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SCREENING OF LACCASE PRODUCER FROM SOIL AND ITS APPLICATIONS

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ABSTRACT

In present work, twenty-eight bacteria and thirty fungi were isolated from soil on Luria Bertani (LB) Agar at 37ºCin 2-3 days, Potato Dextrose Agar (PDA) at 28±2ºCafter 3days of incubation respectively. From bacterial isolates, three bacteria and three fungi showed intense development of brown color using 1%Tannic acid as a substrate in 3-4 days at 37ºCwhich indicates they were laccase producers and are identified as *Pseudomonas aeruginosa, Erwinia chrysantemi, Klebshiella oxytoca, Cladosporium spp.* and two *Aspergillius spp,*. Crude laccase of *Klebshiella oxytoca* was extracted by centrifugation then partially purified using 40% & 70% ammonium sulphate precipitation. Partially purified laccase expressed highest activity 980U/ml at pH 7.5 & 30ºCafter 30min of incubation using 1% tannic acid. Immobilization of partially purified laccase was done by entrapment method using sodium alginate. Partially purified and immobilized laccase showed 97% of crystal violet & 94% neutral red dye degradation in 3 days of incubation as well as meat tenderization. It has antibacterial activity against *S. aureus, S.typhi,* and *Agrobacterium tumifaciens* and showed zone of inhibition ranging from 17mm-20mm. Immobilized laccase also synthesized silver nanoparticle at 75ºCin 3-4 hrs. Laccase from *Klebshiella oxytoca* in our work have wide applications and can be used in bioremediation.

Keywords: Laccase, Tannic acid, Dyes, Meat tenderization, Antibacterial activity, Silver-nanoparticle.

1. INTRODUCTION

Laccases (E.C.1.10.3.2, p-diphenol: dioxygen oxidoreductase) are a group of multi-copper containing enzymes that catalyze one-electron oxidation of phenolic compounds with concomitant reduction of oxygen to water [1]. They were first discovered in the lacquer of the Japanese tree *Toxicodendron vernicifluum* (formerly *Rhus venicifera*), from which the name laccase was taken. Later, it was shown that laccase enzymes were widely distributed in nature. The first bacterial laccase was found in the plant-root-associated bacterium, *Azospirillum lipoferum*. [2].

Laccase is one of the most actively investigated enzymes for the remediation of environmental pollutants. Laccase (p-diphenol: oxygen oxidoreductase), the most important number of lignolytic system, is multi copper enzyme belonging to the group of blue-copper proteins. Laccases are secreted by the family of fungi known as "white rots"[3]. Laccase is widely distributed in higher plants and fungi and has been found also in insects and bacteria. Recently a novel polyphenol oxidase with laccase like activity was mined from a metagenome expression library from bovine rumen Microflora [4].

Laccases find wide commercial applications within food industry, pulp and paper industries, textile industry, synthetic chemistry, cosmetics, soil bioremediation and biodegradation of environmental phenolic pollutants and removal of endocrine disruptors [1].

Laccase is generally found in higher plants and fungi but, recently it was found in some bacteria such as S. *lavendulae, S. cyaneus*, and *Marinomonas mediterranea*. In fungi, laccases appear more than the higher plants. Basidiomycetes such as *Phanerochaete chrysosporium, Theiophora terrestris*, and *Lenzites, betulina* and white-rot fungi such as *Phlebia radiate, Pleurotusostreatus* and *Trametes versicolour* also produce laccase. Many *Trichoderma species* such as *T. atroviride, T. harzianum, and T. longibrachiatum are* the sources of laccases. Laccase from the *Monocillium indicum* was the first laccase to be characterized from Ascomycetes which shows peroxidase activity. *Pycnoporus cinnabarin* produces laccase as ligninolytic enzyme while *Pycnoporus sanguineus* produces laccase as phenol oxidase. In plants, laccase plays a role in lignifications whereas in fungi it has been implicated in delignification, sporulation, pigment production, fruiting body formation, and plant pathogenesis [5].

The aim of present study is to isolate laccase producer from soil, study their optimization parameters and useful applications for society.

