Production Optimization and Partial Purification of Laccases from Bacterial Consortium

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Abstract- Laccase belongs to the family of blue multi-copper oxidases that has three domain structures and usually contains four copper atoms. An attempt was made to screen, optimize, production and partially purify laccase enzyme produced from consortium of laccase producing Pseudomonas aeruginosa and Pseudomonas fluorescens. Guaiacol and acetate buffer were used to assay laccase production. Laccase activity was highest when operated at the following conditions, 72 h incubation, 40°C temperature, and pH-7, 2% Glucose as carbon source and 2% peptone as nitrogen source in the production medium. The enzyme was partially purified by ammonium sulfate precipitation and dialysis method.

Key words: Laccase, Pseudomonas aeruginosa, Pseudomonas fluorescens, enzyme activity, optimization

I. INTRODUCTION

accases are the most numerous members of the multi-copper protein family, which also includes tyrosinases, monoxygenases, and dioxygenases. Phylogenetically, these enzymes have developed from small sized prokaryotic azurins to eukaryotic plasma proteins ceruloplasmin (Claus 2003). They contain four histidine-rich copper-binding domains, which coordinate the types 1–3 copper atoms that large number of studies have been produced on the mechanisms of oxidation of non-phenolic substrates (Baiocco *et al.*, 2003). The use of naturally occurring differ in their environment and spectroscopic properties (Messerschmidt and Huber, 1990). The bacterial azurins, *e.g.*, crystallized rusticyanin from *Thiobacillus ferrooxidans*, which contain only type 1 copper, can be regarded as the precursor protein of laccases (Hough *et al*. 2001). Laccases are the model enzymes for multi-copper oxidases and participate in (1) cross-linking of monomers, (2) degradation of polymers, and (3) ring cleavage of aromatic compounds (Kawai *et al.* 1988). For catalyzing the oxidation of non-phenolic substrates, laccase requires the presence of a mediator in the medium. A mediator is a small molecule that behaves like an ‘electron between laccase and substrate and these small molecular-mass compounds are converted into sFig: radicals by means of enzymatic oxidation. They act as redox mediators and oxidize other compounds that, in principle, are not substrates of laccase. From the description of the first laccase mediator, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) to more recent use of the—NOH-type synthetic mediator, including 1-hydroxybenzotriazole (HBT), violuric acid (VLA) and N-hydroxyacetanilide (NHA), a

advantage (Camarero *et al*., 2005). The enzyme possesses great biotechnological potential because of its wide reaction capabilities as well as broad substrate specificity. Promising applications include biosensors for drug analysis and phenols in tea (Ghindilis *et al.* 1992; Peter and Wollenberger, mediators would present environmental and economic

1997), polymer synthesis (Huttermann *et al.* 2001), textile-dye bleaching (Claus *et al.*, 2002), bioremediation (Murugesan 2003; Wesenberg *et al.*, 2003), fungicidals (Spillman, 2003) pulp bleaching (Palonen and Viikari, 2004), clarification of juices and wines (Ygshinwa, 2004).

Laccases is a multicopper blue oxidase capable of oxidizing ortho- and para-diphenols and aromatic amines by removing an electron and a proton from a hydroxyl group to form radicals. However, during the last few years of work on laccase applications include development of oxygen cathode in biofuel cells (Barton *et al.,* 2001), biobleaching of kraft pulp (Srebotnik and Hammel, 2000), decolorization of synthetic dyes (Baldrain, 2006), organic synthesis (Pilz *et al*., 2003), laundary cleaning (Gouka *et al*., 2001), bioremediation (Mayer and Staples, 2002; Wu *et al.*, 2008), biosensors (Vianello *et al.,* 2006), labelling in immunoassays, drug analysis, clarification of juices and wines, design of laccase fungicidal and bactericidal preparations (Jahansen, 1996).

II. MATERIALS AND METHODS

Procurement of bacterial culture

Baceterial cultures of *Pseudomonas auruginosa* and *Pseudomonas fluorescens* was procured from the Microbial Culture Collection Bank (MCCB), Department of Microbiology and Fermentation Technology (MBFT), Jacob School of Biotechnology and Bioengineering (JSBB), Sam Higginbottom Institute of Agriculture Technology and Sciences (SHIATS).

Maintenance of bacterial culture

The cultures of *Pseudomonas auruginosa* and *Pseudomonas fluorescens* were routinely subcultured and maintained in Nutrient agar (Peptone: 5g, Beef extract: 3g, Sodium chloride 5g, Agar-agr: 20g, pH: 7.2, distilled water: 1000ml.) slants, incubated at 28°C for 24-48h.

