at the strain level. A DNA-DNA hybridization technique needs to be performed in order to identify the place of the PK4 in the taxonomy at the species level. Hence the isolate PK4 was identified as *Bacillus* sp. and named as *Bacillus* sp. PK4 (Figure 3b).

Optimization of laccase production by one-factor-at-a-time method

In one-factor-at-a-time method pH, temperature,



Figure 3b. *Bacillus* sp. PK4 showing reddish brown halo in nutrient broth supplemented with 0.1% guaiacol indicating laccase production.

inoculum size, agitation, carbon and nitrogen source, the inorganic salts namely KCl, BaCl₂, MnSO₄, ZnSO₄ and FeSO₄ and inducers such as ABTS, guaiacol, 2,5, xyldine, veratryl alcohol, catechol and copper sulphate were optimized. The induction of laccase production by copper addition, with respect to time, was also studied. Under each set of experimental condition, the growth and laccase activity were determined.

Growth kinetics and laccase production

The time course of the extracellular and intracellular

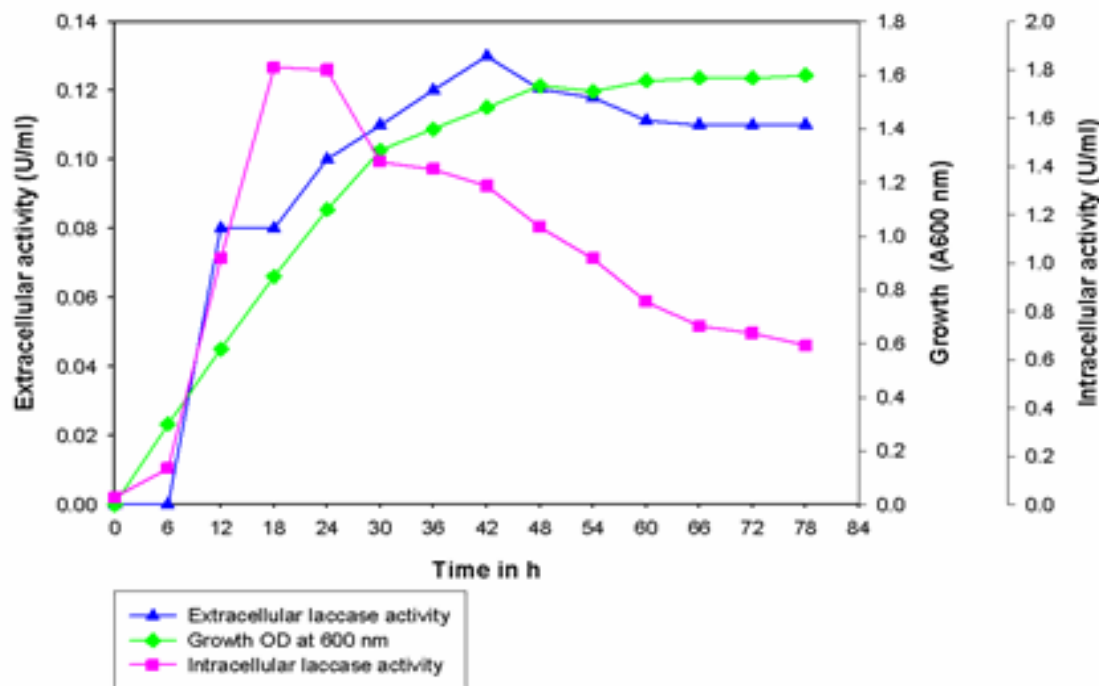


Figure 4. Growth, intracellular and extracellular laccase production of *Bacillus* sp. (PK4 at different time intervals Values are the mean of triplicates).

laccase activity was determined using DMP as a substrate over a period of 78 h. The isolated strain reached its highest intracellular activity of 1.81 U/ml at 18 h and 24 h and highest extracellular activity of 0.13 U/ml at 42 h. As depicted in Figure 4, the lag phase of the strain was short and the growth then followed the exponential or log phase up to 48 h and reached stationary phase (Figure 4). It is evident that the strain had maximal intracellular activity during the log phase of the growth and maximum extracellular activity in the early stationary phase. The extracellular enzyme activity was found to be higher throughout the stationary phase. These results clearly indicated that the isolated strain had started producing and secreting the enzyme laccase during the log phase of the growth itself. The maximum extracellular laccase activity was detected at 72 h for *B. safensis* DSKK5 (Singh et al., 2014) and 96 h for *Bacillus tequilensis* SN4 (Sondhi et al., 2014). However, Telke et al. (2011) showed that *Bacillus* sp. ADR secretes a maximum extracellular laccase at the end of 24 h of incubation using nutrient broth.