2. MATERIAL AND METHODS

2.1. Isolation, Screening and identification of laccase producing bacteria and fungi

Five soil samples were collected from garden area, industrial area, bio fertilizer soil, coconut root soil & lake area of the Aurangabad, Maharashtra, India. Initial isolation of bacteria and fungi were done from soil samples on nutrient agar and potato dextrose agar (PDA) by employing standard serial dilution method. The laccase producing fungi and bacteria were screened based on the growth on PDA media (at 30ºC for 3-4 days) and Luria Bertani (LB) media (at 37ºC for 24hrs.) respectively containing tannic acid as a substrate. PDA plates and LB plates were observed for growth and development of brown colored zone in plates containing tannic acid [6]. Most efficient laccase producing bacteria were identified by biochemical and morphological characteristic as prescribed by Bergey's Manual of systematic bacteriology volume-2(1984).

2.2. Laccase production (Bacteria and Fungi)

For laccase production, 2% bacterial inoculum of 0.1 O.D.600nm [7] and 2 discs 6mm disc of 48-72hrs old fungal culture were inoculated into 25 ml of production medium Luria-Bertani (LB broth) & Potato dextrose broth containing 1% (2mM) tannic acid as a substrate at 37ºC & 30ºC for 2/3 days on a rotary shaker at 100 rpm respectively [8]. Bacterial and fungal cells were removed by centrifugation under cooling condition (4ºC) at 10,000 rpm for 20 minutes and supernatant obtained was used as a crude laccase enzyme, and assay was carried out as per method [6] with some modifications. Tannic acid (2mM), in phosphate buffer (pH 7.0) was used as substrate. The reaction mixture contained 3ml acetate buffer, 1ml tannic acid and 1ml crude enzyme and one tube without enzyme as a control were incubated at 37ºC for 15min, then absorbance was measured at 450nm. One unit (U) of laccase was defined as a change in absorbance of 0.001min^{-1} [1].

2.3. Partial purification of laccase

Most proficient laccase producer bacterial cell free extract after centrifugation was subjected to precipitation by adding solid ammonium sulphate (40% and 70%) at 4ºC for 24 h. The precipitate obtained was dissolved in minimum amount of phosphate buffer (pH 7.5) dialyzed overnight against the same buffer and then concentrated on sucrose [9]. Laccase activity of each sample was analyzed and further preceeded for optimization.

2.4. Optimization parameters for laccase activity

2.4.1. Effect of pH, temperature, substrate and incubation period on laccase activity

To determine the effect of pH, the laccase activity was measured with 2mM tannic acid as a substrate dissolved in different buffers with varying pH range (pH 4.5 to 8). The buffer system used in present work as sodium acetate buffer (pH 4 to 5) phosphate buffer (pH 6 to 8) and effect of temperature was studied at different temperature range 15, 30, 37, 45, 55 and 65ºC by using 2mM substrate. Optimum substrate concentration for highest laccase activity was determined in phosphate buffer of pH 7.5 with varying substrate concentration 1mM, 2mM, 4mM, 6mM and 8mM tannic acid and effect of incubation period was studied by incubating the sample at different incubation periods from 5 min, 10min, 15 min, 20 min, 30 min, 40 min, 50 min. and 60 min and laccase activity was measured under standard assay condition as per protocol [6].

2.5. Effect of Metal-ions and Organic solvents

To determine the effect of metal-ions on laccase activity, the enzyme was incubated in presence of metal-ions such as 1mM sodium sulphate, EDTA, Magnesium sulphate, Mn_2SO_4, mercuric chloride, copper sulphate, 1% DMSO, zinc sulphate. Enzyme was removed after 30min. and optical density was measured at 540nm [10]. Laccase activity measured in the absence of any inhibitor or metal ions was taken as 100% relative activity.

2.6. Immobilization of laccase

For immobilization, 500mg sodium alginate was dissolved in 25ml hot water and allowed to cool, to it 2 gm. freeze dried laccase was added and homogenized slurry was made. Using dropper slurryd 2% $CaCl_2$ solution in drop wise manner was added. Beads of sodium alginate entrapped with laccase were formed and allowed to harden in 2% $CaCl_2$ for 1 hr. at 4ºC [11]. To check laccase activity in immobilized form, beads were removed from $CaCl_2$, washed 3-4 times with distilled water and preceeded for assay. 4-5 beads of immobilized laccase were added in 1ml phosphate buffer (pH 7.5) and preceeded as described earlier [6].

2.7. Dye Degradation

The ability of bacterial laccase to degrade dyes was analyzed by adding different concentrations of the laccase such as 0.5%, 1%, and 2% in 0.01gm/ml crystal violet, neutral red tubes and incubated at 30°C. Dyes degradation by laccase was checked by taking O.D. at 540nm after incubation periods 1day, 2days and 3days, compared with control (without laccase) [8].

