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Production of highly thermo-tolerant laccase from novel thermophilic bacterium *Bacillus* sp. PC-3 and its application in functionalization of chitosan film

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In this study, a novel thermophilic bacterial strain was isolated from Tattapani hot spring located in the Chhattisgarh state of India. The laccase was produced via submerged fermentation and purified by ammonium sulfate precipitation and anion exchange chromatography up to 13.7 fold. The 16S rRNA gene sequence and biochemical analysis revealed that the bacterial isolate is *Bacillus* sp. strain PC-3. The activity of extracellular crude laccase was determined to be 11.2 U/mL using 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as a substrate. The SDS-PAGE revealed that the enzyme consists of single subunit with molecular size of 36 kDa. The laccase exhibited the maximum enzyme activity at temperature of 60°C and pH 7. Moreover, the laccase retained 99.1% of its original activity for 180 min and exhibited half-life of 3.75 h at 60°C. Similarly, the laccase retained 95% activity at pH 7 for 240 min and displayed significant activity at wider pH range. In addition, the laccase was used for functionalization of chitosan film and characterized for antioxidant and antimicrobial activity. Interestingly, the functionalized chitosan film showed the improved antioxidant and antimicrobial activity.

[**Key words:** Thermophilic bacteria; Laccase; Thermostable enzyme; Chitosan; Functionalization; Antioxidation; Antimicrobial]

Laccases belongs to multi-copper oxidoreductase enzyme (E.C. 1.10.3.2; benzenediol: oxygen-oxidoreductase), which has broader substrate affinity over groups of compounds such as phenolic compounds, aniline compounds, aromatic amines and some recalcitrant environmental pollutants (1,2). Laccase have potential applications in various industrial processes such as decolorization of textile dyes, polymer synthesis, wine and beverage stabilization, detoxification of industrial effluents and biodegradation of environmental pollutants (3). Moreover, laccase was utilized as catalyst for the production of anti-cancerous and antibiotic drugs, ingredients for cosmetics and hair coloring (4,5). Laccases are primarily distributed in bacteria, fungi, plants and insects. However, fungi have been extensively studied worldwide for laccase production (6). The main drawback of fungal laccase is that they are not stable at high temperature (>50°C). Therefore, the laccase production is currently targeted towards the exploration of thermophiles. The discovery of thermophiles will be beneficial for the development of highly thermotolerant enzyme for industrial application (7). Generally, the thermophilic enzymes provide several advantages over mesophilic enzymes including resistant to chemical denaturants, high alkalinity or extreme acidity, higher reaction rates, less susceptible to microbial contaminations and better substrate diffusion (8). Several suitable bioprocess applications exist for thermophilic enzymes. These specifically refer to their applicability in degradation of lignocellulosic biomass, degradation of wheat straw lignin, pulp bleaching and dye decolorization (8–10). However, limited thermophilic microbes have been explored for the production of laccase enzyme.

In recent times, the exploration of thermophilic bacterial strain for the production of thermostable laccase is getting significant attention. So far, thermo-tolerant laccases have been produced from various thermophilic bacterial strains such as *Thermobifida fusca*, *Bacillus tequilensis* SN4, *Geobacillus thermocatenulatus*, *Brevibacillus* sp. (Z1), *Anoxybacillus gonensis*, *Thermus thermophilus*, *Meiothermus ruber*, *Bacillus pumilus* and *Aquifex aeolicus*. However, these strains were utilized for the limited applications namely oxidation of dye intermediates, bleaching of softwood pulp, removal of textile dyes and oxidation of xenobiotic compounds (11–20).

Nowadays, synthesis of bioactive compounds and its processing using laccase has received important attention. These include antibiotics, therapeutic drugs, anti-inflammatory drugs, antioxidants and functionalization of polymers (4). Biological polymers such as chitosan provide an excellent antioxidant and antimicrobial properties (21). Functionalization of chitosan film with polyphenols like tannic acid was found to improve its antioxidant and antimicrobial properties against several pathogens (22). So far, fungal laccase has been exploited for the synthesis of bioactive compounds particularly for functionalization of chitosan film.

Considering the above research limitation and avenues, this work attempts to extend the research towards the exploration of novel thermophilic bacterial strain for the production of themotolerant laccase. In this work, a novel thermophilic bacterial strain was isolated from Tattapani hot spring located in Chhattisgarh, India. The laccase was produced via submerged fermentation and purified by ammonium sulfate precipitation followed by ion

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exchange chromatography. The stability of enzyme at different temperatures and pH was investigated. Eventually, the enzyme was utilized for the functionalization of chitosan film to improve its antioxidant and antimicrobial activity.

MATERIALS AND METHODS

Materials 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,6-dimethoxyphenol (DMP) and PCR master Mix were procured from Sigma–Aldrich (St. Louis, MO, USA). Guaiacol, tannic acid, chitosan and pre-stained protein ladder were purchased from HiMedia (Mumbai, India). Macro-prep DEAE media was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). TrackIt 1 Kb Plus DNA ladder was procured from Invitrogen (Carlsbad, CA, USA). Acryamide and bis-acrylamide were obtained from Loba Chemical (Mumbai, India). All the chemicals are of analytical grade.