Effect of pH

Each microbial species has its own characteristic pH range at which it grows and produces the maximum enzyme. From Figure 5, it is clear that the optimum pH for laccase production and growth of the isolated strain

was 7.5. No growth was observed at pH 3.0 to 5.0. The laccase production and bacterial growth were found to increase with an increase in pH up to 7.5 and a gradual decrease was observed from pH 7.5 to 10.0. However, the enzyme production at pH 7.0 (0.19 U/ml), pH 7.5 (0.20 U/ml) and pH 8.0 (0.18 U/ml) was quite comparable. Figure 5 also depicted that the growth and enzyme production by the isolate were higher in the alkaline pH when compared to that at acidic pH. The ability of the isolate to produce enzymes at alkaline pH makes it suitable for bioremediation and various industrial applications. It must be noted that the bacterial strain was isolated from paper mill effluent, which had the pH in the alkaline range (pH 8.6) and probably this might be the reason for the ideal growth of the strain at neutral to alkaline pH. In line with the results of the present study, the optimum pH for laccase production by bacteria isolated from different environmental source was reported to be in the range of 7 to 8 (Singh et al., 2009; Kaushik and Thakur, 2014; Sondhi et al., 2014)

Effect of inoculum size

From Figure 6 it could be inferred that laccase production increased with increase in inoculum size up to 10% and decreased with the further increase in inoculum size of 15 and 20%. The maximum laccase production (0.38 U/ml) was obtained at 10% inoculum size. The bacterial

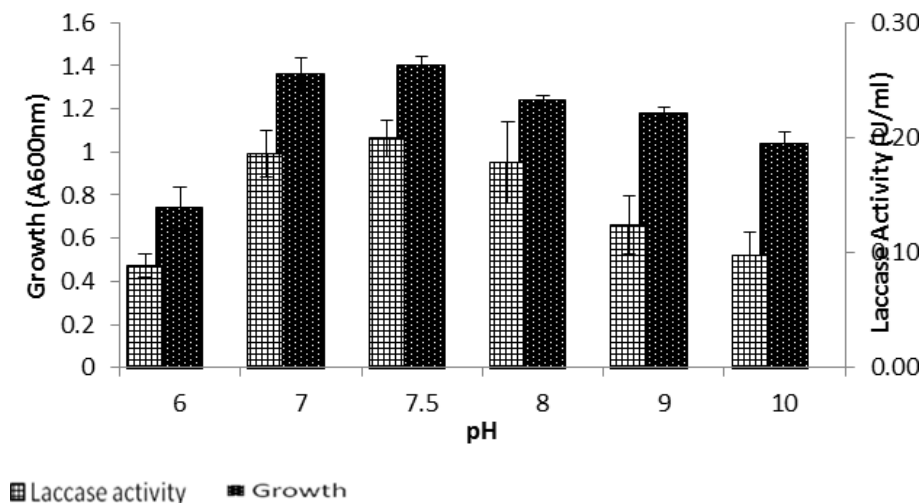


Figure 5. Growth and laccase activity of *Bacillus* sp. PK4 at selected pH (Values are the mean \pm SD of triplicates).

