

Production and Partial Purification of Laccase Produced by *Bacillus* species Isolated from Contaminated Soil

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ABSTRACT: Laccases, a group of multi-copper oxidases, catalyzes oxidation of wide variety of naturally occurring as well as synthetic compounds like phenolic, diamines and aromatic amine compounds. Laccases are widely distributed among plants, insects, fungi and bacteria. In present study, indigenous bacteria capable of producing laccase enzyme was isolated and screened from soil and sediment samples. Among these bacterial strains AS1, with maximum laccase production of 3.348 U/ml after partial purification, was selected and identified as *Bacillus spp.* on the basis of biochemical characteristics. Effect of various physico-chemical parameters like pH, temperature, carbon, nitrogen and copper supplementation on laccase production by bacterium was investigated. Dye decolorization potential of laccases was studied on Reactive Red 152 and Coralene Yellow C4G azo dyes. Partial purification of laccase produced by bacterium under optimum condition was done by ammonium sulphate precipitation and membrane dialysis method.

KEYWORDS: Bacterial Laccases, Dye decolorization, *Bacillus* species, Lignocellulosic waste, Multi-copper oxidases.

I. INTRODUCTION

In recent years, enzymes are found to be of great importance in industries, and laccases are among those which are widely found in nature. Laccases (p-benzenediol: oxygen oxidoreductase EC 1.10.3.2) are a family of multi-copper oxidase enzymes distributed among variety of bacteria, fungi, insects and plants [25]. Laccases are glycosylated polyphenol oxidases that contain 4 copper ions per molecule and carry out oxidation of phenolic and related compounds to oxygen and water [5, 10]. Laccases catalyzes the oxidation of various organic and inorganic compounds, like diphenols, polyphenols, methoxy-phenols, diamines, substituted phenols, and aromatic amines, hydroxyindols, benzenethiols, carbohydrates, and inorganic/organic metal compounds [11, 13, 14]. They also oxidize other substrates such as aromatic amines, syringaldazine, 2, 2-azinobis (3-ethylbenzothiazoline-6-sulphonate) (ABTS), 1-hydroxybenzotriazole (HBT), 2,2,6,6-tetramethylpiperidineoxy (TEMPO), violuric acid to form free radicals [4, 19].

Laccases play very important roles in food industry, paper and pulp industry, textile industry, synthetic chemistry, cosmetics, soil bioremediation and biological degradation of phenolic pollutant compounds in environment. These enzymes are also used for pulp delignification, degradation of pesticides and insecticides, water detoxification textile dye transformation, food technological uses and for biosensor and analytical purposes [9]. They also play a key role in detoxification and degradation of toxic environmental pollutants and are useful for bioremediation applications [5].

Despite of wide occurrence in plants, laccases have not been used or characterize so far because their detection and purification is difficult. In fungi, they play role in pigment formation, detoxification and lignin degradation [3, 28]. In bacteria, laccases are having their role in melanin production, detoxification of copper, spore coat resistant and are involved in morphogenesis [24]. The bacterial laccases are having many advantages over classical fungal laccases due to their highly efficient expression, higher thermostability, stability at high chloride concentrations and wide pH range useful for pulp bleaching and detergent [8, 23]. The present study was aimed to isolate and characterize laccase

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producing indigenous bacteria and to establish optimal physiological parameters for laccase production and to investigate dye decolorization potential of laccases thus produced.

II. MATERIALS AND METHODS

Soil and effluent samples were collected from chemically contaminated sites near the vicinity of Surat city. Samples were collected in sterile plastic jar and stored at 4°C until further use. Samples were enriched by inoculating 1 ml of sample in 50 ml sterile nutrient medium (HI Media Pvt. laboratories, Mumbai, India). The flasks were incubated at 30 °C for 48 hours at 120 RPM. After enrichment samples were screened for bacterial isolates.

Isolation and Screening of Laccase Producing Bacteria:

A loop full of enriched sample was streaked on nutrient agar plates and pseudomonas isolation agar plates and incubated at 37°C for 24-48 hours. Plates were observed for morphologically different bacterial isolates.

