APPLICATION OF PARTIALLY PURIFIED LACCASES FROM *PSEUDOMONAS FLUORESCENS* ON DYE DECOLOURIZATION

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ABSTRACT

The present work focuses on screening, optimizing the process parameters to achieve the maximum production of extracellular laccases and application of laccase on dye decolourization. Guaiacol was used as substrate for screening of laccase activity. The optimization study revealed that the laccase activity was highest when operated at the following conditions, 72 h incubation, 40°C temperature, pH-7, 2% glucose as carbon source and 2% sodium nitrate as nitrogen source in the production medium. Percentage of decolourization was observed to be 36.09% of congo red dye at 96 h. Laccase enzyme produced by Pseudomonas fluorescens is capable of decolourizing congo red dye and can be used in controlling environmental pollution.

Keywords: Laccase, Pseudomonas Fluorescens, Enzyme Activity, Optimization, Dye Decolourization

I. INTRODUCTION

Laccases (E C 1.10.3.2) are multicopper enzymes belonging to the group of blue oxidases. Structurally, laccase belongs to the multi-copper oxidase (MCOS) that have three domain structure and usually contain four copper atoms. A great number of dyes are used in textile industry. Among all the dyestuff, the azo dye congo red is considered the most used in industry, representing 70% of total dyes produced per year (Jadhav *et al.,* 2009). Bacteria and fungi are widely used for decolorization of textile dyestuff and textile industry wastewater. The biodegradation ability of bacteria is associated with its intracellular and extracellular oxidoreductive enzyme system such as laccase, azoreductase and NADH-DCIP-reductase (Telke *et al.,* 2009). Laccase-dye system is undoubtedly one of the most promising alternative methods for the treatment of harmful azo dyes.

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), biosensors (Vianello *et al.,* 2006), labelling in immunoassays, drug analysis, clarification of juices and wines, design of laccase fungicidal and bactericidal preparations (Jahansen, 1996).

Research on laccases has intensified in recent years, with particular focus on their catalytic versatility and oxidative activity in the absence of any requirement for additional reactive compounds (in contrast to peroxidases). In addition,

the possibility to clone these enzymes and to expand their range of oxidation through redox mediators offers considerable biotechnological potential. The use of low-molecular weight compounds as mediators has particular merit because once they are oxidized by laccases to stable radicals, these radicals may continue oxidizing other compounds, including those not used directly as substrates by the enzyme. The application of laccases to the textile industry is particularly important. In fact, 90% of reactive textile dyes entering activated sludge sewage treatment plants pass through unchanged and are discharged into rivers (Abadulla *et al.*, 2000). Although several combined oxic and anoxic treatments have been reported to enhance the degradation of textile dyes, the generation of carcinogenic amines from azo dyes (the most widely used dyes in the industry) through anoxic processes poses a serious health hazard. In addition, the colored industrial effluents significantly reduce oxygen solubility in receiving waters and are thus an important environmental hazard. Laccases and laccase-mediator systems therefore offer the potential to oxidatively degrade a wide range of aromatic compounds, providing an alternative to conventional treatments (Kandelbauer and Gübitz, 2005).

II. MATERIAL AND METHODS

2.1 Screening for laccase production

Laccase production by *Pseudomonas fluorescens* was confirmed using a Nutrient agar media containing 0.01% guaiacol as indicator compound. A reddish brown colour zone was observed around the colonies after incubation at 30ºC for 7 days.

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

2.2.1 Effect of temperature

The effect of temperature on laccase production, the production medium was inoculated with 1 ml culture of *Pseudomonas fluorescens* and incubated at temperatures *viz.* 20, 30, 40 and 50ºC for 24-48 h with pH 7. Laccase assay was measured at 530 nm.

2.2.2 Effect of pH

The effect of pH on laccase production was carried out by incubating the flask containing 100 ml production media inoculated with 1 ml culture of *Pseudomonas fluorescens* at different pH 5, 7, 9 and 12 for 24-48 h. Laccase assay was measured at 530 nm.

2.2.3 Effect of incubation period

In order to find the optimal time of incubation for the maximum laccase production, 100 ml production medium was prepared with optimized pH and inoculated with 1 ml culture of *Pseudomonas fluorescens* and incubated at optimized temperature for 24-48 h. The sample was withdrawn at time intervals *viz.* 0, 24, 48, 72, 96 and 120 h each time. Laccase assay was measured at 530 nm.