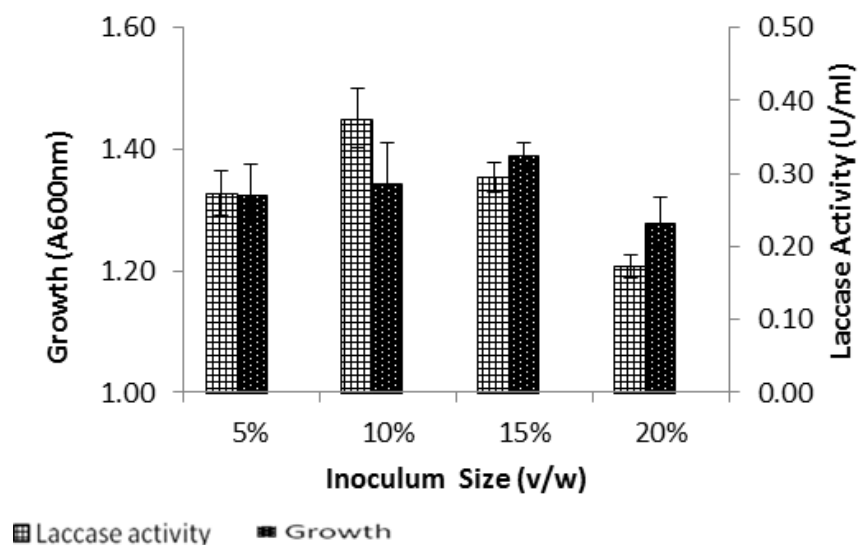


Figure 6. Growth and laccase activity of *Bacillus* sp. PK4 with selected inoculum size (Values are the mean \pm SD of triplicates).

biomass was also increased with increase in inoculum volume and the maximum was reached at 15%. Further increase in inoculum size drastically reduced the growth. One of the important criteria determining the laccase production on an industrial scale is inoculum size. A higher inoculum volume results in the rapid utilization of the substrate and thereby lowering the yield and vice versa (Deb et al., 2013). The rapid exhaustion of the nutrients might be the reason for decreased bacterial growth and enzyme production at higher inoculum size. Accumulation of toxic metabolites might also subsequently reduce the enzyme production.

Effect of temperature

The optimum temperature for the growth and laccase production was found to be 37°C. The temperature 27 and 47°C were found to decrease both laccase production and bacterial growth. This is at par with the reports of a recent study on *Bacillus safensis* by Singh et al. (2014) who demonstrated that the optimum temperature for maximum enzyme production was 37°C (Figure 7). Niladevi et al. (2009) had reported in their study that the optimum temperature for laccase production for *Streptomyces psammoticus* as 33°C.

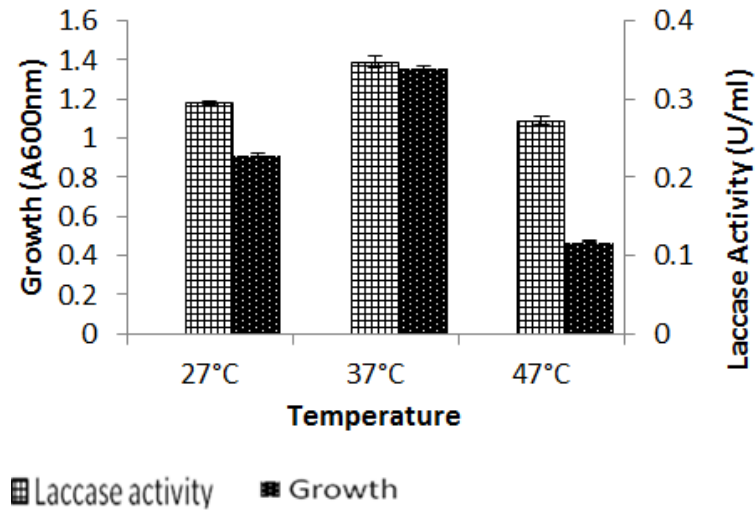


Figure 7. Growth and laccase activity of *Bacillus* sp. PK4 at different temperatures (Values are the mean \pm SD of triplicates).