Isolation and screening of laccase producing bacteria The samples in the form of water, soil and pebbles were collected from various places of Tattapani hot spring, Chhattisgarh, India. The obtained samples (temperature of 92°C and pH 8.4) were preserved at 4°C until further use. After appropriate dilutions with sterile water, the samples were enriched on nutrient broth by incubating at 55°C for 24 h. The enriched samples were spread onto nutrient agar medium and incubated at 55°C for 24 h. Based on the color and morphology; the different bacterial colonies were streaked separately and purified several times. The obtained pure bacterial cultures were stored at 4°C until further experimentation.

The thermophilic bacterial isolates were screened for laccase activity via simple plate assay using guaiacol (5 mM) as a substrate. The bacterial isolates were spotted on nutrient agar plates supplemented with guaiacol and incubated at 37°C for 48 h. The appearance of reddish brown color indicates the lacasse producing ability of the isolated species. For confirmation, the same bacterial strains were screened again using DMP as a substrate. During the experiment, 1% DMP was mixed with agar and poured into the sterile petri plate. After solidification, the circular wells were prepared and filled with 100 μL of crude laccase and incubated for 24 h at 50°C. The formation of orange color zone confirms the presence of laccase enzyme.

Identification of bacterial isolate The identification process involves three steps namely extraction of genomic DNA, PCR amplification and gene sequence analysis. To extract the genomic DNA, the bacterial isolate was grown in 10 mL of nutrient broth and allowed to incubate overnight at 55°C. The broth was centrifuged for 10 min at 10,000 rpm to separate the cells. The bacterial suspension was resuspended and washed twice with the distilled water. The DNA was extracted from bacterial cells according to the Marmur's method (23).

The amplification of 16S rRNA gene was done using forward primer F27 and reverse primer 1492R having sequence (5′-AGAGTTTGATCATGGCTCAG-3′) and (5′-CACGGATCCTACGGGTACCTTGTTACGACTT-3′), respectively. Initially, the genes were denatured for 5 min at 94°C and 30 PCR cycles of 94°C, 56°C and 72°C for 1 min each. Then, the elongation was performed for 7 min at 72°C (24). Subsequently, the amplification of 16S rRNA gene was confirmed by agarose gel electrophoresis (25). The PCR product was sequenced with universal primers targeting 16S rRNA genes.

The obtained sequence was compared with NCBI database sequences using BLAST tool to identify the sequence similarity (http://www.ncbi.nib.gov/blast). The closest bacterial strain and percent of similarity were obtained from BLAST result. Finally, the phylogenetic tree was prepared using online phylogeny tool (http://www.phylogeny.fr/) based on neighbor-joining method.

Characterization of bacterial isolate The morphological and physiological characteristics include color, gram reaction, shape, spore formation and motility was evaluated (26). Biochemical tests such as catalase, oxidase, indole, citrate, MR-VP, nitrate reduction tests, gelatin hydrolysis and fermentation of sugars were performed (27). Antibiotic sensitivity of the bacterial isolate was also studied for chloramphenicol and ampicillin. All the biochemical test reagents were obtained from Himedia.

Production and purification of laccase Nutrient broth (50 mL) supplemented with 2 mM $CuSO_4 \cdot 5H_2O$ was inoculated with 500 μL of overnight culture of isolate for submerged production of laccase. The pH of the medium was 7 and the culture was maintained at 55°C for 36 h at 120 rpm. Aliquots from fermentation broth were taken and centrifuged for 10 min at 8000 rpm. The supernatant containing laccase was considered as extracellular crude enzyme and assayed using ABTS as a substrate. On the other hand, the intracellular laccase activity was also estimated. For this, the broth was centrifuged and obtained pellet was suspended in 3 mL of 100 mM phosphate buffer (pH 7.0). Then, sonication was conducted at 50 % amplitude for 5 min at 1 s/cycle using probe sonicator (Oscar Ultrasonic, Mumbai, India) to extract the intracellular proteins. To remove the cell fragments, the suspension was centrifuged at 8000 rpm for 20 min at 4°C and supernatant was considered as intracellular enzyme.

The ammonium sulfate precipitation and ion-exchange chromatography were performed for the purification of extracellular crude laccase. The ammonium sulfate precipitation was done at the intervals of 0–20%, 20–40%, 40–60%, 60–80% and 80–100% using the standard table reported elsewhere (28). The sample with maximum precipitation was centrifuged for 15 min at 10,000 rpm. The pellet was then resuspended in sodium phosphate buffer of 50 mM (pH 6.0) and was dialyzed against the sodium phosphate buffer of 20 mM (pH 6.0). Ion exchange chromatography was performed to further purify the sample. DEAE cellulose was used as stationary phase in a column (1.5 × 14 cm) with a bed volume of 20 mL. Initially the column was equilibrated using sodium phosphate buffer of 50 mM (pH 6). A sample volume of 1 mL was added in the column and eluted using gradient NaCl (0–1 M) as a mobile phase. Different fractions were collected and assayed for laccase activity. The fraction having maximum activity was pooled.