2.2.4 Effect of carbon source

In order to find the suitable carbon source on laccase production, 100 ml production medium supplemented with 2% different carbon sources *viz.* glucose, sucrose, mannitol, maltose was prepared with optimized pH and autoclaved. The production media was inoculated with 1 ml culture of *Pseudomonas fluorescens* followed by incubation at optimized temperature at optimized time interval. Laccase assay was measured at 530 nm.

2.2.5 Effect of nitrogen source

In order to find the suitable nitrogen source on laccase production, 100 ml production medium supplemented with optimized carbon source and 2% different nitrogen sources *viz.* peptone, ammonium chloride, sodium nitrate, ammonium sulfate was inoculated with 1 ml culture of *Pseudomonas fluorescens* followed by incubation at optimized temperature for optimized time interval. Laccase assay was measured at 530 nm.

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

2.3 Production and Partial purification at optimized culture conditions

For laccase production, 10 ml Nutrient broth was inoculated with loopful culture of *Pseudomonas fluorescens* and incubated at 30ºC at 120 rpm. The culture was served as seed culture after 24 h for further inoculation. A 100 ml sterile production media was prepared containing 2% optimized carbon and nitrogen source with optimized pH according to the composition given by Unyayar *et al.*, (2005) sterlized and inoculated with 5% seed culture (v/v) and was incubated at optimized temperature for optimized time interval.

2.4 Extraction of crude enzyme

After incubation the cells were harvested by centrifugation at 10,000 rpm at 4ºC for 10 min. The clear supernatant obtained was used as crude enzyme and was stored in vials for further use.

2.6 Determination of crude and partially purified 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).

Calculation:-

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

Where,

Vt = final volume of reaction mixture (ml)

Vs = sample volume (ml)
€ = extintion co-efficient of guaiacol = 6740/M/cm.

2.7 Partial purification of Laccases

Partial purification of laccase enzyme was done through Ammonium sulfate precipitation and Dialysis. Protein content was estimated at each step of purification.

2.7.1 Ammonium sulfate precipitation

The crude laccase was filtered through Whatman No.1 filter paper and the resulting filtrate was concentrated by freeze-drying and kept at 4ºC. Protein was precipitated using ammonium sulfate from the crude laccase. Solid ammonium sulfate was added slowly to the crude extract isolate to give 70% saturation and the solution was stirred gently for at least 1 h at 4ºC and was left to stand overnight. The precipitate was collected by centrifugation at 8000 rpm for 1 h at 4ºC. The supernatant was discarded and the pellet was dissolved in 0.01 M phosphate buffer (pH 7) (Barda and david, 1949).

2.7.2 Dialysis

Pellet was dissolved in 0.01 M phosphate buffer with pH-7 and was dialysed against the same buffer overnight at 4ºC.

2.8 Estimation of protein content of crude and partially purified laccases

1 ml of NaOH solution was taken in a test tube and heated upto 100ºC. 1 ml protein sample was taken and mixed in the above solution and was left for 4-5 minutes. 5 ml reagent D was mixed and was left for 10 minutes at room temperature. 0.5 ml of Folin-Ciocalteau reagent was added and left for 30 minutes. The absorbance of the solution was measured at 530 nm (Lowery *et al.*, 1951).

2.9 Dye decolorization assay by partially purified laccases

The decolorization of Congo red was investigated by partially purified laccase produced from *Pseudomonas fluorescens.* Stock solutions of the dye was prepared by dissolving 0.001 g of Congo red in 100 ml sterilized distilled water. The reaction was initiated with laccase (0.1 ml) followed by addition of 1 ml Congo red and then the content were incubated at 37ºC under mild shaking conditions. Control sample was prepared in parallel without bacterial laccase under identical conditions. All measurements were done in triplicate. The absorption spectrum of dye was measured at 0, 24, 48, 72, 96 h using a Colorimeter at 600 nm. The effect of dye decolorization was determined by the decrease in absorbance under the maximum wavelength of the dye, respectively. The efficiency of decolorization was expressed in terms of percentage (Zhao *et al.*, 2011).

$$\text{Decolourization}(\%) = \frac{\text{initial absorbance} - \text{observed absorbance} \times 100}{\text{Initial absorbance}}$$

III. RESULTS AND DISCUSSION

3.1 Screening of isolates for laccase production

Reddish brown colour zone was observed around the colonies of *Pseudomonas fluorescens* (Fig:1) indicating the positive reaction for the presence of laccase enzyme. The result obtained is in contrast with Aruna *et al.*, (2012) in which white rot fungi was screened for laccase activity on Nutrient agar plate containing 0.01% guaiacol that showed reddish brown zone around the colony of the fungi and confirmed that it was laccase producing.