Isolates were primarily screened for laccase production by plating them on Luria Bertani (LB) agar medium (HI Media Pvt. laboratories, Mumbai) supplemented with Guaiacol (0.01%) (HI Media Pvt. laboratories, Mumbai). The laccase production by isolates was confirmed by presence of brown color zone, surrounding the bacterial growth. Isolates with brown color zone was selected and subjected to secondary screening. For secondary screening, a loop full of bacterial suspension was inoculated in 50 ml of sterile LB broth supplemented with 1% fructose and 0.01% CuSO₄ [26] and incubated at 30°C at 125 rpm for 96 h. Laccase activity was checked daily by method described below.

Laccase Activity Assay (ABTS assay) [3]:

5 ml of sample was removed aseptically and centrifuged at 10,000 rpm for 10 min at 4 °C. Laccase activity was measured using ABTS {2, 2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)} as a substrate for Laccase. The reactive mixture consists of 1.5 ml sodium acetate buffer (1 mM, pH 5.0), 1.5 ml ABTS (0.5 mM) and 1.5 ml cell free supernatant. The absorbance was noted down at 420 nm using UV/Visible Shimadzu-UV-3600 Plus Spectrophotometer. The laccase activity in U/ml was calculated by the formula:

$$E. A. = \frac{A \times V}{t \times e \times v}$$

Where, E. A.: Enzyme activity (U/ml), A: Absorbance at 420 nm; V: total mixture volume (ml); t: incubation time; e: extinction coefficient for ABTS (3.6 x 10⁴ M⁻¹ cm⁻¹); v: enzyme volume (ml).

Characterization of Laccase Producing Bacterial Isolates:

Bacterial isolates showing maximum laccase activity were streaked on nutrient agar and sent to Advanced Diagnostic Laboratory, Param doctor house, Lal Darvaja, Near Surat Railway Station, Surat, Gujarat for biochemical characterization. Biochemical tests were carried out on Phoenix Instrument, version: 6.01 A and EpiCenter, version: V6.20A.

Effect of pH and Incubation Temperature:

Effect of initial pH of the media on Laccase enzyme activity was checked. For this initial pH of the Laccase production medium (LPM) (Tryptone 10 gl⁻¹, Yeast Extract 5 gl⁻¹, Sodium Chloride 10 gl⁻¹, Fructose 0.1 gl⁻¹ and CuSO₄ 0.001 gl⁻¹) was adjusted to 5, 6, 7, 8 and 9, 10. 1.5 ml of bacterial suspension was inoculated in LPM and incubated at 30°C at 120 RPM. Samples were checked for laccase activity.

The optimal temperature of laccase production differs greatly from one bacterium to another. 1.5 ml of bacterial suspension was inoculated in LPM and incubated at various temperatures like 20°C, 30°C, 40°C and 50°C at 120 rpm. Samples were removed and checked for laccase activity.

Effect of Carbon and Nitrogen Supplementation:

Additional co-substrates were added individually to LPM like additional carbon supplementation (glucose, sucrose and maltose) and nitrogen supplementation (peptone, urea, sodium nitrate) at various concentrations i.e. 0.2%, 0.5% and

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1% (w/v). For this, 100 ml of LPM was supplemented with different carbon sources in different concentration, inoculated with bacterial suspension and checked for laccase production regularly.

Effect of Copper Supplementation:

The Laccase activity is influenced by the presence and absence of Copper ions [29]. Here, for optimization of copper supplementation LPM was prepared supplemented with different amount of CuSO_4 like 0.005, 0.01, 0.02 and 0.03%. 1.5 ml of bacterial suspension was inoculated in flasks containing different amount of CuSO_4 and incubated at 37°C at 120 rpm. Samples were removed and checked for laccase activity.