Laccase activity was determined at each purification step using ABTS (ε= 36,000 $M^{-1}cm^{-1}$) as a substrate (29). The mixture containing 300 μL of enzyme and 0.5 mM ABTS (final concentration) in sodium acetate buffer of 100 mM (pH 4.5) was incubated at 60°C for 10 min. After that, 0.5 mL of 80% trichloroacetic acid was added to impede the reaction. The oxidation of ABTS was determined by monitoring the absorbance increase at 420 nm. The amount of enzyme involved to oxidize 1.0 μmol of ABTS per min is considered as one unit of enzyme. The concentration of total protein was quantified by Lowry's method using UV–Vis spectrophotometer at a wavelength of 620 nm (30).

Characterization of laccase SDS-PAGE was conducted to determine the relative molecular size of laccase according to the method reported elsewhere (31) using Mini-Protean 2-Gel Electrophoresis system (Bio-Rad, India). The amount of protein sample loaded in the wells (10 % polyacrylamide gel) was 20 μg of crude enzyme and 100 ng of purified laccase. The staining of the gel was done for 1 h in 0.1% solution of coomassie brilliant blue R-250 that contained 10% acetic acid and 50% methanol in the distilled water. The gel was destained in a solution containing 10% acetic acid and 40% methanol in distilled water. The approximate molecular weight of lacasse was estimated using the pre-stained protein ladder.

The optimum temperature was investigated by measuring the laccase activity at various temperatures ranging between 40°C and 80°C at pH 7.0. The thermal stability of laccase was also determined in terms of activity as a function of time. The activity was examined at various temperatures (50°C, 60°C and 70°C) for 240 min at an interval of 30 min.

In order to evaluate the optimal pH of the enzyme, the enzymatic reaction was performed over the pH range from 4 to 9. Similarly, the stability of the enzyme at the pH ranging from 6 to 8 was also studied for 240 min. An aliquot of sample was withdrawn from the reaction mixture at every 30 min and assayed for enzyme activity.

Laccase-mediated functionalization of chitosan In this study, the chitosan film was functionalized using laccase as a mediator to increase its antioxidant and antimicrobial activity. The chitosan film was formed by dissolving 2 g of chitosan powder in 100 mL of acidized water (pH 2). The undissolved chitosan pellet was removed and chitosan solution was poured in petridish and dried at 45°C for 24 h. The film was neutralized for 3 h using 1 M NaOH and cleaned using distilled water and phosphate buffer of 100 mM (pH 6.5). The chitosan film was stored in phosphate buffer at 4°C.

For polyphenol preparation, 10 mM of tannic acid was prepared using phosphate buffer (pH 6.5) and suspended in 25.6 U of purified laccase at 30°C for 24 h under constant stirring condition. The obtained product was centrifuged for 2 h at 10,000 rpm followed by three times washing with water.

The chitosan film was functionalized using the heterogeneous grafting method in which the film was incubated in the solution containing phosphate buffer (100 mM, pH 6.5), polyphenols and purified laccase (25.6 U). The solution was kept under constant stirring at 30°C for 24 h. The film was rinsed thoroughly with ethanol and water. Eventually, the chitosan film was exposed to UV light for 30 min to remove any contamination present in the film (32).

Characterization of functionalized chitosan film Fourier transform infrared spectroscopy (FTIR) analysis of control chitosan film, tannic acid aggregates and functionalized chitosan film was carried out using FTIR spectrometer (Bruker Alpha) to identify the occurrence of functional groups. The absorbance was measured within the range of 650–4000 cm^{-1} with 16 scans.

Antioxidant activity of functionalized chitosan film was measured according to the method reported in the literature (32). The chitosan (1 mg) was mixed with $ABTS^{\bullet+}$ free radical solution (ABTS of 7 mM and potassium persulfate of 2.45 mM). Prior to use, the absorbance of $ABTS^{\bullet+}$ solution was adjusted to 0.700 ± 0.025 at a wavelength of 734 nm using phosphate buffer saline (PBS). The $ABTS^{\bullet+}$ radical inhibition was monitored at 734 nm and percentage of inhibition was determined at the end of 30 min according to the following equation

$$SE\ (\%) = \frac{A_C - A_S}{A_C} \times 100 \quad (1)$$

where SE is the scavenging effect, A_C and A_S represents the initial and final concentration of $ABTS^{\bullet+}$ in the sample, respectively.

Antimicrobial activity of functionalized chitosan film was tested with two different pathogenic bacteria namely *Staphylococcus aureus* and *Salmonella typhimurium*. For this, the pathogens were inoculated separately in to nutrient broth containing control and functionalized chitosan film. The pathogens were grown at 37°C and growth was measured at a regular interval of 1 h for 16 h at a wavelength of

600 nm. The difference in the growth rate of pathogens with and without the functionalized chitosan is the measure of anti-microbial activities.