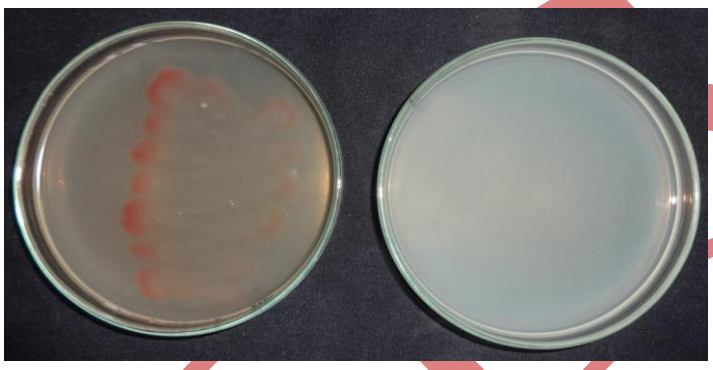


Fig:1 Screening of isolates for laccase production

3.2 Optimization of culture conditions for laccase production

3.2.1 Effect of temperature on laccase activity

The highest laccase activity was observed at 40ºC (0.0356 U/ml) (Fig:2). The present study is in contrast with the study conducted by Ding *et al*., (2012) in which the influence of temperature on laccase activity was studied and reported 60°C as the optimal temperature for laccase activity. Similar kind of study was conducted by Adejoye and Fasidi, (2009) in which effect of temperature on laccase activity was studied and reported highest laccase activity (51.5 ± 2.21 U/ml) at 28°C. Incubation temperature plays an important role in the metabolic activities of microorganisms. The optimal temperature of laccase differs greatly from one strain to other. 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.

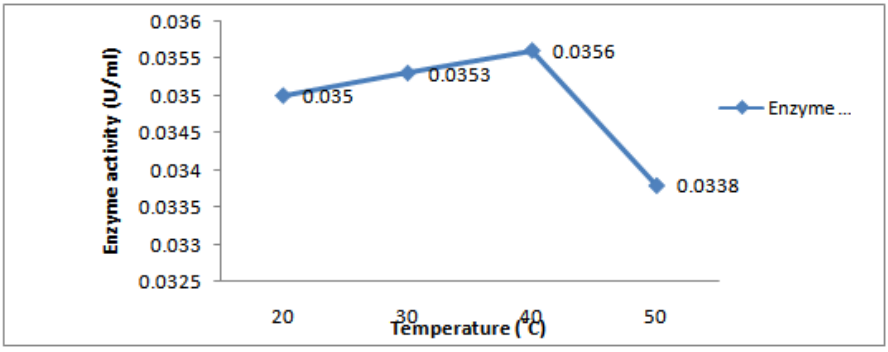


Fig:2 Effect of temperature on laccase activity

3.2.2 Effect of pH on laccase activity

The highest laccase activity was observed at pH 7 (0.0317 U/ml), while lowest laccase activity was observed at pH 12 (0.0267 U/ml). (Fig:3). The present study is in contrast with the study conducted by Ding *et al.*, (2012) in which highest laccase activity was reported at pH 3. The maximum release of laccase activity of 0.24 U/ml from *Ganoderma* sp. was reported at pH 6.0 by Sivakumar *et al.*, (2010). In the similar kind of study conducted by Adejoye and Fasidi, (2009) in which the highest laccase activity of (2.86 U/min) was recorded at pH 5.5. 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. The optimum value of pH varies according to the substrate because different substrate causes different reaction for laccases. Many reports suggested that the bell shaped profile occurs in case of laccase activity. At high pH value, the potential difference between the phenolic substrate and the T_1 copper can increase the substrate oxidation while the hydroxide anion (OH^-) binds to the T2/T3 cooper centre.These effects helps in determining the optimal value of pH for laccase enzyme. The pH is one of the operational parameters that influence the metabolic activity of the organism, playing an important role in protocol optimization for fermentation process.