2.8. Meat tenderization

2.8.1. Determination of Water-holding Capacity

Fresh boneless meat was sliced into several pieces of approximately the same size and weight. The meat was divided into two groups; one group was used for the experiment and the other used as control group. The experiment group consisted of 4 samples and the control group had one sample; C-Control (Sample without laccase), L1 -Sample treated with 1% laccase, L2-Sample treated with 2% laccase, L3-Sample treated with 3% laccase and L4-Sample treated with 4% laccase. 6 g samples of meat (treated with control, 1%, 2%, 3% and 4% of laccase) were stirred for 1 min with 10 ml of 0.6 M sodium chloride (NaCl) solution in a 15 ml centrifuge tube. The tube then was held at 4ºC for 15 min, stirred again for 1 min and centrifuged at 10,000 rpm at 4ºC for 15 min. After centrifugation, the volume of the supernatant was measured and the water-holding capacity of the meat treated with various concentrations of meat was calculated using the formula [12].

WHC (%) = (Initial volume -Volume of supernatant/ Initial volume) x 100

2.9. Silver nanoparticles synthesis

10ml of 1mM of silver nitrate was taken in test tube and 5-6 beads of immobilized laccase were added and incubated at 75ºC for 2-3 hrs and observed for color change, silver nanoparticles were washed by centrifugation at 10000 rpm for 10 min at 4ºC and used further for antibacterial activity [13].

2.10. Antibacterial activity

Antibacterial activity of laccase and silver nanoparticles produced by laccase against the pathogenic test organisms like *Agrobacterium tumificiens, S. aureus, E. coli, S.typhi* was evaluated by agar well diffusion method [14].

3. RESULT AND DISCUSSION

3.1. Isolation and identification of bacteria & fungi from soil samples

Bacteria (28) and 30 fungi were isolated on Luria Bertani agar and potato dextrose agar, respectively after 3-5 days of incubation from the soil samples collected from various different garden soils of Aurangabad, (Maharashtra). Out of the isolated bacteria and fungi, twenty-six bacteria and thirteen fungi were able to oxidize tannic acid present in the LB, PD broth medium respectively, development of brown colored surround-ding to colonies, indicates laccase producers (Fig 1). The most efficient laccase producing bacteria (3) and fungi (3) were further identified as per Bergey's Manual of systematic bacteriology volume-2(1984), VITEC, ABIS software bacteria were found to be *Erwinia chrysanthemi, Pseudomonas aeruginosa* and *Klebsiella Oxytoca* (Fig. 2) and by lacto phenol cotton blue staining, fungi were found to be as *Cladosporium spp*. and *Aspergillius spp.* Brown coloured precipitate development varied from organism to organism. Sagar Desai [8], isolated and characterized the laccase from *Enterobacter spp.* Gochev et al, [1] isolated 4 laccase producing *Trichoderma spp*. from soil sample, while Shraddha et al., [5] reported laccase production, from *S. lavendulae, S.cyaneus,* and *Marinomonas mediterranea.* Vladimir et al., [15] studied physiological regulation of laccase and manganese peroxidase production from *white rot basidomycetes.*

Fig. 1: Zone of Brown Colour due to laccase activity

Fig. 2: *Klebsiella Oxytoca*

3.2. Laccase Assay

After three days of incubation, supernatant obtained was subjected to laccase assay by using tannic acid as a substrate. Out of twenty-six isolates, three isolates showed highest laccase activity. The isolate *Erwinia chrysanthemi* produce 680U/ml, *Pseudomonas aeruginosa* 720U/ml, *Klebsiella Oxytoca* 980 U/ml. showed laccase production after three days of incubation at 37ºC. Fungal isolates *Cladosporium spp*. and two *Aspergillus spp*. produced laccase 676 & 719 U/ml, respectively. *Klebsiella Oxytoca* showed maximum laccase activity i.e.980 U/L, *B. subtilis (*MTCC 2414) laccase showed maximum activity (267±2.64 U/mL) after 96 h [16], while *Pseudomonas putida (*MTCC 7525) produced (94.10 U/ml) at 30ºC and pH 8 after 108 h incubation [10]. In the present study, isolate showed efficient laccase production than the earlier reports cited. The most efficient reproducible result was shown by *Klebsiella oxytoca*, hence for further optimization study *Klebsiella Oxytoca* was used.