RESULTS AND DISCUSSION

Isolation and screening of thermophilic bacteria Thermophilic bacterial strains were isolated from Tattapani hot spring and investigated for their thermal stability at higher temperature. The optimal growth of the selected bacterial strain was observed at the temperature of 55°C which fulfill the condition of a thermophile (33). The selected strains were screened for laccase activity using guaiacol as a substrate. The reddish brown color around the bacterial colonies was observed which showed the laccase activity of bacterial isolate (Fig. 1A). Moreover, the ability of bacterial isolate for production of laccase was confirmed using DMP as a specific substrate that oxidized to give orange color, which was observed around the crude laccase wells (Fig. 1B).

Identification and biochemical characterization of bacterial isolate The gene sequences of bacterial isolate (1061 nucleotides of 16S rRNA) was aligned to compare with the sequences obtained from NCBI database using BLAST tool to identify the possible genera of the isolate based on the homology. The BLAST result displayed that the bacterial isolate has 99% similarity with 16S rRNA sequence of *Bacillus* sp. PC-3 (MG988279). Fig. 1C illustrates the phylogenetic tree prepared by using neighbor-joining method. It was apparent that the isolated bacterial strain is *Bacillus* sp. strain PC-3.

The morphological, physiological, and biochemical characteristics of the screened isolate were also studied. It was noted that the bacterial isolate is gram-positive, motile and spore-forming. Biochemical tests revealed that the strain is positive for oxidase and catalase tests, citrate utilization, gelatin and starch hydrolysis, methyl red test, whereas negative for indole test and nitrate reduction test. Moreover, it was inferred that the stain successfully utilizes glucose, lactose, sucrose, fructose, maltose, galactose and arabinose. Finally, antibiotic tests indicate that the strain is sensitive for chloramphenicol and ampicillin antibiotics.

Production and purification of laccase The laccase enzyme was produced from *Bacillus* sp. strain PC-3 via submerged fermentation using nutrient broth. The incubation temperature, pH, time and shaking speed were maintained at 55°C, pH 7, 36 h and 120 rpm. In this condition, the protein concentration was found to be 0.601 g/L. The ammonium sulfate precipitation was performed to purify the extracellular crude laccase followed by anion exchange chromatography.

TABLE 1. Purification of laccase.

Purification step	Enzyme activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme	11.2	0.601	18.635	100	1
Ammonium sulphate precipitation	7.6	0.326	23.312	67.85	1.25
Ion-exchange chromatography	1.71	0.0067	255.22	15.26	13.7

The different ammonium sulfate concentrations were tested to precipitate the maximum laccase and it was inferred that the maximum precipitation occurred at 60%. In this step, the enzyme was purified 1.25 times with yield of 67.85%. Next, the enzyme was suspended in ion exchange column having DEAE cellulose and the fractions showing maximum enzyme activity were pooled and further purified 13.7 times with 15.26% yield (Table 1).

The activity of extracellular and intracellular crude enzyme was estimated to be 11.2 U/mL and 1.4 U/mL, respectively. The extracellular enzyme displayed significantly higher activity as compared to intracellular crude enzyme. Therefore, further analysis and investigation were carried out for extracellular enzyme only. The obtained laccase activity (11.2 U/mL) is comparable with the other reported bacterial laccases namely *Thermobifida fusca* (4.96 U/mL), *Bacillus* sp. strain WT (0.01 U/mL), *Streptomyces lavendulae* REN-7 (0.076 U/mL) and *Bacillus* sp. PK4 (2.13 U/mL) (11,24,34,35). Further, the specific activity of crude extracellular enzyme was determined to be 18.635 U/mg. The result with respect to activity, protein concentration and specific activity during purification is presented in Table 1. The enzyme activity and total protein concentration was found to be decreased at each purification step while specific activity was increased.

Characterization of laccase SDS-PAGE was performed to determine the purity as well as molecular weight of laccase enzyme. The SDS-PAGE analysis revealed that the laccase enzyme consists of single subunit with molecular size of 36 kDa (Fig. 2).