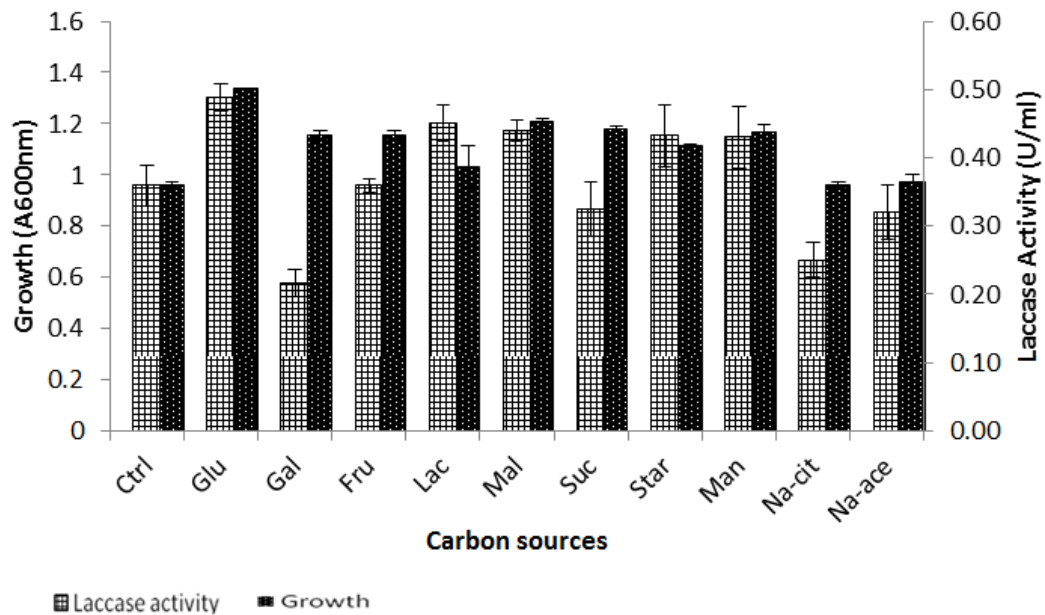


Figure 8. Growth and laccase activity of *Bacillus* sp. PK4 with selected carbon sources (Ctrl – control; Glu – glucose; Gal – galactose; Fru – fructose; Lac – lactose; Mal - maltose; Suc – sucrose; Star - starch; Man - mannose; Na-cit – sodium citrate; Na-ace - sodium acetate (Values are the mean \pm SD of triplicates).

Effect of carbon sources

The type of carbon source in the medium plays a major role in the production of ligninolytic enzymes (Gaulhaup et al., 2002) and also the substance that induces the laccase production in one species may inhibit the same in other species (Elshafei et al., 2012). The carbon source of the growth medium appears to regulate the ligninolytic

enzymes in microbes and the activity of these enzymes can be increased by the choice of the carbon source (Vaithanomsat et al., 2012). The media without carbon source served as control.

Both inorganic and organic carbon sources used in the present study supported the growth of the bacteria (Figure 8). The bacterial strain achieved the maximum growth with glucose (1.34) when compared with the

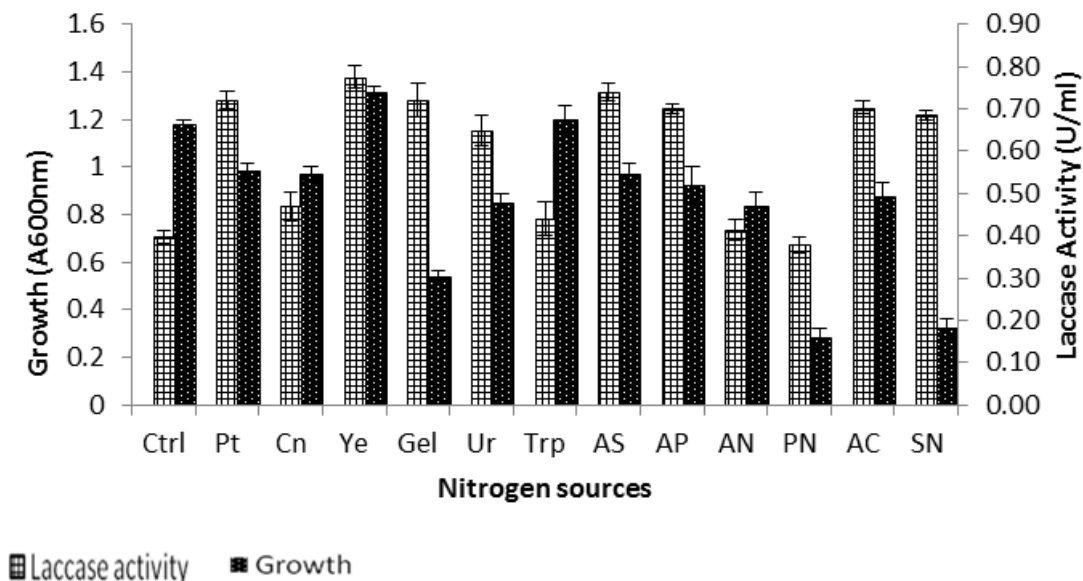


Figure 9. Growth and laccase activity of *Bacillus* sp. PK4 with selected nitrogen sources (Ctrl – Control; Pt – peptone; Cn- casein; Ye – yeast extract; Gel – gelatin; Ur – urea; AS – ammonium sulphate; AP – ammonium phosphate; AN- ammonium nitrate; PN- potassium nitrate; AC – ammonium carbonate; SN – sodium nitrate (Values are the mean \pm SD of triplicates).