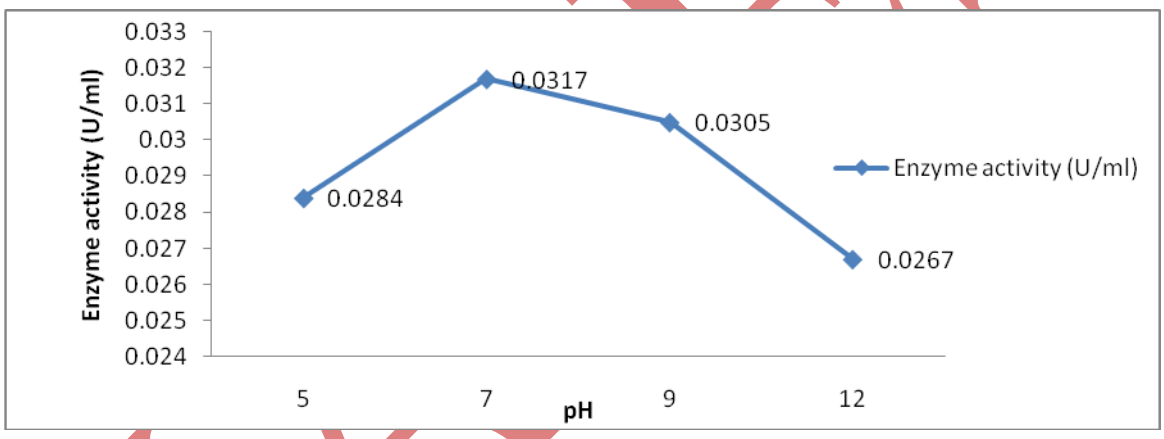


Fig:3 Effect of pH on laccase activity

3.2.3 Effect of incubation time on laccase activity

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 (0.0415 U/ml). (Fig:4). The present study is in contrast with the finding of Sivakumar *et al.*, (2010) in which effect of incubation time on laccase activity was studied and highest activity was observed on 10^{th} day of incubation with *Ganoderma* sp. The similar kind of study was conducted by Desai *et al.*, (2011) in which highest activity by fungi was observed on 6^{th} day of incubation. 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.

