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Original Research Article

Congo red dye decolourization by partially purified laccases from *Pseudomonas aeruginosa*

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Laccase, *Pseudomonas aeruginosa*, enzyme activity, dye decolourization

A B S T R A C T

Laccase is a copper-containing polyphenol oxidase that acts on a wide range of substrates. The present work focuses on screening, optimizing the process parameters to achieve the maximum production of extracellular laccases by *Pseudomonas aeruginosa* and application of laccase on dye decolourization. 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% peptone as nitrogen source in the production medium. The enzyme activity of crude enzyme and partially purified enzyme was observed to be 1.92 and 0.95 U/ml respectively. 42.86% of decolourization was observed at 96 h by laccase enzyme produced by *Pseudomonas aeruginosa*. Laccase enzyme produced by *Pseudomonas aeruginosa* is able to decolorize congo red dye and can be used in controlling environmental pollution.

Introduction

Great number of dyes is used in textile industries. Among all the dyestuffs, the azo dye congo red is considered the most utilised in industries, representing 70% of total dyes produced per year. In recent years, several microorganisms have been investigated for decolourization of dyes, and its effectiveness depends on the adaptability and the selectivity of the microorganisms. (Jadhav *et al.*, 2009).

Laccases (E C 1.10.3.2; benzenedoil : oxygen oxidoreductase) are the family of multicopper oxidases belonging to the group of blue oxidases. Structurally, laccase bolongs to the multi-copper oxidase (MCOS) that require oxygen to oxidize organic compounds, particulary phenols (Piontek *et al.*, 2002) and non-phenolic substrates by one-electron transfer, resulting in the formation of reactive radicals, while reducing molecular oxygen to water (Yaropolov *et al.*, 1994; Solomon *et al.*, 1996). In the environment they are involved in the biodegradation of lignin and humic acids. The enzyme exhibits a broad substrate

specificity, which can be enhanced by addition of redox mediators (Solomon *et al.*, 1996). The main limitation for the extensive industrial application of laccase is its high cost. To attain the production of a large amount of enzyme at low cost, media optimization plays a crucial role. To attain the production of a large amount of enzyme at low cost, media optimization plays a crucial role.

Laccases are classified into two groups in accordance with their source, *i.e.* plant and fungal. However, diphenol oxidases (laccases) have been identified in bacteria (Givaudan *et al.,* 1993) and insects (Barrett *et al.,* 1991). The enzyme is a copper protein and contains four metal ions classified into three types, referred as T1, T2 and T3 (Yaropolov *et al.,* 1994; Solomon *et al.,* 1996). The T1 copper is responsible for blue colour of the enzyme and has a characteristic absorbance around 610nm. The T2 copper cannot be detected spectromatically; however, it generates a characteristics EPR signal (Solomon *et al*., 1996). The bi-nuclear T3 copper is diamagnetic.

The first reported laccase was in 1883 from *Rhus vernicifera,* the Japanese lacquer tree. Laccases were discovered largely in plants and fungi (Thurston, 1994; Mayer and Staples, 2002). However, bacterial laccase was first repored in 1993 (Givaudan *et al.*, 1993). Laccase like activity has been found in *Escherichia coli, Streptomycetes, Azopirillum lipoferum, Mariomonas Mediterranea, Bacillus sphaericus* and *Bacillus subtilis*.

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 (Baldrian, 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 (Johansen, 1996).

Materials and Methods

Screening for laccase production

Laccase production by *Pseudomonas aeruginosa* 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.

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

This was done by varying culture conditions that effect laccase production to determine the optimum conditions for the production of laccase from *Pseudomonas aeruginosa.* Production medium was used for analysis.

Effect of temperature

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

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 aeruginosa* at different pH 5, 7, 9 and 12 for 24–48 h at optimized temperature. Laccase assay was measured at 530 nm.

Effect of incubation period

In order to find the optimal time of incubation for the maximum laccase production, 100ml production medium was prepared with optimized pH and inoculated with 1ml culture of *Pseudomonas aeruginosa* 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.