3.3. Optimization parameters for laccase activity

Optimization studies include effect of pH, temperature, substrate and incubation period on laccase activity. All the tests were run in triplicates. The effect of pH on laccase activity was studied at pH range of 4.5 to 8, in present study. Laccase from Klebsiella *Oxytoca showed* laccase activity at all pH, optimum laccase activity was at pH 7.5 (978U/ml) and lowest activity at pH 4.5 (680U/ml) (Fig. 3) after 30 min of incubation, which indicate laccase activity was present in the slightly alkaline medium. Similar result was shown by Ambrin et al., [3] in their work, *P. ostreatus* showed laccase activity over a wide range of pH 5-11. Highest laccase activity of *P. putida* was found at pH 8 within 96 h at 40ºC [10]. Temperature is a parameter which is important for enzyme stability. Laccase shows highest activity at 30ºC (980 U/ml) (Fig.4) and lowest at the 15ºC (193U/ml) (Fig.4).

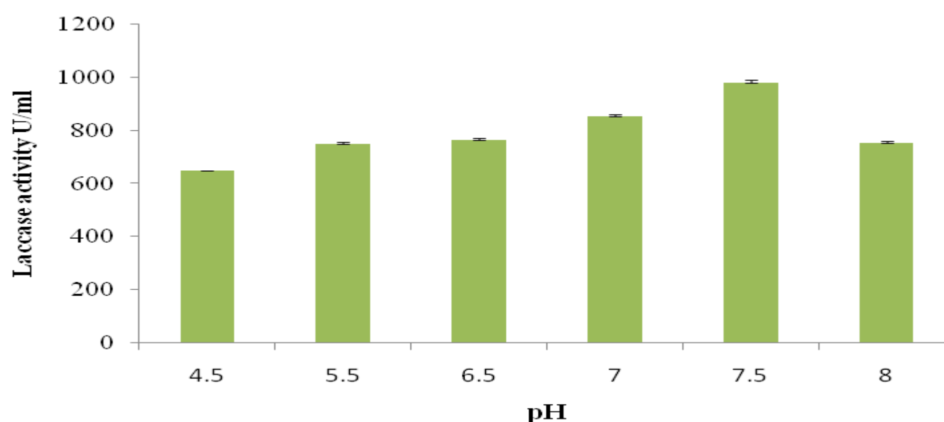

Fig. 3: Effect of pH on Laccase activity

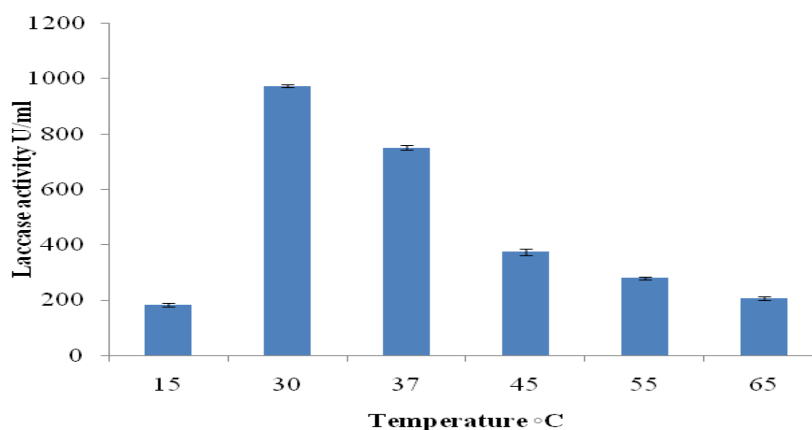

Fig. 4: Effect of temperature on Laccase activity

Sagar Desai [8] observed maximum laccase production at 30ºC temperature by *Enterobacter spp.* while Ambrin et al, [3] in his study reported that laccase from *P. ostreatus* have highest laccase activity at 50ºC.

The effect of substrate concentration on laccase production using *B. subtilis* (MTCC 2414) revealed that rice bran and wheat bran showed the maximum enzyme activity of 134.8±4.75 U/mL and 117.6±4.23 U/mL, respectively [16].

In the incubation parameter, laccase was subjected to a different incubation period, maximum laccase activity was shown by *Klebsiella Oxytoca* at 30 min (960 U/ml) (Fig.6) while laccase from *Bacillus subtilis* (MTCC 2414) showed maximum enzyme activity (267±2.64 U/mL) at 96 h [16].