Screening of Pseudomonas aeruginosa and Pseudomonas fluorescens for Laccase production

Cultures of Pseudomonas aeruginosa and *Pseudomonas fluorescens* and their consortia were screened for production of lacasses enzyme on Nutrient agar plates supplemented with 1% guiacol. The bacterial cultures and consortia were streak inoculated on the plate and incubated for 4-5days at 28 ºC. An uninoculated plate was used to check contamination. Appearance of red-brown colouration of colony indicated positive lacasse production by the bacteria and consortia.

Preparation of consortia of Pseudomonas aeruginosa and Pseudomonas fluorescens for Laccase production

A consortium was prepared in Nutrient broth medium seeded with equal aliquots of overnight grown broth culture of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens incubated* at 28˚C for 24 hours. The grown cultures were tested for any contamination throufh pour plate technique. The broth was then used as seed or starter culture for laccase enzyme production.

Optimization of culture conditions for enzyme production (Ding et al., 2012)

Varying parameters were analyzed for the optimization of laccase production by the consortia *viz.* influence of temperature, pH, incubation time, carbon source in medium, nitrogen source in the medium. Influences of these parameters were used to determine the lacasse activity of the consortia growing in the liquid submerged production medium. Laccase activity was determined at 530 nm. Uninoculated medium served as media control.

Effect of temperature

The effect of temperature on laccase production was determined at varying temperatures *viz.* 20, 30, 40 and 50ºC maintained during the incubation periodof laccase production by the consortia. To 100 ml production medium (pH: 7), 1% inoculum of consortia was added and incubated for 24-48h. Laccase activity was determined at 530 nm. An inoculated medium served as media control. Thereafter, lacasse activity was measured at 530nm.

Effect of pH

Effect of pH on laccase production by the consortia was determined at optimized temperature for 24-48 h while varying the pH values of the medium *viz.* 5, 7, 9 and 12. Laccase activity was determined at 530 nm. Uninoculated medium was served as media control.

Effect of incubation period

Effect of incubation time on laccase production was evaluated at optimized temperature and pH. Lacasse production flasks were subjected to different incubation time *viz.* 0, 24, 48, 72, 96 and 120 h. Laccase activity was determined at 530 nm. Uninoculated medium was used as media control.

Effect of carbon source

Effect of carbon sources on laccase production was done using 100 ml production medium supplemented with 2% different carbon sources *viz.* Glucose, Sucrose, Mannitol, Maltose prepared in Erlenmeyer flask (250 ml) at optimized pH, temperature (ºC) and incubation time (h). The production media was inoculated with 1% inoculum. Laccase activity was determined at 530 nm. Uninoculated medium served as media control.

Effect of nitrogen source

effect of nitrogen sources on laccase production was determined using 100 ml production medium supplemented with optimized carbon source and 2% varying nitrogen sources *viz.* Peptone, Ammonium chloride, Sodium nitrate, Ammonium sulfate prepared in Erlenmeyer flask (250 ml) with optimized pH and autoclaved. The production media was inoculated with 1% inoculum of consortia followed by incubation at optimized temperature and time interval. Laccase activity was determined at 530 nm. Uninoculated medium was served as media control.

Laccase production and partial purification

Consortia was developed and was transferred to 100 ml sterile production media containing 2% optimized carbon and nitrogen source was prepared with optimized pH according to the composition given by Unyayar *et al.,* (2005) and incubated at 30ºC (120 rpm). A sterilized and inoculated with 5% seed culture (v/v) and was incubated at optimized temperature for optimized time interval. After incubation the cells were harvested by centrifugation at 10,000 rpm at 4ºC for 10 min. The clear supernatant was filtered through Whatman No.1 filter paper. The clear supernatant was stored at 4ºC and used for purification. The laccase purification was performed according to the protocol described by (Barda and David, 1949).

Determination of Laccase activity

Laccase activity was determined by measuring the oxidation of guaiacol at 530 nm. The reaction mixture was containing 10 mM guaiacol and 100 mM acetate buffer (pH 5). Absorbance for blank was measured at 470 nm while that of test samples was measured at 530 nm. The change in the absorbance of the reaction mixture with guaiacol was monitored for 10 min of incubation. Enzyme activity was measured in U/ml which is defined as the amount of enzyme catalyzing the production of one micromole of coloured product for min per ml (Jhadav *et al.,* 2009). Protein concentration was determined by the method of (Lowery *et al.,* 1951) with bovine serum albumin.