Effect of carbon source

In order to find the suitable carbon sources for maximum laccase production by *Pseudomonas aeruginosa* 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 1ml culture of *Pseudomonas aeruginosa* followed by incubation at optimized temperature for optimized time interval. Laccase assay was measured at 530 nm.

Effect of nitrogen source

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

The enzyme activity was measured using the following formula:

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

Production and partial purification at optimized culture conditions

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

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.

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 100mM acetate buffer (pH 5). Absorbance for blank was measured at 470nm while that of test samples was measured at 530nm. 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:-

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

Where,

Vt = final volume of reaction mixture (ml)

Vs = sample volume (ml)

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

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.

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 1h at 4ºC and was left to stand overnight. The precipitate was collected by centrifugation at 8000 rpm for 1h at 4ºC. The supernatant was discarded and the pellet was dissolved in 0.01 M phosphate buffer (pH 7) (Barda and david, 1949).

Dialysis

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

Estimation of protein content of crude and partially purified laccases

1 ml of NaOH solution was taken in a test tube and heated up to 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).

Dye decolorization assay by partially purified laccases

The decolorization of Congo red was investigated by partially purified laccase produced from *Pseudomonas aeruginosa*. Stock solutions of the dye was prepared by dissolving 0.001g of Congo red in 100ml sterilized distilled water. The reaction was initiated with laccase (0.1ml) followed by addition of 1ml 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).

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

Results and Discussion

Screening of isolates for laccase production

Reddish brown colour zone was observed around the colonies of *Pseudomonas aeruginosa* (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. The reddish brown zone formed around the colony is due to the oxidation- reduction reaction.

Optimization of culture conditions for laccase production

Effect of temperature on laccase activity

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.2). The present study is in contrast with 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. Adejoye and Fasidi, (2009) reported maximum 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

Effect of pH on laccase activity

The highest laccase activity was observed at pH 7 (0.0341 U/ml), while lowest laccase activity was observed at pH 12 (0.0287 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 help 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.

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 (0.0459 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.

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 an 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.

Effect of nitrogen source (2%)

Among inorganic nitrogen sources, the highest laccase activity was observed with peptone (0.0382 U/ml) (Fig.6). The present study is in contrast with Ding *et al.*, (2012), Sivakumar *et al.,* (2010) and Adejoye and Fasidi, (2009). Earlier reports indicated that yeast extract supported for the higher laccase production. Nitrogen plays key role in laccase production, while the organic nitrogen source gave high laccase yields. Nitrogen plays key role in laccase production, the nature and the concentration of nitrogen in the culture medium for growing the organism are essential for laccase production. 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.

Purification of laccase

The crude enzyme was precipitated by ammonium sulfate precipitation up to 70% saturation with a total activity of 0.059 U/ml and 0.82 mg/ml of protein. After ammonium sulfate precipitation, the final purification by dialysis the fraction showed 0.028 U/ml enzyme activity and 0.55 mg/ml 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.

Decolourization of Congo red by laccase enzyme

In the present study, the decolourization of congo red by laccase enzyme produced by *Pseudomonas aeruginosa* was studied at varying time interval from 0–96 h.

The decolourization percentage obtained by laccase enzyme produced from *Pseudomonas aeruginosa* at 0 h (9.52%), 24 h (14.29%), 48 h (28.57%), 72 h (38.09%) and 96 h (42.86%). The percentage of decolourization was gradually increasing from 0 h to 96 h. The maximum decolourization of congo red by laccase enzyme was observed at 96 h (42.86%). (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*. In a similar kind of study conducted by Govindward *et al.*, (2011) in which 62, 38 and 80% decolorization was observed by *Pseudomonas sp.* SUK1, *Aspergillus ochraceus* NCIM-1146 and consortium-PA respectively, within 24 h.