All the parameters were studied in triplicate (Values are mean±SD of three independent determinations).

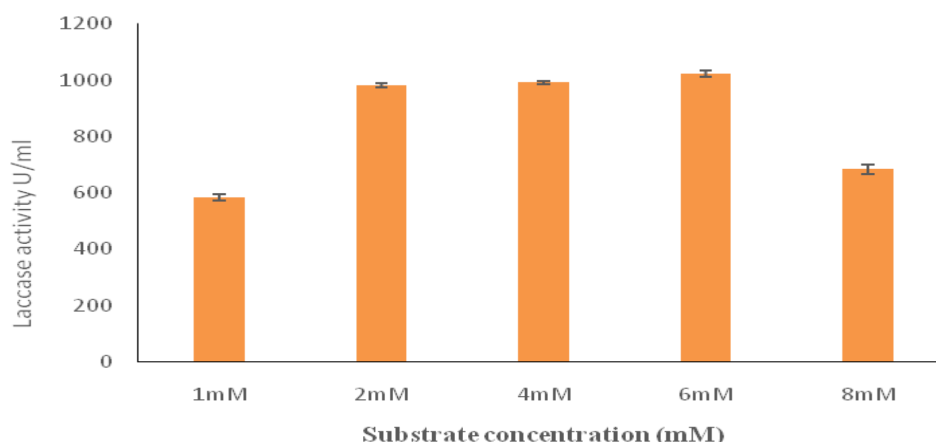

Fig. 5: Effect of substrate concentration on Laccase activity

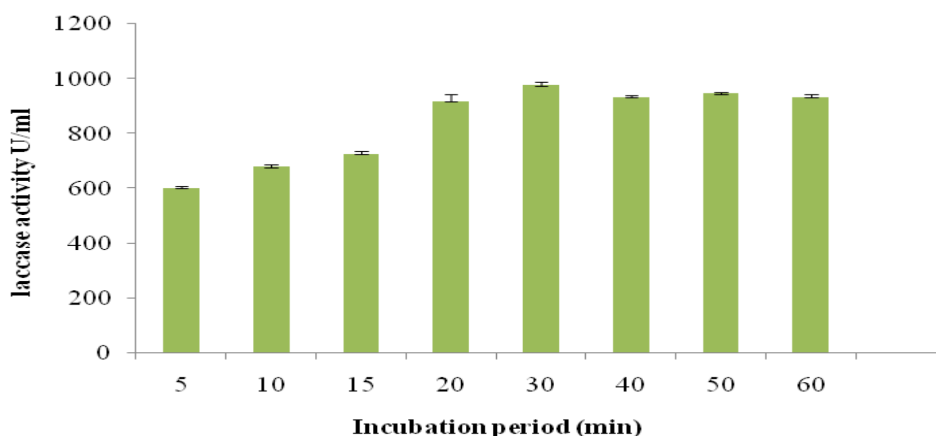

Fig. 6: Effect of incubation period on laccase activity

3.4. Effect of metal ions and organic solvent

The laccase activity was strongly inhibited by PMSF, which are well-known inhibitors of serine proteases. This suggested that a serine was involved in the catalytic activity [17]. In the presence of Cysteine, $MnSO_4$ and $CuSO_4$ laccase activity was enhanced 40%, 135%, 119% respectively, while DMSO, $ZnSO_4$, act as potent inhibitors, inhibiting 94% and 95% activity, respectively. Some of some metal ions show negligible effect on laccase activity such as $HgCl_2$, Na_2SO_4 (Fig. 7). Kuddus et al., [10] reported that Hg^{2+} ion show inhibitory effect at 10 mM conc. retaining 27% activity while pCMB show complete inhibition at 10 mM. Metal ion $CuSO_4$ enhanced maximum laccase activity of 141.4±6.64 U/mL followed by $FeSO_4$ (123.5±4.59 U/mL). But, $MgSO_4$, $CuCl_2$, and $HgCl_2$ were found to inhibit the laccase activity to a greater extent [16].