Decolorization of Azo Dyes:

For finding out dye decolorization potential of laccase enzyme a slightly modified protocol [18] was adopted. The reaction mixture contained 50 mM phosphate buffer (pH 8), crude enzyme and Reactive Red 152 and Coralene Yellow C4G dyes at various concentrations (100, 200, 300, 400 and 500 ppm) along with mediator ABTS. The decolorization was monitored spectrophotometrically at 490 nm using UV-VIS Spectrophotometer (Shimadzu-UV-3600 Plus). Crude enzyme was obtained from cell free extracts of selected isolates. Percentage dye decolorization was calculated according to formula:

$$D = \frac{100 \times (A_{ini} - A_{obs})}{A_{ini}}$$

Where, D: Decolorization (%); A_{ini} : Initial Absorbance; and A_{obs} : Observed Absorbance.

Partial Purification of Laccase Produced Under Optimum Influencing Condition [20]:

Laccase production was carried out under submerged condition in LPM along with the optimized carbon and nitrogen sources such as 1% glucose and 1% peptone, respectively at pH 9. Selected bacterial isolate was inoculated in the medium and incubated at 30°C at 120 RPM for 96 hours. The contents of the flask were centrifuged at 10,000 RPM for 10 min at 4°C. Thus obtained supernatant was concentrated by fractionated precipitation of 80% ammonium sulphate saturation. The flask was resuspended in 100 ml of 50 mM glycine-NaOH buffer (pH 9.0). After centrifugation, the samples were extensively dialyzed against phosphate buffer (50 mM, pH 8.0). The resultant crude enzyme was dissolved in phosphate buffer and checked for laccase activity.

III. RESULTS AND DISCUSSION

Isolation and Identification of Laccase Producing Bacteria:

The enriched samples were streaked on nutrient agar plates and pseudomonas isolation agar (PIA) plates and incubated. After incubation 42 different bacterial colonies were isolated and primarily screened for laccase production. Out of these, 08 showed brownish zone surrounding the colonies indicating guaiacol oxidation and subjected to secondary screening. Similar media was used by other researchers [1, 6] for screening of laccase producing bacteria from industrial wastewater. In secondary screening, bacterial isolate AS1 was selected showing maximum laccase activity of 0.541 U/ml after 96 hours. (Figure: 1). On the basis of cultural and biochemical conditions, selected isolate was identified as *Bacillus* species.

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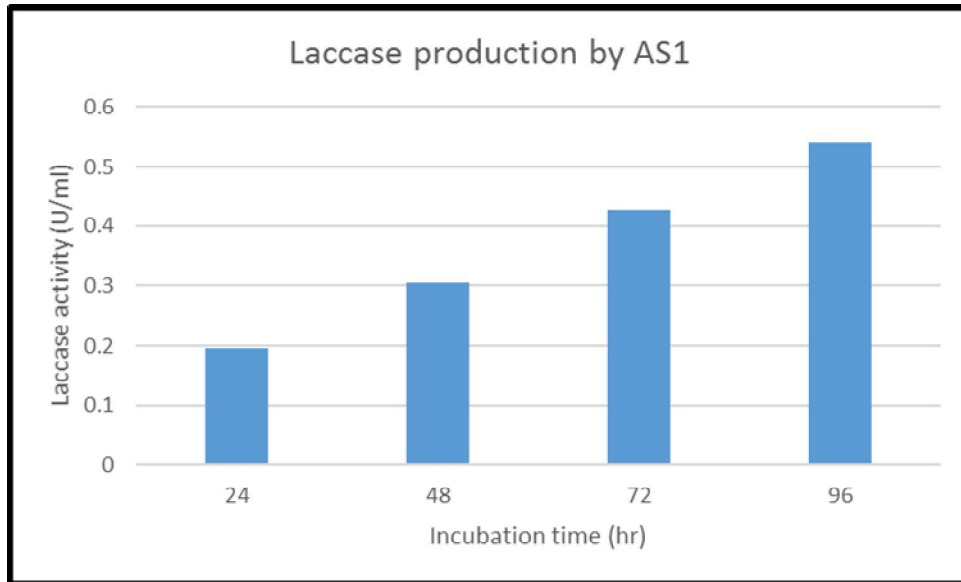


Figure 1. Laccase Production by Bacterial Isolates under Submerged Condition

Optimization of Physico-Chemical Parameters for Laccase Production:

The growth conditions for laccase production by isolated bacterium were optimized like pH of the production medium, incubation temperature, and co-substrates like glucose, maltose and lactose. The pH of the medium strongly affects many enzymatic reactions and transport of compounds across the cell membrane.