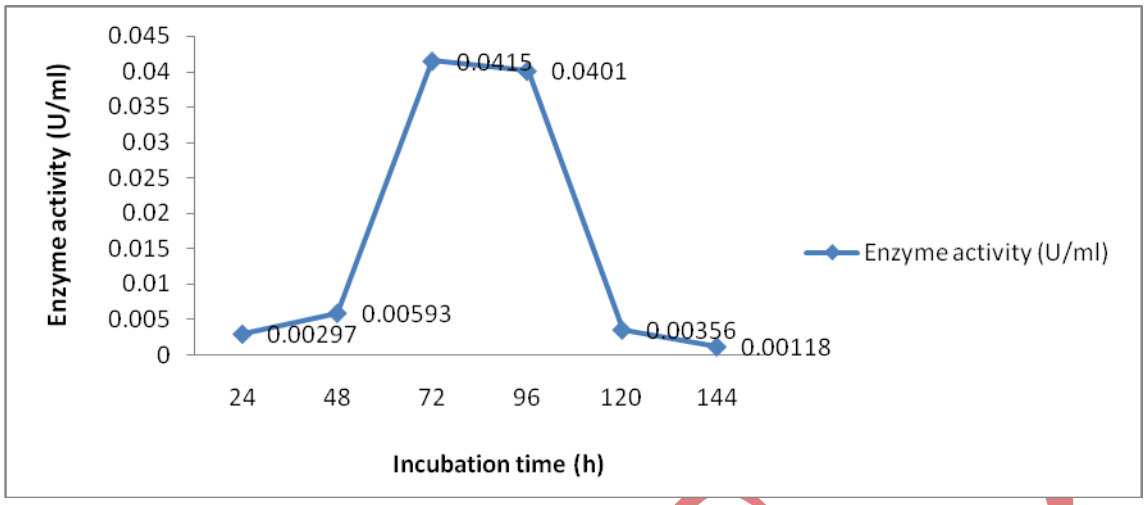


Fig:4 Effect of incubation time on laccase activity

3.2.4 Effect of carbon source (2%)

Overall the highest laccase activity was observed with glucose. (Fig.5). The present study is in agreement with study conducted by Ding *et al.*, (2012) in which five sugars (glucose, maltose, lactose, sucrose, and starch) were tested; 20 g/l glucose was most as effective sole carbon source, resulting in the highest laccase production. While the present study is in contrast with the study conducted by Sivakumar *et al.*, (2010) and Adejoye and Fasidi, (2009). It has been reported that the carbon source is the most important factor in laccase production, and that the addition of suitable amount of other sugar to the culture media has a influence on laccase synthesis. Among the carbon source, glucose is readily utilizable substrate which would promote laccase production. Medium containing glucose showed the highest laccase activity as enzymes are substrate specific. Since glucose is a monosaccharide which is easily broken down 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.

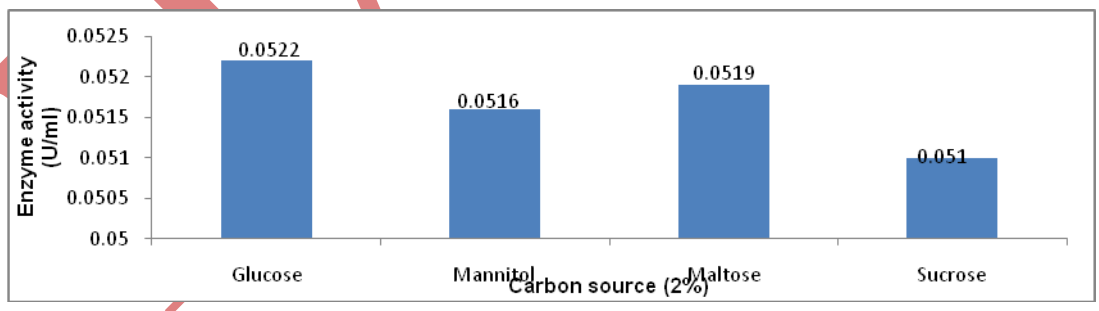


Fig:5 Effect of carbon source (2%)

3.2.5 Effect of nitrogen source (2%)

The highest laccase activity was observed with sodium nitrate. (Fig:6). The present study is in contrast with Ding *et al.*, (2012), Sivakumar *et al.*, (2010) and Adejoye and Fasidi, (2009). Nitrogen plays key role in laccase production, while the organic nitrogen source gave high laccase yields. The nature and the concentration of nitrogen in the culture

medium for growing the organism are essential for laccase production Medium containing sodium nitrate showed the highest laccase activity as enzymes are substrate specific. It has already been demonstrated that substrates that are efficiently and rapidly utilized by the organism results in high levels of laccase activity.

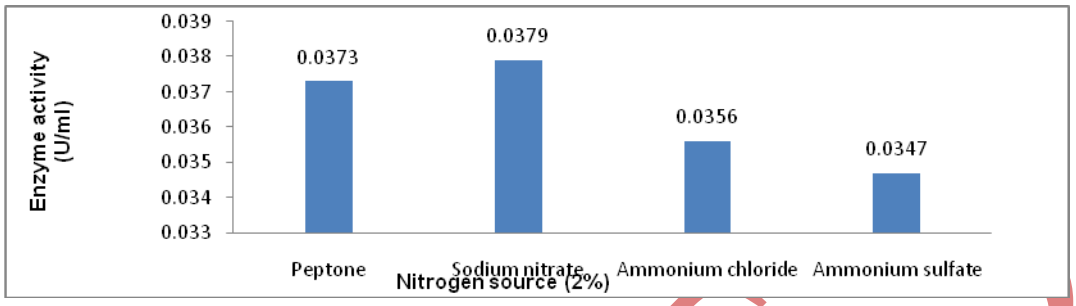


Fig:6 Effect of nitrogen source (2%)

3.3 Purification of laccase

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 0.063 U/ml and 0.67 mg/ml of protein. After ammonium sulfate precipitation, the final purification by dialysis the fraction showed 0.032 U/ml and 0.38 mg/ml of protein. As in present study, similar laccase activity was reported by several workers (Abou-Mansour *et al.* 2009; Aruna *et al.* 2012 and Jhadav *et al.* 2009) and purification of laccase enzyme was done by using ammonium sulfate and dialysis method.