control (0.96). However, the inorganic source sodium citrate and sodium acetate did not increase the growth of the culture when compared to control.

The control sample gave the laccase activity of 0.36 U/ml. The maximum laccase production was supported by glucose in the medium (0.49 U/ml) followed by lactose (0.45 U/ml), maltose (0.44 U/ml), starch (0.43 U/ml) and mannose (0.43 U/ml). The galactose (0.22 U/ml) and sucrose (0.32 U/ml) were found to lower the laccase production when compared with the control. The inorganic carbon sources sodium citrate (0.25 U/ml) and sodium acetate (0.32 U/ml) were also found to decrease the laccase production.

The presence of glucose augmented both the laccase production and growth among the various tested carbon sources as compared to control. This is in accordance with the results of studies on *Bacillus* sp. carried out by Kaushik and Thakur (2014) which suggested that dextrose was the best carbon source when compared to sodium acetate, sucrose and sodium citrate. However, Kaira et al. (2015) reported that fructose, maltose, sucrose, xylose, galactose, cellulose, and starch supported the maximum laccase production compared to glucose as sole carbon source in *Serratia marcescens*.

Effect of nitrogen sources

The nitrogen is primarily metabolized to produce amino acids, protein, nucleic acid and cell wall components in microbes. The nature and concentration of the nitrogen source are the most important factors for the production

of ligninolytic enzymes in white-rot fungi (Kunamneni et al., 2007). And also in bacteria, production of the economically important enzyme depends on the type and concentration of the nitrogen source (Shivanand and Jayaraman, 2009). The effect of various organic and inorganic nitrogen sources at 0.5% level on laccase production and growth of the isolated strain was studied and the results are shown in Figure 9.

Figure 9 portrays that all the selected nitrogen sources in the study supported the growth of the isolate and maximum growth was achieved with yeast extract (1.35). The growth of the bacterial isolate with different nitrogen sources gave decreasing order in terms of optical density at 600 nm as given below:

Yeast extract > peptone (0.99) > casein (0.97) = ammonium sulphate (0.97) > ammonium phosphate (0.93) > ammonium carbonate (0.88) > urea (0.85) > ammonium nitrate (0.84) > gelatin (0.54), sodium nitrate (0.32) > potassium nitrate (0.31).

With regard to laccase production, yeast extract (0.77 U/ml), ammonium sulphate (0.74 U/ml), peptone (0.72 U/ml) and gelatin (0.72 U/ml) were found to be the best nitrogen sources. Nearly a two-fold increase of laccase production was observed in the presence of these nitrogen sources when compared with control (0.36 U/ml). The other tested nitrogen sources, namely ammonium phosphate, ammonium carbonate, sodium nitrate, urea, ammonium nitrate, and casein gave a higher amount of the enzyme when compared with the control and it ranged between 0.41 U/ml and 0.70 U/ml