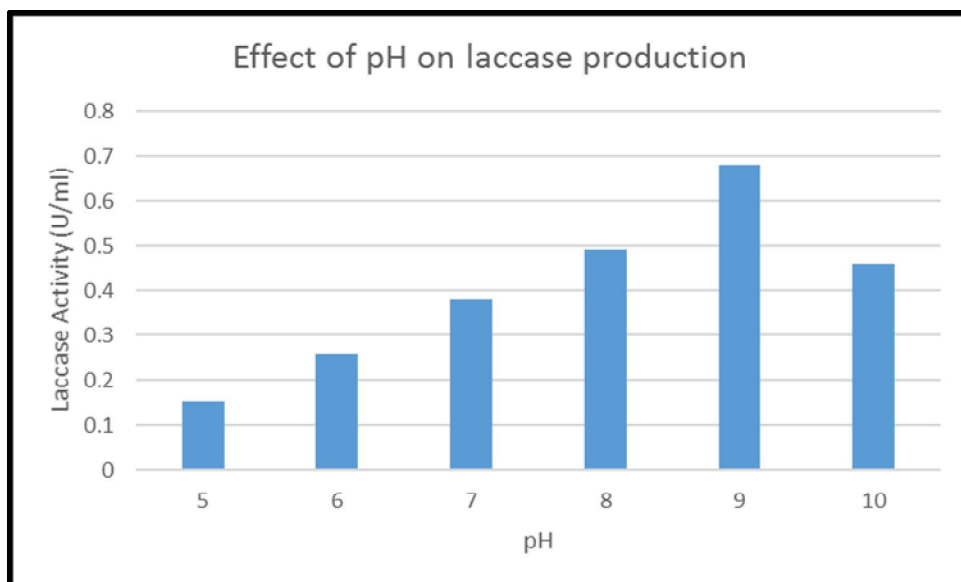


Figure 2. pH Optimization for Laccase Production by Isolated Bacterium

The results of pH optimization suggested that isolated bacterium showed highest Laccase activity of 0.681 U/ml at pH 9 (Figure: 2). Our findings were supported by the work of Kuddus [15]. They also found maximum laccase production at pH 9 & 8 by bacteria isolated from sawdust and dairy effluent. In contrast to that, researchers obtained maximum laccase activity at pH 7 by mixed bacterial consortium [21]. The result of pH optimization showed that pH in alkaline range was the ideal condition for laccase production by both selected isolates. The initial media pH shifted towards the

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higher pH as the enzyme production started. The ability of the bacterium to grow and produce laccases in media of high pH values makes this enzyme more suitable for industrial applications.