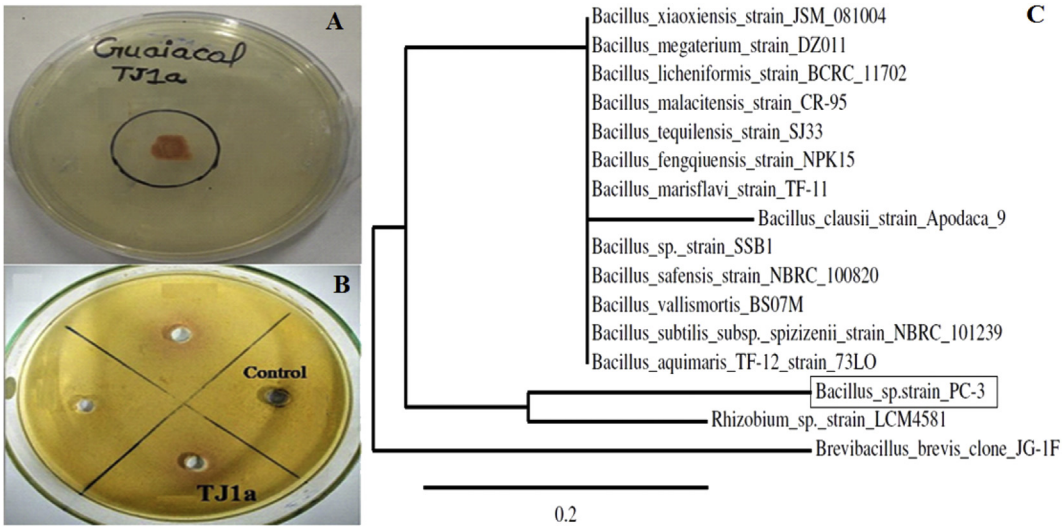

FIG. 1. Screening of bacterial strain using (A) guaiacol, (B) DMP, and (C) phylogenetic tree.

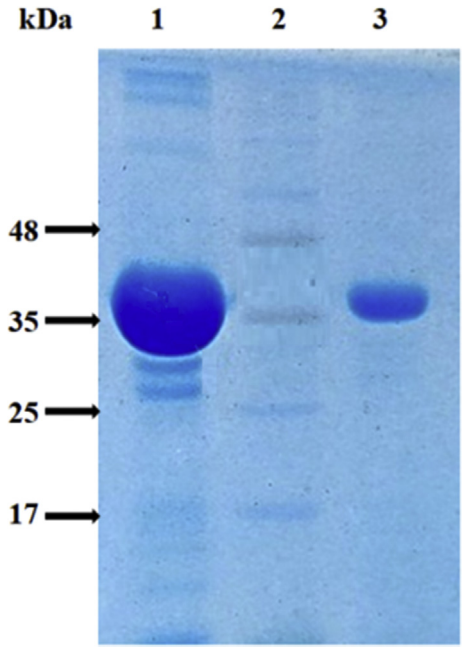

FIG. 2. SDS-PAGE. Lanes 1, 2 and 3 represent the extracellular crude laccase, protein marker, and ion exchange chromatography purified laccase, respectively.

The obtained value of molecular mass almost comply with the other reported bacterial laccase such as *Bacillus tequilensis* SN4 (32 kDa) and *Kurthia huakuii* LAM0618 (29.3 kDa) (12,10). However, laccase produced from *Thermus thermophilus* HB27 (53 kDa), *Bacillus pumilus* (58 kDa) and *Bacillus* sp. SL-1 (65 kDa) revealed higher molecular mass (17,19,36).

The optimum temperature was examined by measuring the laccase activity at different temperatures ranging from 40°C to 80°C at pH 7. Fig. 3A illustrates the variation of enzyme activity with varying temperature. The optimum temperature for maximum enzyme activity was observed to be 60°C. It was apparent that the enzyme activity increased with temperature up to optimum temperature (60°C) and further decreased with increase in temperature. Similar trend of result has been reported for the laccase from bacterial strain *A. gonensis* P39 (16). For the most part, optimal temperature for production of thermostable laccases lies in the range of 50–80°C (Table 2).

Thermal stability of enzyme was determined to identify its suitability for prolonged industrial applications. The activity of the enzyme was determined at three different temperatures of 60°C, 70°C and 80°C for 240 min. Fig. 3B depicts the laccase activity with respect to time at different temperature. It was observed that the laccase retained 99.1% of its original activity for 180 min at 60°C and further decreased about 70.2% of its activity for 240 min. The half-life ($T_{1/2}$) of the laccase was calculated to 3.75 h at 60°C which is comparatively higher than those bacterial laccases reported in the literature. The thermostable laccase obtained from *γ-proteobacterium* JB exhibited half-life of 30 min at 60°C (37). Similarly, thermostable laccase of *Bacillus pumilus* displayed half-life of 3.5 h at 60°C (19). However, thermostable laccase obtained from *Bacillus tequilensis* SN4, *Thermus thermophilus* HB27 and *Aquifex aeolicus* possessed higher half-life but their activity is relatively lesser than the laccase reported in this work (12,17,20). Further, it could be mentioned that the laccase retained 17.85% of its initial activity at 70°C for 120 min. From this study it was concluded that the laccase was thermally stable at 60°C for 180 min.

The optimum pH for laccase activity was studied in the range of pH 4 to 9 at optimized temperature (60°C). The optimum pH was measured to be 7 for maximum laccase activity (Fig. 3C). The enzyme activity increased up to pH 7 and then decreased with an increase in pH. Moreover, considerable enzyme activity was also observed at pH ranging between 5 and 9 (6.2 and 6 U/mL, respectively). Therefore, it was concluded that the laccase showed activity in wider pH range (pH 5–9). This indicates the applicability of enzyme in different pH condition for industrial application.

The laccase stability measured at different pH values with respect to incubation time is illustrated in Fig. 3D. As seen, the laccase retained around 95% its initial activity at pH 7 for incubation period of 240 min, whereas the laccase retained its activity of 77% and 70% at pH 8 and 9, respectively, for complete incubation time. Therefore, it was considered that the laccase was more stable at pH 7. From this study, the optimum pH for laccase stability was selected as pH 7.