The following formula was used for determination of enzyme activity.

$$\text{Enzyme activity (u/ml)} = \frac{\Delta A_{530}\ \text{nm/min} \times V_t \times \text{dilution factor}}{€ \times V_s}$$

Where,

Vt = final volume of reaction mixture (ml)

Vs = sample volume (ml)

€ = extintion co-efficient of guaiacol = 6740/M/cm.

III. RESULTS AND DISCUSSION

Lacasse assay for screening of consortia

The consortia showed a positive lacasse assay on Nutrient agar medium supplemented with 1% guaiacol after incubation for 48h (Plate:1).

Optimization of culture conditions for laccase production:

The highest laccase activity was observed at 40ºC (0.0388 U/ml) while slight decrease in enzyme activity was observed at 50ºC (0.0382 U/ml). (Fig: 1). Highest laccase activity was observed at pH 7 (0.0341 U/ml) while lowest laccase activity was found at pH 12 (0.0287 U/ml), (Fig: 2). A gradual increase in the enzyme activity from 24 h towards 72 h was observed. After 72 h of incubation time, a decrease in the trend of enzyme activity was observed. Overall the highest laccase activity was observed at 72 h (0.049 U/ml) (Fig: 3). Overall the highest laccase activity was observed with glucose (Fig: 4). Overall the highest laccase activity was observed with peptone. (Fig: 5).