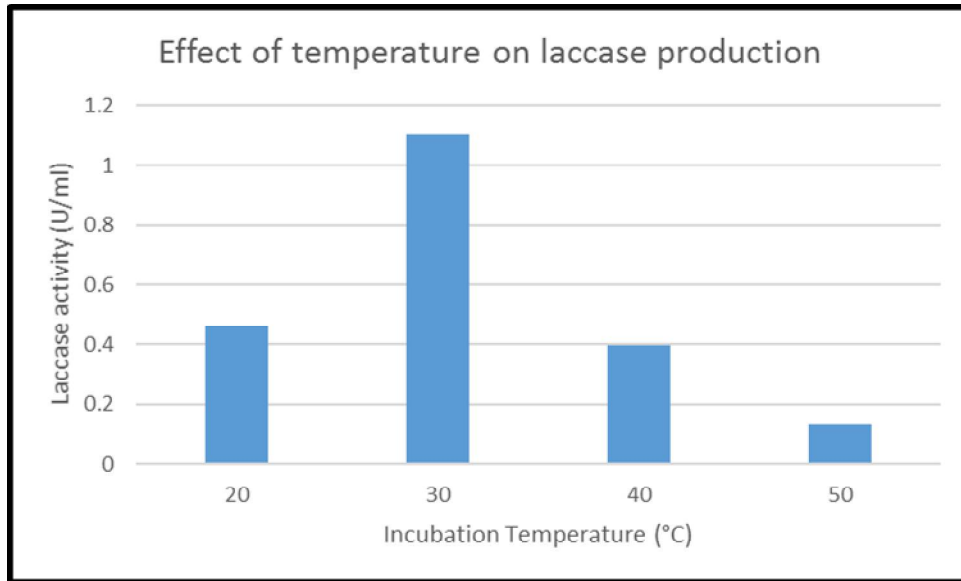


Figure3. Temperature Optimization for Laccase Production by Isolated Bacterium

It is obvious that temperature has a profound effect on enzyme production. In this study, maximum laccase activity of 1.102 U/ml was obtained at 30°C as showed in the (Figure: 3). The same was also observed by workers, wherein maximum laccase production at 30°C temperature by *Streptomyces lavendulae* [16].

Effect of Co-Substrate Supplementation:

Three different carbon sources such as glucose, sucrose and maltose were tested at different concentrations of 0.2%, 0.5% and 1% (w/v) for laccase production by isolated bacterial strain.

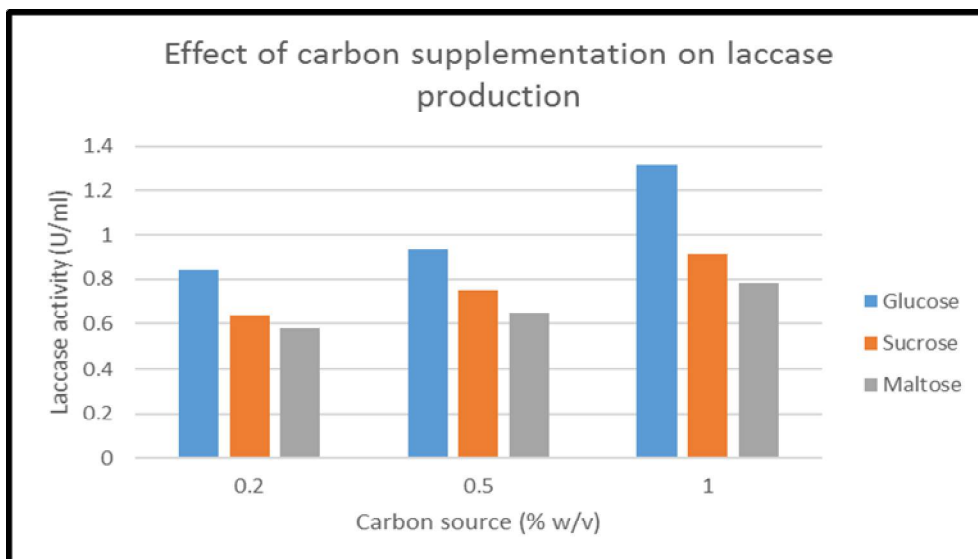


Figure 4. Effect of Carbon Supplementation on Laccase Production by Isolated Bacterium

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Among these Carbon sources, 1% glucose supported maximum laccase activity of 1.315 U/ml (Figure: 4). Similar results were also observed by many other scholars as glucose enhancing laccase production using bacterial isolates [17, 21]. Whereas, other researchers [7, 20] observed maximum laccase activity using sucrose as additional carbon source using bacterial isolates.

Three nitrogen sources like peptone, urea and sodium nitrate were tested at concentration of 0.2%, 0.5% and 1% (w/v) for laccase production by isolated bacterial strain.