The overall characteristics of laccase obtained from *Bacillus* sp. strain PC-3 and a comparison of obtained results with other reported literature are presented in Table 2. Therefore, it was concluded that the characteristics of obtained laccase are comparatively better than most of the other laccases reported in the literature (10–12,15–17,19,20,24,34–43).

Characterization of laccase-mediated functionalized chitosan film The images of chitosan and functionalized chitosan films are depicted in Fig. 4A and B.

Fig. 4F illustrates the FTIR spectrum of tannic acid products, control chitosan and functionalized chitosan film, which confirmed the chitosan functionalization with tannic acid aggregates. In case of tannic acid products, the absorption band was observed between 3400 and 2500 cm^{-1} which confirmed the presence of various hydroxyl groups of polyphenols (oxidized product of tannic acid). Those bands were appeared due to bending (–OH) and stretching (C–OH) vibrations of poly-hydroxyl compounds, substituted benzene ring and aromatic compounds. The stretching band (C=O) at 1716 cm^{-1} represented the carbonyl group presence in the tannic acid aggregates. The strong peak at 1616 cm^{-1} due to –OH bending was slightly broad, which is consistent with the literature (44). Moreover, peaks situated in the region of 900–650 cm^{-1} displays the release of bonded gallic acid dimers, the substituents on benzene rings (45).

FTIR spectrum of control chitosan showed bands with strong absorption in the range of 3000–3500 cm^{-1}, which indicated the –OH and –NH stretching. Moreover, the signals at 1655, 1589, 1392 cm^{-1} are due to different modes of residual *N*-acetyl groups namely amide I, II, and III, respectively. The absorption band at 1159 cm^{-1} and 1124 cm^{-1} denoted the characteristic peaks of chitosan saccharide structure. The peak at 1159 cm^{-1} indicated the anti-symmetric stretching of C–O–C bridge, whereas the peak at 1124 cm^{-1} showed skeletal vibration of C–O stretching (46,47).

FTIR spectra of functionalized chitosan film exhibited the presence of polyphenols of tannic acid characteristic bands which provides the evidence of absorbance of tannic acid into the chitosan film without covalent bonding (32). The absorption peaks between 3000 and 3500 cm^{-1} indicated the hydroxyl group of gallic acid residue, which was absent in the control film. H-bond formation was resulted in lowering the frequency value of amide I of control chitosan, i.e., 1655 cm^{-1} to 1635 $cm^{-1}$for functionalized chitosan.

Anti-oxidant properties of functionalized chitosan film Antioxidant capacity of functionalized chitosan film was tested using $ABTS^{\bullet+}$ decolorization assay technique. Fig. 4C depicts the antioxidant capacity of control chitosan and functionalized chitosan film as a function of time. It was observed that the functionalized chitosan film has a strong inhibitory activity over the oxidation of $ABTS^{\bullet+}$ cation radicals. Moreover, an enhanced antioxidant capacity of functionalized chitosan film was persist over 100 min as compared to native chitosan film. Further, the