Fig.1 Screening of isolates for laccase production

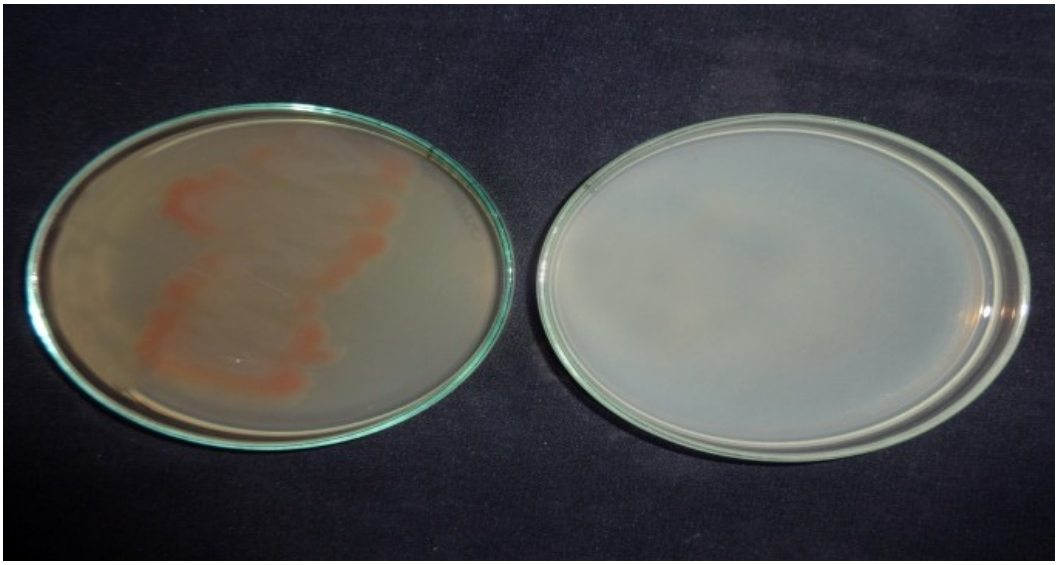
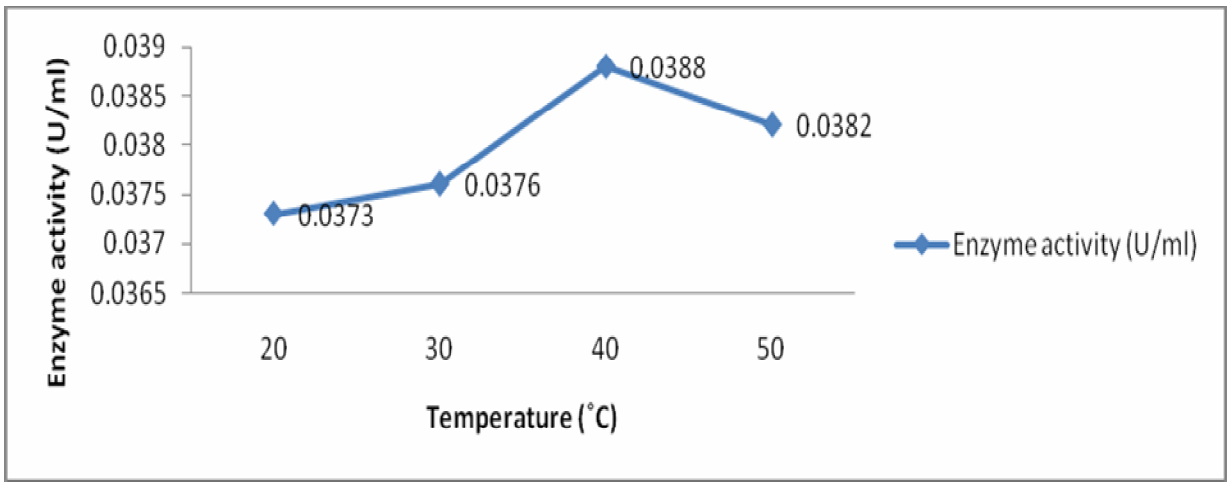


Fig.2 Effect of temperature on laccase activity.