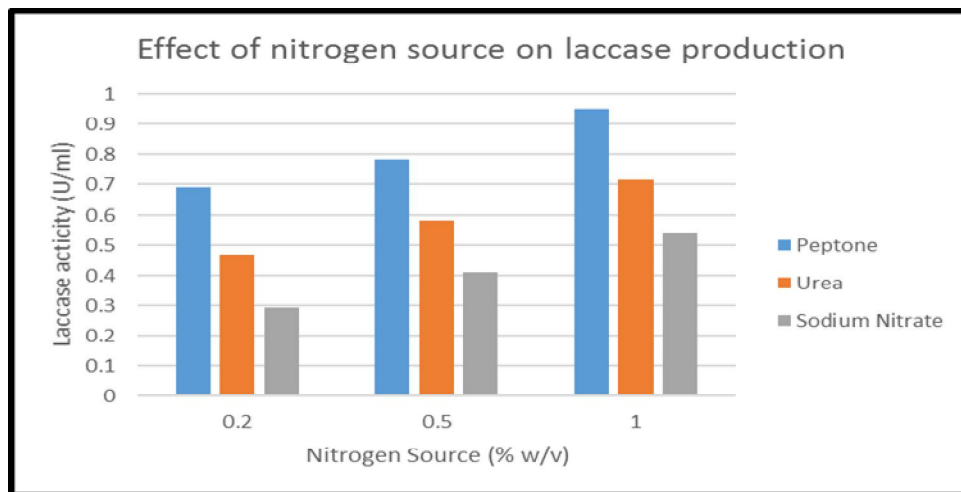


Figure 5. Effect of Nitrogen Supplementation on Laccase Production by Isolated Bacterium

Among these, 1% peptone supported maximum laccase activity of 0.952 U/ml (Figure: 5). Narayanan [20] also found similar results as they reported highest enzyme activity by *Bacillus subtilis* MTCC 2414 using 3% peptone as additional nitrogen source. Similar results were also obtained by Peter *et al.*, (2014) as they found peptone supports maximum laccase activity using mixed bacterial consortium. In contrast to these findings, many other scholars like Demissie and Kumar [7] reported maximum laccase activity using yeast extract.

Effect of Copper Supplementation:

Role of copper in laccase production has also been studied. As laccases are copper oxidases, copper plays important role in medium and is also used as inducer.

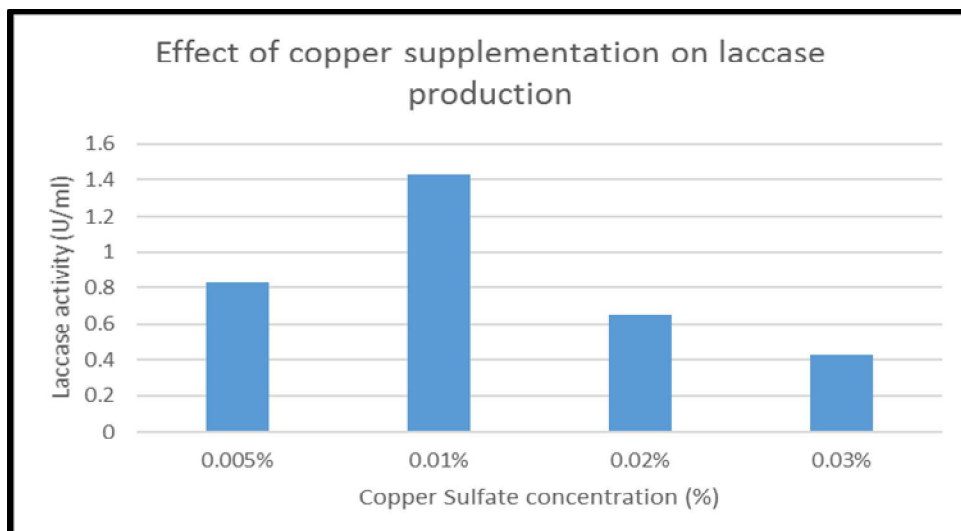


Figure 6. Effect of Copper Supplementation on Laccase Production by Isolated Bacterium

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Results of copper supplementation showed that, copper ions enhanced the laccase production by isolated bacterium with maximum laccase activity of 1.429 U/ml with 0.01% of copper sulfate supplementation (Figure. 6). Copper has been reported to enhance bacterial and fungal laccase production by many scholars [12, 20, 27].