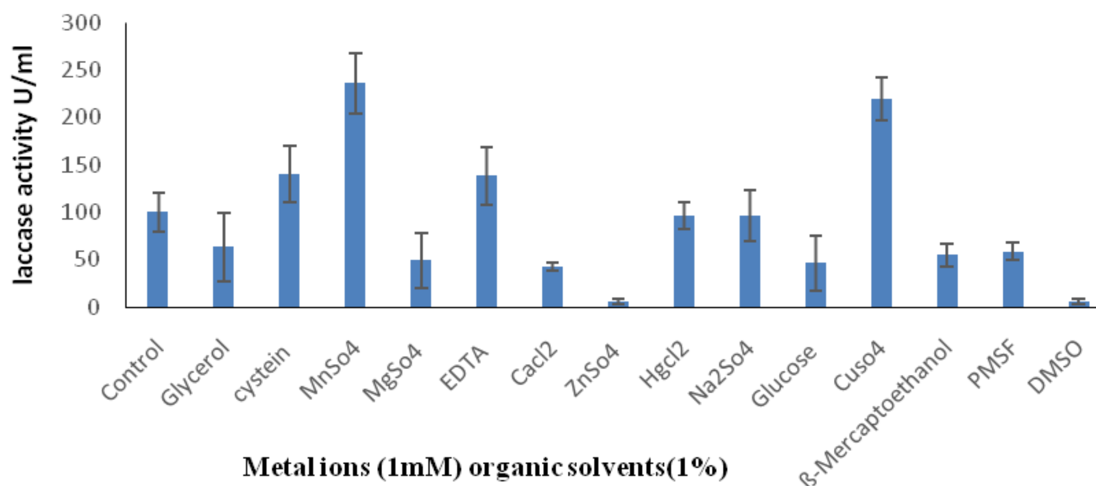

Fig. 7: Effect of metal ions and organic solvent on laccase activity

3.5. Immobilization of laccase

Laccase immobilization was done by the sodium alginate method (Fig. 5). After immobilization, laccase activity was found to be 784±12 U/ml which was found to be reduced as compared to the free enzyme 976±10 U/ml. This immobilized laccase was used in the dye degradation, silver nano particle synthesis, same was done by Zabin et al., [18], in their work they used calcium alginate beads, copper alginate beads, calcium alginate-chitosan beads and sol-gel matrix.

Fig. 8: Immobilized Enzyme

3.6. Dye Degradation

Dye degradation was observed by visual observation. Degradation of dye was carried out by crude extract, partially purified and immobilized laccase. In the present study, partially purified laccase from *Klebsiella Oxytoca* showed maximum degradation activity as compared to others. The best degradation by laccase was shown against the Crystal violet and Neutral red (Fig. 9). The dye degradation was clearly seen after incubation period and the optical density also decreases with respect to the incubation periods (Fig.10). 60% crystal violet, 88% neutral red were degraded after 24 hrs . (First day), as incubation increases degradation was found to be increasing and after three days of incubation 94% and 97% dyes degraded respectively (Fig.10). De-colorization of dye (Bromophenol Blue, Congo red, Brilliant green, Crystal violet) and effluents (Paper effluent A, Paper effluent B and Textile effluent) by using laccase isolated from *P. putida*, [10]. Similarly, dye degradation by using laccase extracted from *Pleurotus Ostreatus* was done on 2 dyes Red F3B, T Blue G [3].