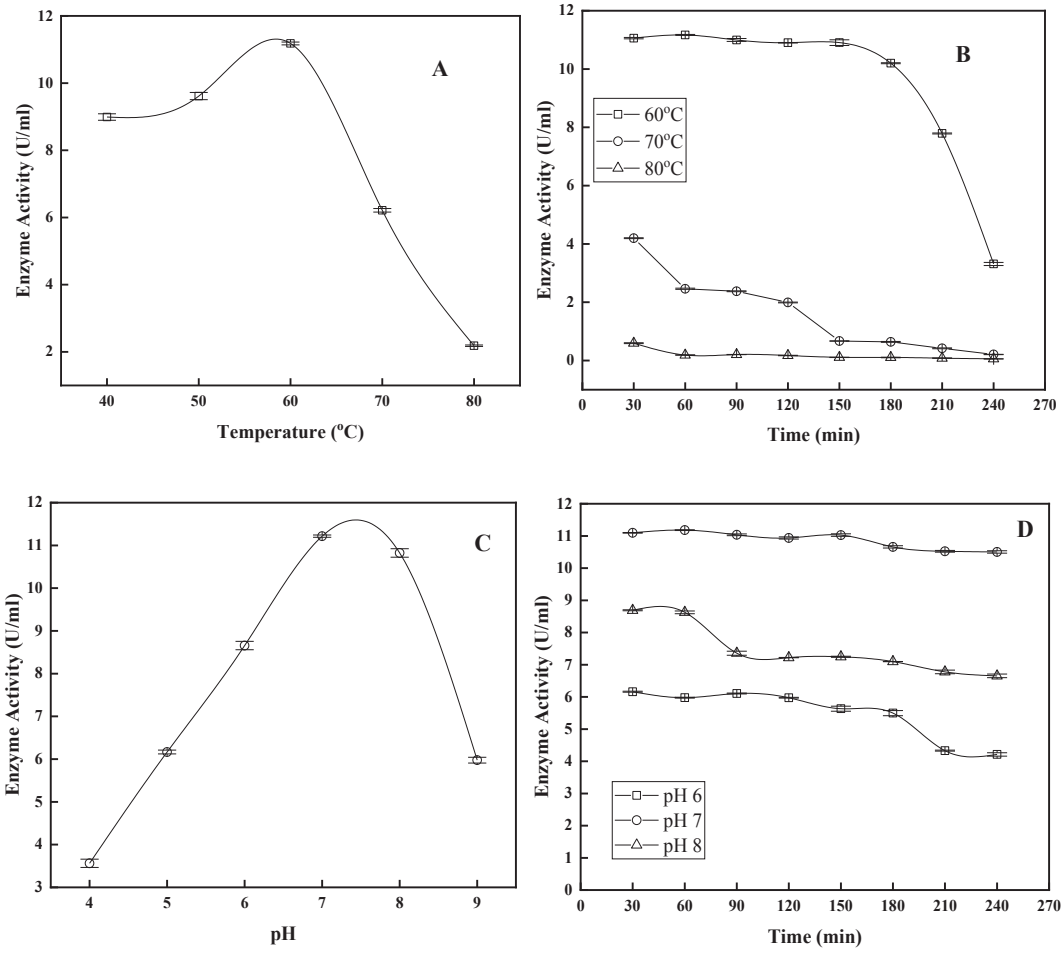

FIG. 3. (A) Variation of enzyme activity with temperature, (B) thermal stability of enzyme with respect to time at different temperatures, (C) variation of enzyme activity at various pH, and (D) pH stability with respect to time at different pH.

TABLE 2. Comparison of laccase characteristics with other reported laccase.

Bacterial isolate	Optimal temperature (°C) and pH	Enzyme activity (U/ml)	Specific activity (U/mg)	Molecular weight (kDa)	Half-life ($T_{1/2}$) of the enzyme	Reference
Azospirillum lipoferum	70, 6.0	–	0.9	97.8	70°C for 43 min	38
Bacillus subtilis	75	–	1.25		80°C for 2 h	39
Streptomyces lavendulae REN-7	70	0.076	0.028	68.7	70°C for 100 min	34
Thermus thermophilus HB27	92, 5–6	–	–	53	80°C for 14 h	17
Aquifex aeolicus AAC 07157.1	75	–	–	57.8	490 min at 80°C	20
γ-Proteobacterium JB	55, 6–7	–	3.94	120	120 and 30 min at 55 and 60°C respectively	37
Haloferax volcanii	45	0.17	29.4	75–80	50°C for 31.5 h	40
Bacillus pumilus	70	–	–	58	0.28, 0.34, 3.5 h at 60°C in different buffers	19
Thermobifida fusca	50	4.96	3.98	24.7	4.7 h at 90°C	11
Brevibacillus sp. Z1	50, 6.5–8.5	55.86	16.5	110	–	15
Bacillus tequilensis SN4	85	1.44	–	32	4 h, 3 h and 1 h at 75°C, 80°C and 85°C respectively	12
Bacillus sp. SL-1	70	–	13	~65	60 min and 20 min at 70°C and 80°C	36
Anoxybacillus gonensis P39	60, 5.0	50.07	21.96	160	–	16
Bacillus sp. PK4	80, 7.5	2.13	–	–	60 min at 80°C	35
Bacillus sp. strain WT	55	0.01	0.002	180	–	24
Bacillus pumilus MK001	80	–	73	58.85	–	41
Klebsiella pneumoniae	70	–	7.12	55.6	–	42
Kurthia huakuii LAM0618	85	–	0.63	29.3	3 days at 80°C	10
Lactobacillus plantarum J16	85	–	0.54	~62.5	–	43
Bacillus sp. strain PC-3	60, 7.0	11.2	18.635	36	3.75 h at 60°C	Present work