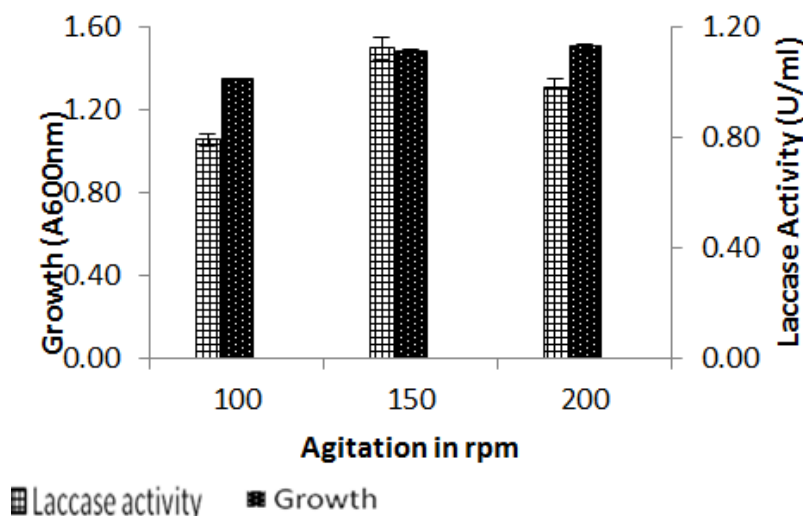


Figure 10. Growth and laccase activity of *Bacillus* sp. PK4 with selected agitation rate (Values are the mean \pm SD of triplicates).

except potassium nitrate (0.28 U/ml). It can also be noted that inorganic nitrogen source sodium nitrate gave inverse relationship for bacterial biomass and laccase production (Figure 9). Further, these results also confirmed that both the organic and inorganic nitrogen sources can augment the laccase production.

Madhavi and Lele (2009) stated that nitrogen sources effectively regulate the laccase production compared to a carbon source. In agreement with these reports, the present study also show a 2-fold increase in laccase production on nitrogen source optimization compared to carbon source optimization. Niladevi et al. (2009) reported yeast extract as the best nitrogen source for the laccase production for the strain *Streptomyces psammoticus* whereas Kaushik and Thakur (2014) showed that tryptone induces the maximum laccase production compared to yeast extract in *Bacillus* sp. In fungi, laccases are activated during the secondary metabolic phase, which is triggered by the depletion of nitrogen source. The results of the present study and previous reports suggested that nutritional supplement that effectively enhances the laccase production depends on the individual microbes and specific growth conditions.

Effect of agitation

The effect of agitation on the growth and laccase production of the isolated strain was determined and the results are shown in Figure 10. From Figure 10, it is evinced that growth of the isolated strain increased with increase in speed of agitation. However, the laccase production was highest at 150 rpm (1.12 U/ml) compared to lower agitation 100 rpm (0.79 U/ml) and higher agitation 200 rpm (0.98 U/ml). The increase in agitation

rate of 200 rpm did not increase the production, but gave the maximum bacterial growth; probably at higher agitation rate the enzyme structure might be changed.

However, at lower agitation rate there was a drastic reduction in enzyme production indicating the insufficient mixing of culture media and supply of oxygen. The amount of dissolved oxygen in the fermentation medium is greatly influenced by the speed of agitation. Excessive agitation results in a higher mechanical force, which may produce the cell destruction and uptake of nutrients, thereby lowering the enzyme production (Purwanto et al., 2009).

Therefore, optimizing the agitation of fermentation media is a very important factor to provide sufficient oxygen, mixing and uptake of nutrients by the microbe. The findings of the present study are in line with the reports of other similar studies indicating the maximum production of laccase at 150 rpm shaking condition for the strains *Streptomyces lydicus* (Mahmoud et al., 2013) and *Streptomyces psammoticus* (Niladevi and Prema, 2008).

Effect of inducers

The extracellular laccases are constitutively produced in small amounts, however, their production can be considerably stimulated by the presence of inducers mainly aromatic or phenolic compounds related to lignin or lignin derivatives. From Figure 11, it is noted that all the selected inducers supported the growth of the bacteria except 50% alcohol, which reduced the growth in terms of biomass. Among all the selected inducers, copper sulphate increased the growth to a greater extent when compared to the control.