r_{xy} =0.213, Positive Correlation

Fig.3 Effect of pH on laccase activity

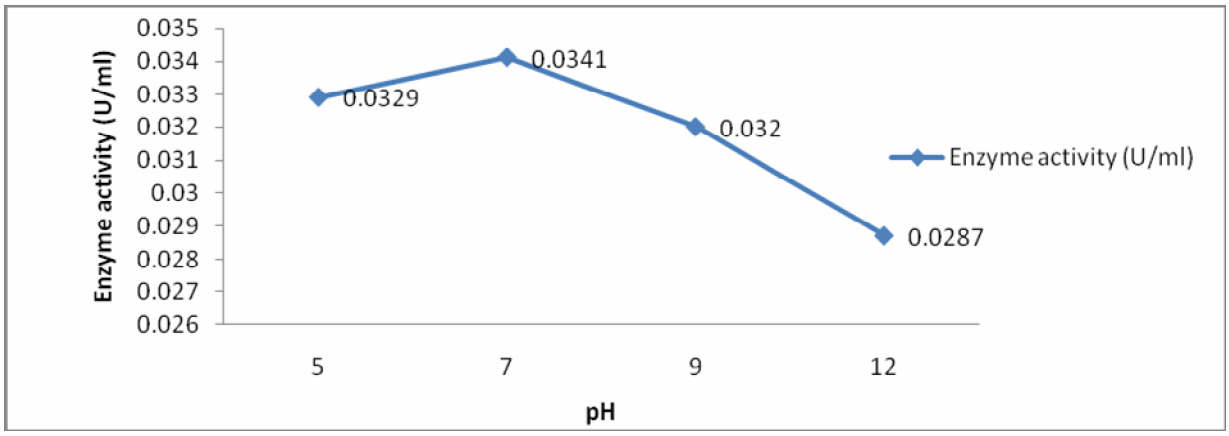


Fig.4 Effect of incubation time

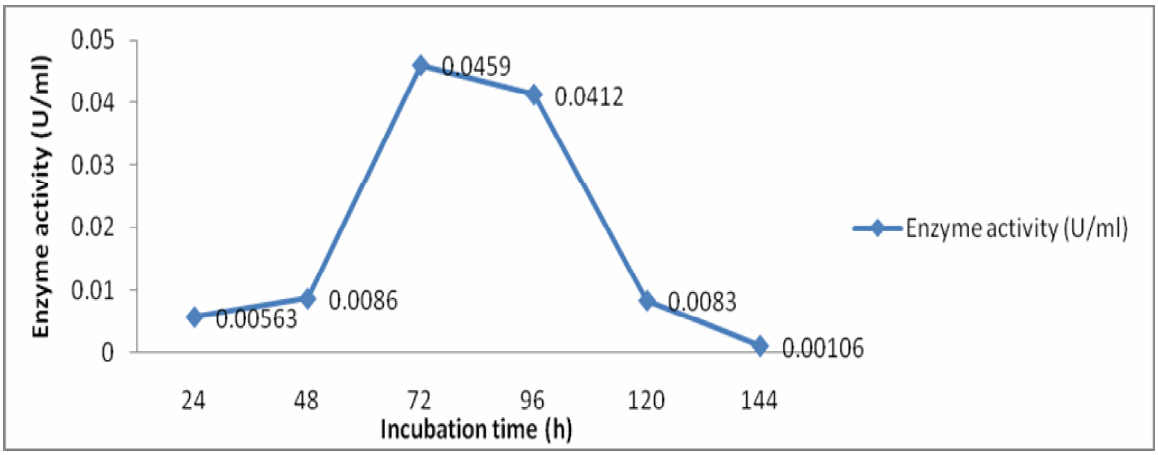


Fig.5 Effect of carbon source (2%)