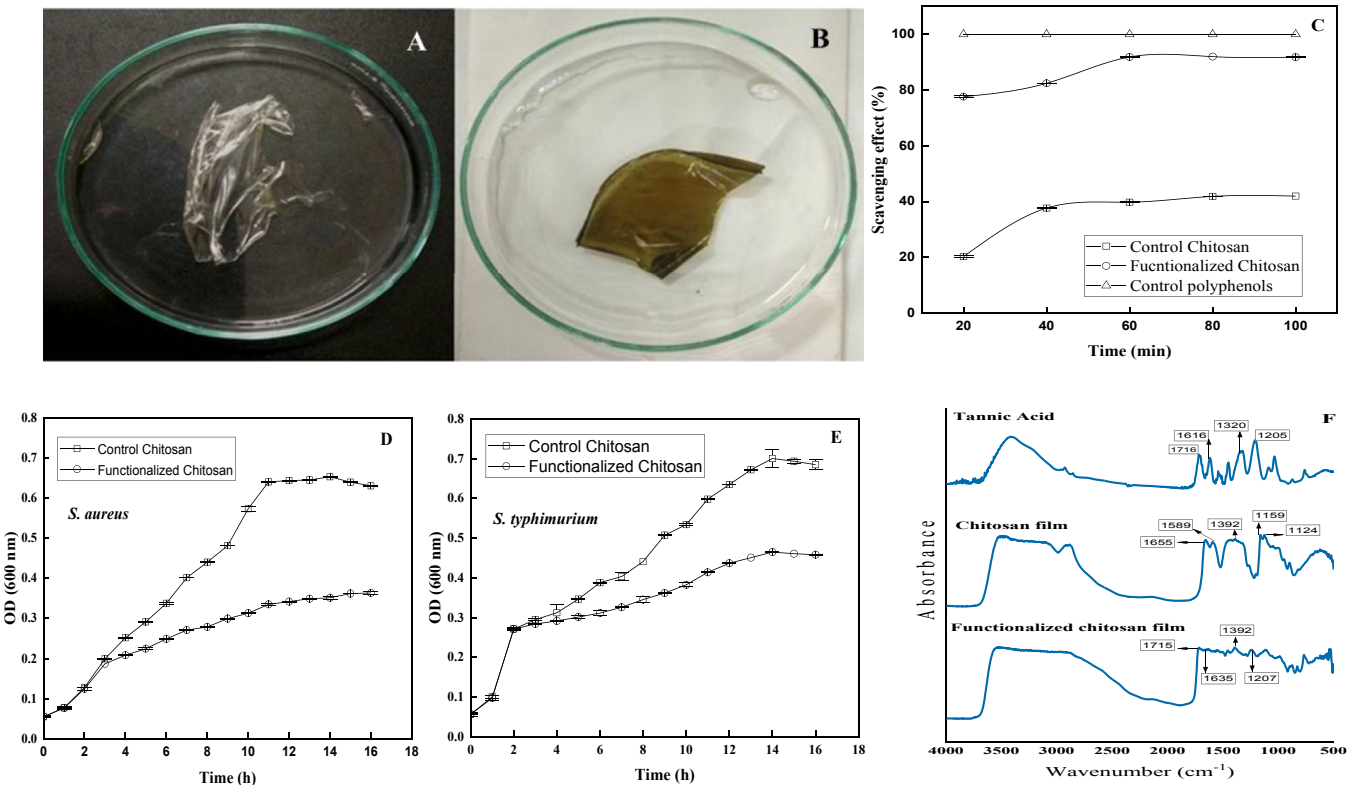

FIG. 4. (A) Control chitosan film, (B) functionalized chitosan film, (C) antioxidant activity of functionalized chitosan film, (D) growth pattern of *Staphylococcus aureus* in functionalized chitosan, (E) growth pattern of *Salmonella typhimurium* in functionalized chitosan, and (F) FTIR analysis of tannic acid aggregates, chitosan film and functionalized chitosan film.

scavenging capacity was increased with time and reached its maximum value after 80 min. In general, antioxidant capacity was primarily depends on the accessibility of reductones. The amorphous nature of the laccase oxidized product of tannic acid could influence the accessibility of reductones. Moreover, the presence of hydroxyl groups of laccase oxidized product of tannic acid might also enhanced the anti-oxidant capacity. About 50% improvement in antioxidant capacity was observed with functionalized chitosan film than control chitosan. Similar type of work has been reported by Bozic et al. (32) in which the antioxidant capacity was enhanced using homogenous and heterogenous methods catalyzed by the commercial laccase.

Anti-microbial properties of functionalized chitosan film The antimicrobial susceptibility of functionalized chitosan film was tested using two different pathogenic bacteria namely *Staphylococcus aureus* and *Salmonella typhimurium*. Fig. 4D and E presents the growth kinetics of pathogens in control and functionalized chitosan film. The growth of pathogens was found to be decreased after 2 h of incubation with the functionalized chitosan film as compared to control film that indicates the improved anti-microbial properties of functionalized chitosan film. Generally, the tannic acid alone can act as an antimicrobial agent (48). It has been hypothesized that phenolic compounds inhibits the calcium and potassium ions transport by disrupting the lipid phase of the cell membrane (49). The free hydroxyl group on the benzene ring plays an important role for the antimicrobial activity of tannic acid. The FTIR data demonstrate that this group remained unaffected during the functionalization process. The similar approach was adopted to investigate the antimicrobial activity of functionalized chiotsan film using bacterial strains such as *Escherichia coli*, *Salmonella entrica*, *Listeria monocytogenes* and *Candida albicans* (32).

In the present study, an extracellular thermostable and pH-tolerant laccase was produced from the thermophile *Bacillus* sp. PC-3, isolated from the Tattapani hotspring, India. The enzyme has the ability to enhance the antimicrobial and antioxidant activity of chitosan by the functionalization of tannic acid aggregates. Therefore, a bacterial laccase was explored for the functionalization of biopolymers as their application was restricted to the degradation of dyes and bioremediation of waste water previously. The laccase molecular weight was determined to be 36 kDa and it showed broader thermal and pH stability with the optimum temperature of 60°C. The enzyme utilized guaiacol, DMP, ABTS and tannic acid as its substrates; therefore it also possesses broader substrate range. Thus, the laccase can be an excellent catalyst for various industrial applications.

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