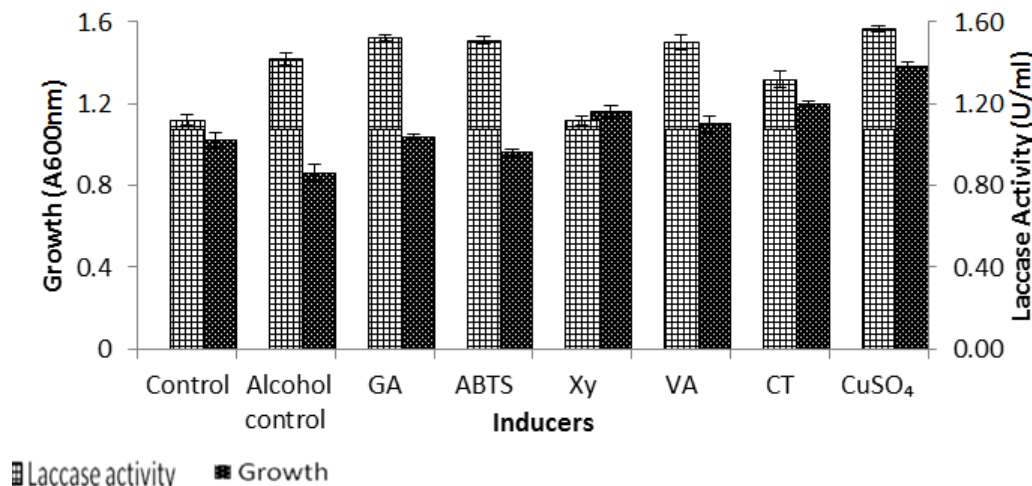


Figure 11. Growth and laccase activity of *Bacillus* sp. PK4 with different inducers GA – Guaiacol; Xy - Xylidine; VA – Veratryl alcohol; CT – Catechol (Values are the mean \pm SD of triplicates).

Guaiacol, ABTS, and veratryl alcohol increased the laccase production to a greater extent when compared with alcohol control. The catechol and 2,5 xylidine reduced the laccase production in comparison with alcohol control. The findings of the present study reveal that copper at a concentration of 0.1 mM induced the laccase production. According to the literature, the amount of copper required to induce laccase activity varies with different bacterial species ranging from 0.1mM to 1 mM (Mahmoud et al., 2013; Santo et al., 2013).

The enhancement of laccase activity in response to various inducers depends on the physiological and genetic makeup of microbial strain. Elisashvili et al. (2010) corroborated that the structure of aromatic compound and concentration play an important role in the synthesis of laccase. In addition, they suggested that enhanced laccase activity might function as a defense mechanism against chemical stress. Mongkolthanasak et al. (2012) also stated that laccase activity of different bacterial strains namely *Rhodococcus* sp., *Enterobacter* sp., *Staphylococcus saprophyticus* and *Delftia tsuruhatensis* could be triggered with different substrates (guaiacol, veratryl alcohol, phenol red and ethidium bromide).

Effect of salts

The production of extracellular enzymes by microbes is substantially increased not only by carbon and nitrogen source but also by trace elements (Fakhfakh-Zouari et al., 2010). The effect of various salts namely KCl, BaCl₂, MnSO₄, ZnSO₄ and FeSO₄ on laccase production and growth of the isolated strain was examined and the result is presented in Figure 12. The MgSO₄ and CaCl₂ at the

concentration of 0.01g/l were present in the original medium which was kept as control.

The presence of KCl, ZnSO₄, and FeSO₄ in the media appeared to enhance the growth of bacteria as compared with the control. However, the presence of BaCl₂ and MnSO₄ resulted in decreased growth (Figure 12). Figure 12 also illustrates that FeSO₄ gave the maximum laccase production for the isolated strain. The ZnSO₄ also increased the laccase production in comparison with the control. KCl was found to have less effect on laccase production for the isolated strain. From the figure, it is also evident that MnSO₄ did not have an effect on laccase production, whereas BaCl₂ had shown the negative impact on laccase production for the isolated strain. Niladevi et al. (2009) reported that trace element solution has ZnSO₄, FeSO₄ and MnSO₄ as the major influencing factor for laccase production. Fonesca et al. (2010) affirmed that Fe and Cu ions induce the laccase production through translational and post-translational regulation.

Effect of copper-induced time

The effect of copper addition (0.1 mM) at different time intervals 0, 2, 4, 6 and 8 h was studied. The results are recorded in Table 2. The addition of copper at the 0th h (2.13 U/ml) effectively increased the laccase production compared to the addition of copper after 2 h (1.72 U/ml), 4 h (1.22 U/ml), 6 h (1.19 U/ml) and 8 h (1.18 U/ml). The copper may effectively induce the production of laccase at the initial stage of growth of the bacteria compared to the later stages. Copper is an essential micronutrient for most living organisms and required for assembling copper proteins, which are involved in the oxidation and reduction reactions. Copper acts both as an inducer and