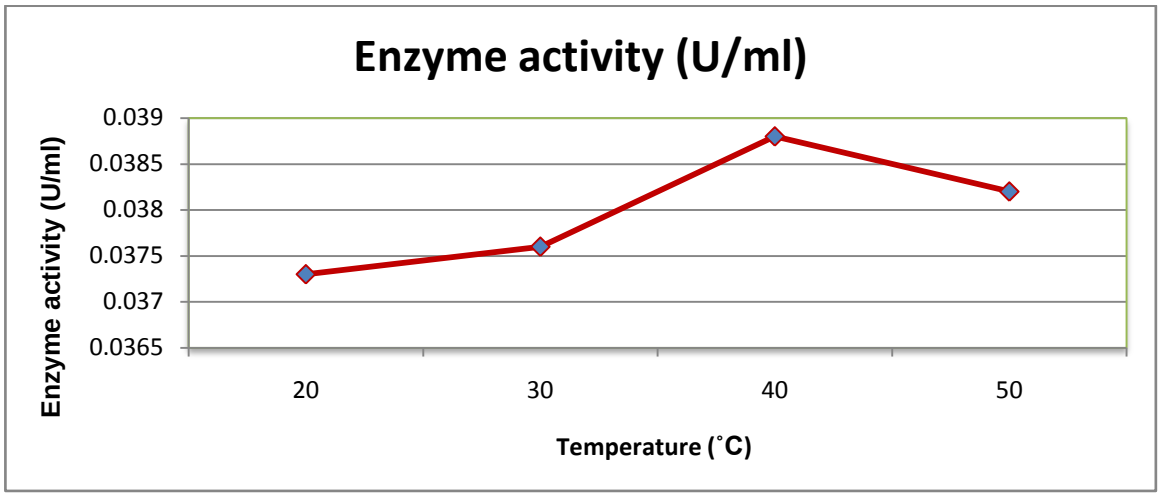

Fig: 1 Effect of temperature on laccase activity

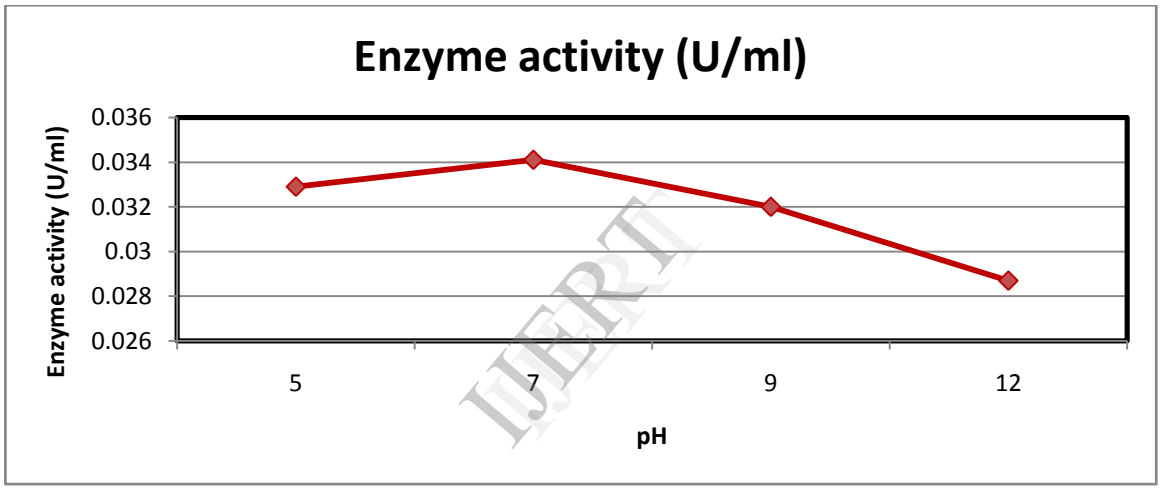

Fig: 2 Effect of pH on laccase activity

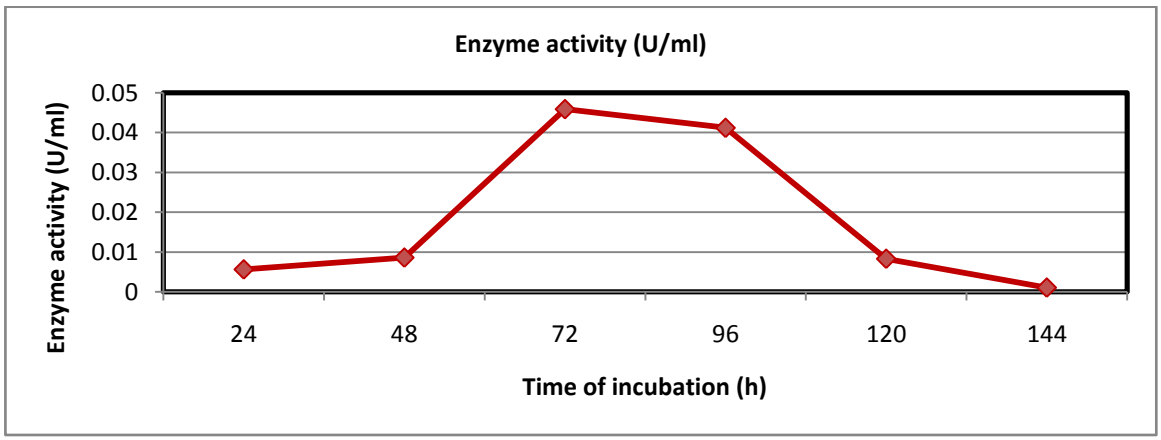

Fig: 3 Effect of incubation time on laccase activity

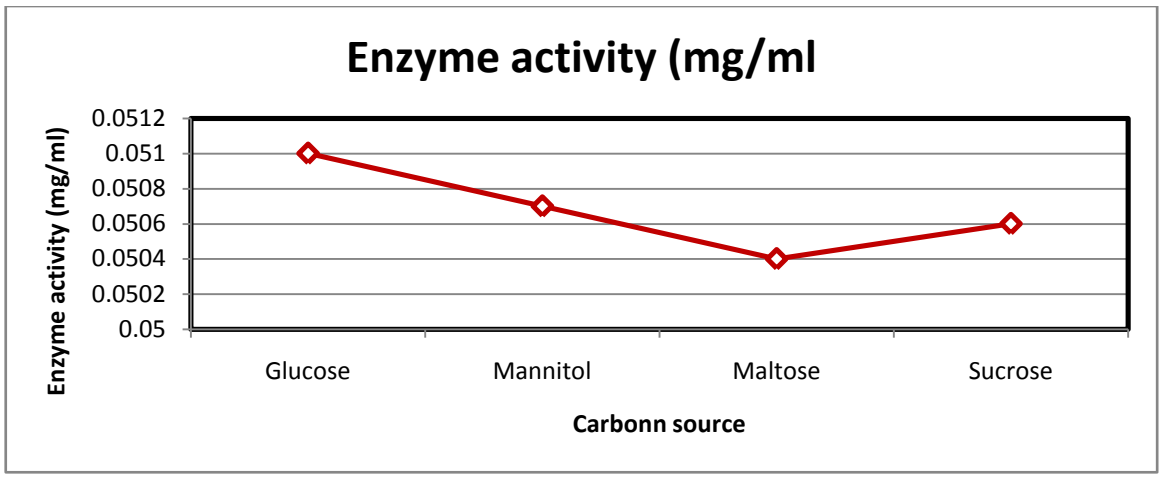

Fig: 4 Effect of carbon source (2%) on laccase activity

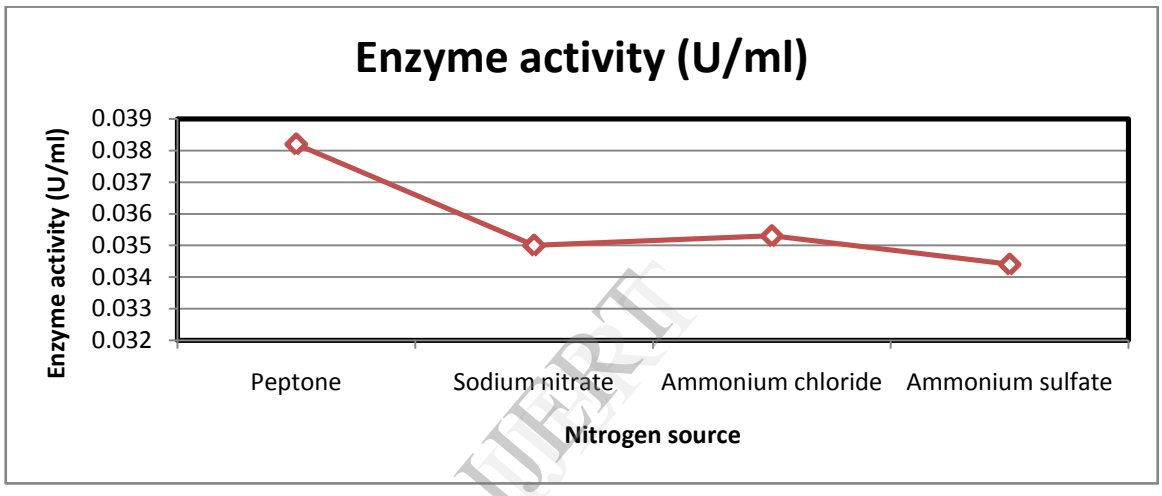

Fig: 5 Effect of nitrogen source (2%) on laccase activity

Laccase activity of partially purified lacasses from consortia

Culture filtrate was obtained from the medium. Partial purification of laccase was carried by two method ammonium sulfate and dialysis. The crude enzyme was precipitated by ammonium sulfate precipitation up to 70% saturation with a total activity of 0.059 U/ml with 0.82 mg/ml of protein. After ammonium sulfate precipitation, the final purification by dialysis the fraction showed 0.028U/ml enzyme activity 0.55 mg/ml protein. As in present study, similar laccase activity was reported by several workers (Abou-Mansour *et al.* 2009; Jhadav *et al.* 2009) and purification of laccase enzyme was done by using ammonium sulfate and dialysis method.

IV. DISCUSSION

Rich *et al*., 2013 explained the laccase thermostability. Laccase from *A. pullulans* strain NRRL Y-2568 exhibited a temperature optimum of 35–45 ◦C, while strains from clade 5 showed optima of 50–60 ◦C. Laccases from strains in clade 5 were further compared with those from the lignindegrading fungi *T. versicolor* and *P. cinnabarinus* for temperature stability. Laccases from *A. pullulans* retained at least 90% of maximal activities for 1 h at 50˚C, while those from *T. versicolor* and *P. cinnabarinus* lost 90% of their maximal activities under these conditions. Thus, laccases from *A. pullulans* appear to be relatively thermostable. Jordan *et al*., 2009 reported improved stability was observed for both spherezyme preparations over that of the free laccase. The most pronounced improvement in thermal stability was noted for PEI coated spherezymes. The non-immobilised Denilite® laccase was stable from neutral to alkaline pH during the time course of the experiment (not shown), while limited stability was observed at acidic pH. Difficulties in assays at pH greater than 6