Decolorization of Azo Dyes:

Dye degradation potential of laccase produced by isolated bacterium was studied using different concentrations of Reactive Red 152 and Coralene Yellow C₄G azo dyes purchased from local textile markets of Surat, Gujarat. For this assay, supernatant from laccase production flask was mixed with 50 mM phosphate buffer (pH 8), dyes of various concentrations along with mediator ABTS, and absorbance was measured at 490 nm.

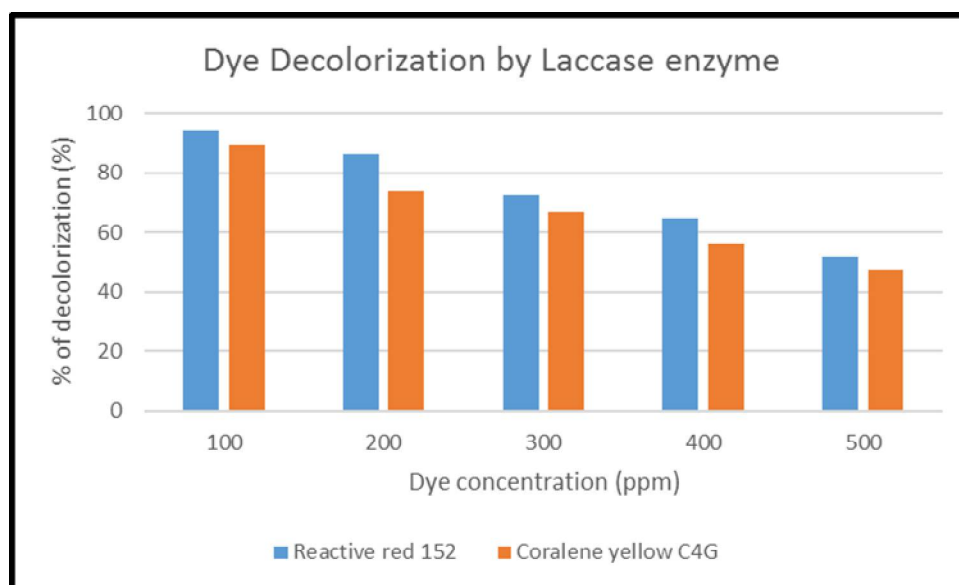


Figure 7. Dye Decolorization Potential of Laccase Produced by Isolated Bacterium

Results of dye decolorization suggests that laccase enzyme is able to decolorize both Reactive Red 152 and Coralene Yellow C₄G azo dyes with 94.2 % and 89.5% of decolorization, respectively (Figure. 7). These results are in agreement with dye decolorization study findings of many other researchers [2, 22]. These findings suggest that the laccase produced by isolated bacterium has good potential for dye degradation that can be exploited for large scale studies.

Partial Purification of Laccase:

During the first course of purification step, the crude supernatant obtained after production was subjected to 80% saturation of ammonium sulphate precipitation and laccase activity was assayed. The results of laccase activity showed the maximum laccase activity of 2.248 U/ml. After dialysis it was found to be 3.348 U/ml. A total of 1.87 mg/ml of protein was estimated at the end of partial purification of enzyme.

IV. CONCLUSION

In the present study, production and partial purification of laccase from *Bacillus* species under submerged fermentation conditions was carried out. Enzyme production was found to be maximum in presence of 1% glucose, 1% peptone and 0.01% CuSO₄ at 30 °C, pH 9. Maximum laccase activity of 3.348 U/ml was observed after dialysis and partial purification. Laccase enzyme showed good dye decolorization potential with maximum dye decolorization of 94.2 % and 89.5% both Reactive Red 152 and Coralene Yellow C₄G azo dyes, respectively. We recommend scale up studies for the production of laccase enzyme for process of dye decolorization at certain industrial level.

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