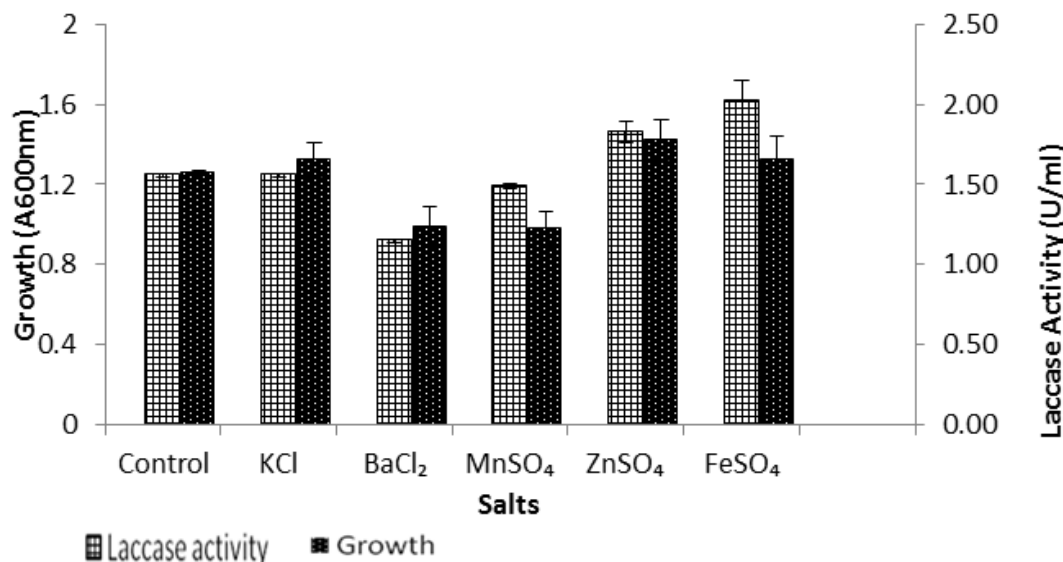


Figure 12. Growth and laccase activity of *Bacillus* sp. PK4 with selected salts (values are the mean \pm SD of triplicates).

Table 2. Effect of copper-induced time.

| Copper-induced time (h) | Laccase activity (U/ml) | Growth at A ₆₀₀ nm |
|-------------------------|-------------------------|-------------------------------|
| 0 | 2.13 \pm 0.08 | 1.20 \pm 0.01 |
| 2 | 1.72 \pm 0.17 | 1.22 \pm 0.03 |
| 4 | 1.22 \pm 0.04 | 1.22 \pm 0.01 |
| 6 | 1.19 \pm 0.07 | 1.19 \pm 0.02 |
| 8 | 1.18 \pm 0.06 | 1.18 \pm 0.01 |

Values are the mean \pm SD of triplicates.

as a micronutrient and has the potential to raise the laccase production considerably (Bakkiyaraj et al., 2013).

However, the presence of copper in higher concentration is extremely toxic to microbial cells (Trevors and Cotter, 1990). The extent of laccase production enhancement also depends on the inducer concentration and its time of addition (Gainfreda et al., 1999). Strong (2011) also stated that the addition of inducer prior to inoculation is more effective compared to the addition of inducer when the biomass is actively growing. From Table 2 it is also evident that there is no difference in terms of growth of bacteria with respect to the time of addition of copper. The results are in line with Zheng et al. (2013), who avowed that the addition of copper at a different time interval (0 to 6 h) in *Proteus hauseri* ZMd44 has no influence on cell growth.

The optimization of media components and growth conditions resulted in 2.13 U/ml compared to unoptimized media (0.18 U/ml) that is, 11.8 fold increase in laccase production was achieved.

Conclusion

The study demonstrates the presence of thermostable laccase producing bacteria in the selected environmental samples. The bacterial strain which produced laccase with higher thermostability was from paper mill effluent and it was identified as *Bacillus* sp. PK4. The media conditions optimized for laccase production by *Bacillus* sp. PK4 using one factor one time method resulted in 11.8 fold increase. Therefore, the laccase from the isolated strain can be used efficiently in bioremediation of industrial effluents and wastewater treatment.

Conflict of interest

The author declares that they have no conflict of interest.

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