were encountered for the spherezyme preparations as the particles demonstrated agglomeration under these conditions, and this resulted in nonhomogenous oxidation of the substrates. This agglomeration appeared to be time dependant and may be due to electrostatic bridging by the buffer used for the assay. The improved physical mixing of the solutions possible at larger volumes (10 ml) alleviated this problem. Cai *et al*., 2010 revealed the laccases stability at wide range of ph and temperature. The optimal pH values were observed in the acidic region (from 2.5 to 5.5), and the optimal temperatures were almost above 50˚C. LacI and LacII were observed to have a broad reaction temperature range (0–80˚C). Incubation temperature plays an important role in the metabolic activities of microorganisms. Increase and decrease in temperature lead to the gradual decrease in protein products. In the present in investigation there was a gradual increase in enzyme activity from 20-40°C and at 50°C the enzyme activity decreases. pH is one of the important factor for the growth and morphology of microorganisms, they are sensitive to the concentration of hydrogen ion present in the medium. This may be attributed to the change in pH may alter the three-dimensional structure of the enzyme. Since each substrate supported a particular pH for maximum enzyme secretion and the enzyme instability at very high or very low pH. The incubation time plays an important role in the growth of microorganisms and enzyme secretion. Enzyme production increases with time till 72 h after that enzyme production decreased due to depletion of macro and micronutrients in the production medium. It has been reported that the carbon source is the most important factor in laccase production, and that the addition for sui amount of other sugar to the culture media has a benign influence on laccase synthesis. Medium containing glucose showed the highest laccase activity as enzymes are substrate specific. Since glucose is a monosaccharide which is easily broken down is utilized by the microorganism. It has already been demonstrated that substrates that are efficiently and rapidly utilized by the organism results in high levels of laccase activity. Nitrogen plays key role in laccase production, while the organic nitrogen source gave high laccase yields. Medium containing peptone showed the highest laccase activity as enzymes are substrate specific. Peptone is the simplified source of protein and can be readily uptake by the microorganism. It is a source of protein, amino acids for microbial growth. It has already been demonstrated that substrates that are efficiently and rapidly utilized by the organism results in high levels of laccase activity. Jordaan *et al.*, 2009 showed improved chemical and physical stability of laccase considering various influencing parameters *viz*. temperature and pH. High thermostability, alkaline pH optimum and halotolerance are properties that distinguish prokaryotic laccases from eukaryotic laccases, which adds to their potential as robust industrial biocatalysts. *T. thermophilum* laccase has optimum activity at 92˚C, with a half-life of 14 h at 80˚C and retains 60% activity after incubation for 10 min at 100˚C. McoA from *A. aeolicus* has optimum activity at 75˚C and retains activity at 85˚C for 9 h and 90˚C for 5 h. McoP from *P. aerophilum* is optimal at 85 8C with a half-life of inactivation of 6 h at 80˚C. CotA from *B. subtilis* has maximum activity at 75 ˚C; at 80˚C, wild type CotA has a half-life of 4 h whereas recombinant CotA from E. coli has a half-life of 2 h. Other thermostable bacterial laccases include those from *S. cyaneus* (Topt = 70˚C) and laccases from *S. coelicolor*, *S. ipomoea*, *B. sphaericus*, and the bovine rumen, all having optimal activity at 60˚C. The majority of bacterial laccases have optimal activity on SGZ in the pH range 5.5–8.4; for fungal laccases the optimum pH lies within the range 3.5–5.0. Activity on ABTS is optimum within the pH range 4–6 for bacterial laccases; *S. psammoticus* has optimum activity at an exceptionally high pH of 8.5; most fungal laccases show a lower pH optimum (pH 2–5). High pH stability has been demonstrated in bacterial laccases from *S. ipomoea* (pH 7) and *S. cyaneus* (pH 5–8). Spores of *Bacillus* SF had a half-life of 120 h over the pH range 5–7, compared with that of 13 h of the fungal laccase from *T. hirsuta* at pH 4.5. *S. psammoticus* laccase has 40% higher activity in 0.8 M NaCl and retains ABTS activity up to concentrations of 1.2 M NaCl. *S. ipomoea* laccase retains 100% activity in 1 M NaCl at pH 8.0 and 94% activity at pH 8.0 in the presence of 10 mM sodium azide. Stimulation of activity in 0.1 M NaCl has been observed for laccases from *S. maltophilia* and *B. halodurans*. Halotolerance of fungal laccases has rarely been reported except in the case of *Flavodon flavus*, a marine fungus.

V. CONCLUSION

Conclusively, optimized laccase production was achieved at 40˚C at pH 7.0 with incubation time of 72 h in the Laccase production medium supplemented with 2% Glucose (C-source) and 2% peptone as nitrogen source. The partially purified laccase from the consortium had 0.55mg/ml proten contentthat showed 0.059 U/ml laccase activity. The consortium yielded a high amount of lacasse at optimized condition. Further purification of laccase could be done to know the accurate laccase production and activity. The purified laccase is applicable for dye decolourization, could be used as a bactericidal and fungicidal agent, for laundry cleaning purpose, for immunoassays, drug analysis and many more.

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