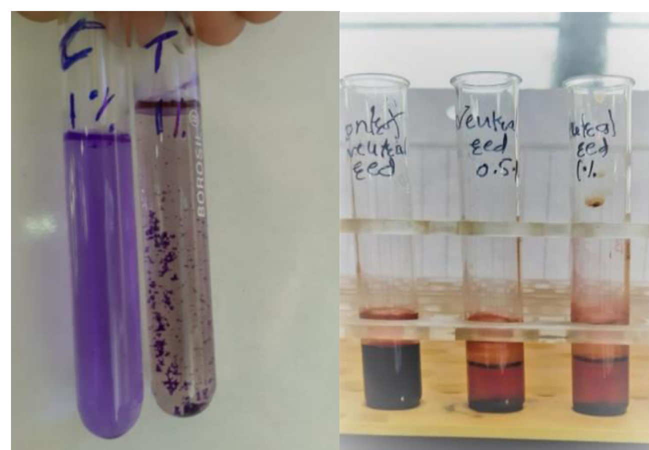

Fig. 9: Crystal violet and Neutral red degradation by laccase

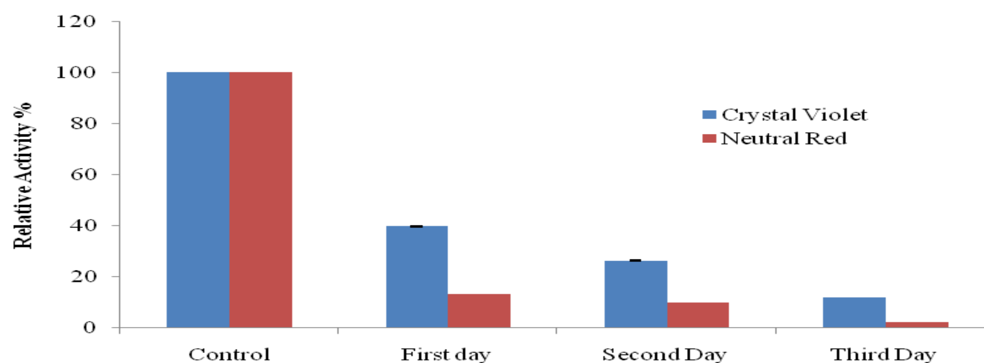

Fig. 10: Dye degradation by Laccase

3.7. Meat Tenderization by laccase

The water holding capacity (WHC) of meat is very important since physical properties such as colour, texture and firmness are partially dependent on the WHC. The water-holding capacity of the meat treated with various concentrations of laccase was calculated using the formula (Table 1) [12]. As compared to control, WHC capacity was found to be increased in presence of laccase. 1% the concentration of laccase showed highest WHC.

3.8. Silver nanoparticle synthesis

On adding, 4-5 immobilized laccase to the 1mM of silver nitrate solution, color of reaction mixture was found to be changed from colorless to reddish brown after an incubation of 2hr. at 75°C indicates the enzymosynthesis of silver nanoparticles. Biosynthesis of silver nanoparticles was confirmed by UV-Vis spectra, showing the highest absorption peak at 410nm and further used as an antibacterial agent against *S. aureus, S. typhi, Agrobacterium tumefaciens, E. coli*, *Pseudomonas*. Nelson et al., [19] in their work reported same production of silver nanoparticle using laccase from *Trametes versicolor* and characterized the nanoparticle by UV-Visible spectra in the range of 440-600nm and XRD.

3.9. Antibacterial activity

Antibacterial activity of laccase and biosynthesized silver nanoparticles was tested against *S. aureus S. typhi, Agrobacterium tumefaciens, E. coli*, *Pseudomonas*, by agar well diffusion method. Laccase was significantly shown to have antibacterial activity against bacterial isolates *S. aureus, S. typhi, Agrobacterium tumefaciens* observed by the clear zone of inhibition which ranges from 17 nm to 24 mm Highest zone of inhibition was 24mm which was against *Agrobacterium* and lowest against the *E.coli* (17mm), while Agbaje et al., [20] reported that, the AgNPs showed selective antimicrobial activities against the ten clinical bacterial isolates tested. AgNPs synthesized induced a maximum inhibitory zone of 20 mm at a concentration of 141μg/ml for AgNPs. Therefore, the appreciable antibacterial property demonstrated by the biosynthesized AgNPs.

Table 1: Meat tenderization

Samples (concentration)	Initial volume (ml)	Volume of supernatant (ml)	Water holding capacity (%)
Control	**7.5**	**5.4**	**28**
1%	**7.5**	**4.3**	**42**
2%	**7.5**	**4.5**	**40**
3%	**7.5**	**4.7**	**37**
4%	**7.5**	**4.8**	**36**

4. CONCLUSION

In the present study, purified enzyme from *Klebsiella Oxytoca* using Tannic acid as substrate showed maximum laccase activity (980 U/ml) and stability at temperature 30°C, pH 7.6, and 6mM substrate concentration in 30 min incubation period. Partially purified laccase efficiently degraded 94% of crystal violet and 97% of neutral red was degraded after 3 days of incubation at 37°C. Silver nanoparticles were also enzymosynthesized. It also has proficient antibacterial activity against *S. aureus, S. typhi,* and *Agrobacterium tumifaciens* ranging from 17mm-24mm and can be effectively used in meat tenderization. To the best of our knowledge, this is the first report on bacteria *Klebsiella Oxytoca* producing laccase and its use for decolorization of synthetic dyes.

Therefore, this strain can be used to decolorize and detoxify the industrial effluents and help in wastewater treatment.

5. ACKNOWLEDGEMENTS

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Conflict of interest

None declared

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