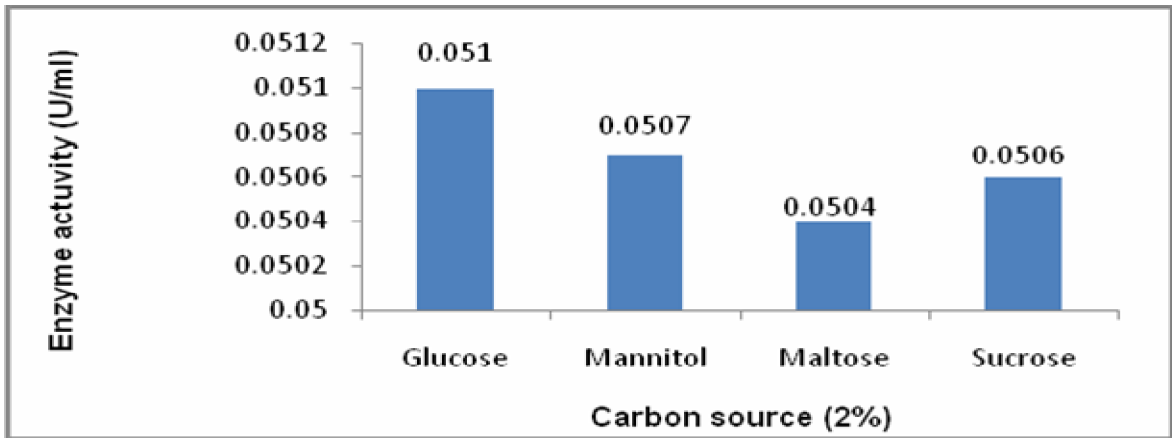


Fig.6 Effect of nitrogen source (2%)

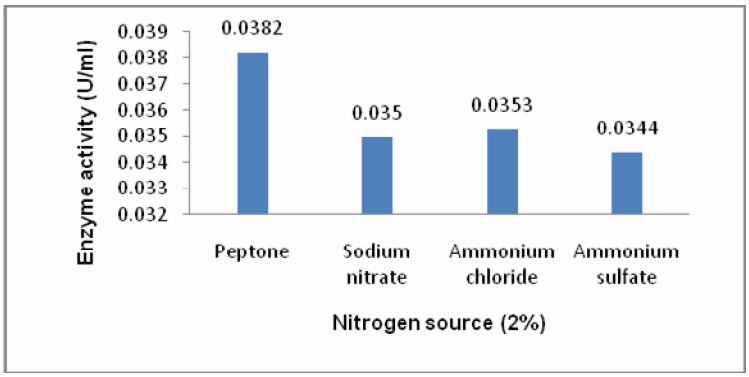
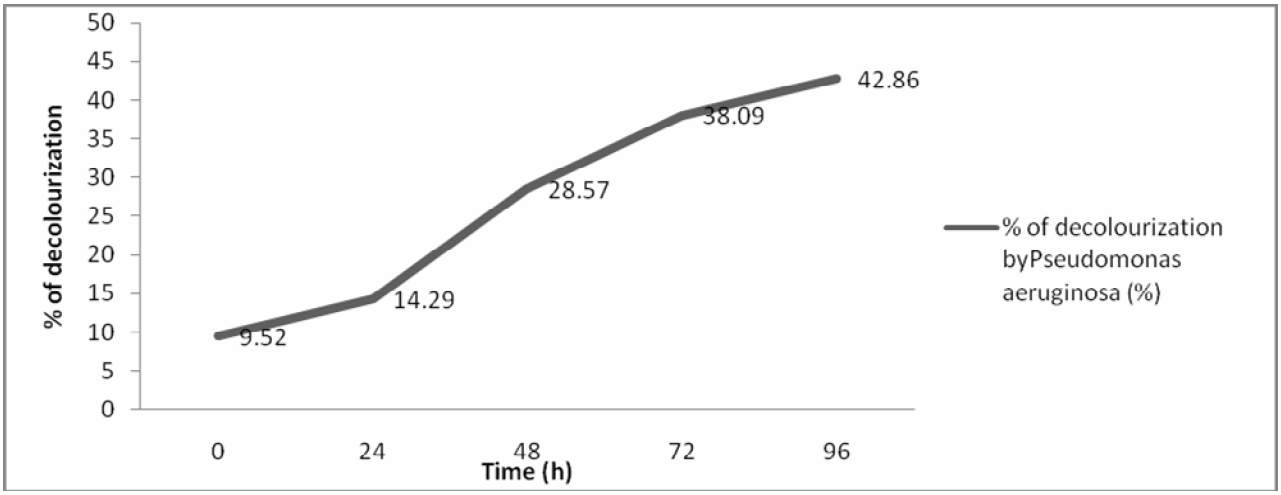


Fig.7 Decolourization (%) of Congo red by laccase enzyme



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