Table:1 Estimation of Protein content in crude and partially purified laccase

Isolate	**Crude enzyme (mg/ml)**	**Partially purified enzyme (mg/ml)**
Pseudomonas fluorescens	0.67	0.38

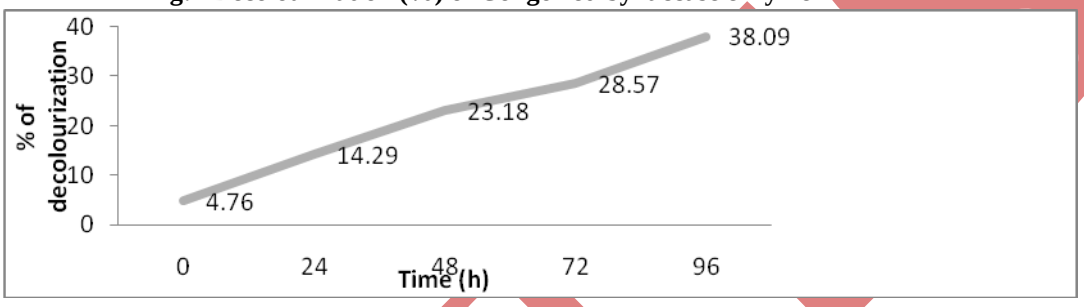
3.4 Decolourization of Congo red by laccase enzyme

In the present study, the decolourization of congo red by laccase enzyme produced by *Pseudomonas fluorescens* was studied at varying time interval from 0-96 h. The decolourization percentage obtained by laccase enzyme produced from *Pseudomonas fluorescens* at 0 h (4.76%), 24 h (14.29%), 48 h (23.18%), 72 h (28.57%) and 96 h (38.09%). The percentage of decolourization was gradually increasing from 0 h to 96h. The maximum decolourization of congo red by laccase enzyme was observed at 96 h (38.09%). (Fig:7).The present study is in contrast with the study of Zhao *et al.*, 2011 in which 70% decolourization of congo red was found with spore-bound laccase from *B. sutilis* WD23 in 24 h at 37°C. In previous studies, higher decolurization rates were reported for the laccases from a number of fungi, such as *Sclerotium rolrsii, Trametes modesta, Pleutrotus pulmonarius and Pycnoporus sanguineus*. Similar kind of study conducted by Rehman *et al.*, (2012) in which *Pseudomonas ostreatus* was capable to decolourize 43,

68 and 94% Synazol red NF6BN from the industrial effluent after 10, 20 and 30 days of incubation at room temperature respectively.

Laccases catalyses oxidation of a wide variety of organic and inorganic compounds, including diphenols, polyphenols, diamines, and aromatic amines. One electron at a time is removed from the substrate, and molecular oxygen is used as the electron acceptor (Gianfreda *et al.*, 1999). Thesubstrate loses a single electron and forms a free radical. The unstable radical undergoes further nonenzymatic reactions including hydration, disproportionation, and polymerization (Thurston, 1994). And the formation of the free radicals helps in the decolourization of congo red dye.

Fig:7 Decolourization (%) of Congo red by laccase enzyme



IV. CONCLUSION

Azo dyes commonly cause environmental problems. Physical and chemical methods can be used for remediation, but these methods are costly and sometimes produce hazardous by-products. Biodegradation is an environment friendly and economical way to modify chemical processes and substituents. For example, many bacterial and fungal species are known to be capable of decolorizing dyes. These strains can produce different types of enzymes which catalyze the decomposition of the dyes. Laccase is an example of one of the important enzymes involved in the catalytic decomposition of the dyes.

VI. ACKNOWLEDGEMENT

I extend my sincere thanks to my advisor for her assistance and kind support throughout my dessertation. The authors offer gratuitous thanks to Hon'ble Vice Chancellor, Most Rev. Prof. R.B. Lal, SHIATS, Naini, Uttar Pradesh, India for provision of research conductance. Heartfelt thanks to Head, Department of Microbiology and Fermentation Technology, JSBB, SHIATS, Allahabad, Uttar Pradesh, India for the kind cooperation towards the resssearch.

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