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"EFFECTS OF PHYSIOCHEMICAL PARAMETERS ON PRODUCTION AND ACTIVITY OF LACCASE ENZYME FROM BACILLUS SUBTILIS"

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ABSTRACT

Chlorophenols are important industrial raw materials for producing dye, preservatives, pesticides and chemicals. However, the industrial waste water containing the refractory organics such as chlorophenols is seriously harmful for the environment and human health. Biodegradation of chlorophenols by bacterial species has been studied for long time as it is a rather effective approach. Laccase-mediated removal of chlorophenols is one biochemical process which are low-cost, environmentally friendly and high efficient. Although bacteria individuals can detoxify these materials, if the pollutants are produced on a large scale, then bacteria may be not the ideal organisms to biodegrade of them. It is well-known that laccase-mediated biodegradation systems are succeeding to deal with it. The objective of this study was to evaluate effects of physiochemical parameters on production and activity of laccase enzyme from Bacillus subtilis under liquid state fermentation using different carbon, nitrogen and pH. The different pH of liquid culture for Laccase production from Bacillus subtilis ranging from pH 5.0 to 9.0. The maximum production of enzyme (200 U/ml) and protein 123 mg/ml were observed at pH 8.0. Different carbon and nitrogen also were tested. The carbon, fructose, nitrogen and peptone were supported for the production of laccase from Bacillus subtilis. The optimum pH laccase its oxidation of syringaldazine was 5.0. The optimum temperature of laccase activity was 40 ˚C. Most of laccase activity (>95%) was inhibited by 1mM l-cysteine, 0.1mM DTT, 1mM p-coumaric acid, 1mM koujic acid, and 1mM thioglycolic acid. The major products of 2, 4-Dichlorophenol was able to detect major distinct peaks only for 2, 4-Dichlorophenol degradation.

Key Words: Laccase Enzyme, Bacillus subtilis, Physiochemical Parameters, 2, 4-Dichlorophenol Biodegradation

I.INTRODUCTION

Microbial degradation of 2,4-D is known to occur in warm, moist soil; however, the rate is very dependent on environmental conditions and soil characteristics [1]. The critical role of micro-organisms in the degradation of organic pollutants is well known. Although the microorganisms capable of degradation of organic pollutants and their catabolic pathways have been investigated intensively, information on the microbial degradation of enobiotics in environments of high salinity and alkalinity is still very limited [2]. Several factors influence

laccase production such as type of cultivation (submerged or solid state), carbon limitation, and nitrogen source [3]. The excess glucose and sucrose reduce the production of laccase by obstructing the initiation [4]. This problem of production of enzyme can be improved by using polymeric substrates like cellulose [5]. The optimum temperature range for laccase production is between 25 ºC and 30 ºC [6]. The pre-incubation of enzymes at 40 ºC and 50 ºC greatly increased laccase activity [7]. Laccase produced by *T. modesta* was fully active at 50 ºC and was very stable at 40 ºC but half-life decreased to 120min at higher temperature of 60 ºC [8]. The use syringaldazine as a substrate and determine the effect of pH on enzyme activity in the range of 3.0–8.0 [9]. Laccase extracted from *Stereum ostrea* showed the highest activity at pH 6.0 and 40 ºC temperature [10]. Laccases are generally produced in low concentrations by laccase-producing fungi, but higher concentrations were obtained with the addition of various supplements such as xenobiotic compound to media [11]. Veratryl alcohol is an aromatic compound; its addition to cultivationmedia results in an increase of laccase production [12]. A low concentration of Cu+2 to the cultivation media increases the laccase production 50 times in comparison to basal medium [13]. A new basidiomycete, *Trametes* sp. 420, produced laccase in glucosemedium and in cellobiosemedium with induction by 0.5mMand 6mMo-toluidine [14]. The present study of xenobiotic-degrading extremophiles may also help in evaluating their use in bioremediation of contaminated saline and/or alkaline environments.

II.MATERIAL AND METHODS

2.1. Physico-chemical properties of Laccase

2.1.1. Optimum temperature of purified Laccase

The optimum temperature was determined from 20°C to 90 ºC. Ten µg of partially purified laccase protein was dissolved in 0.1 M sodium acetate buffer and incubated at different temperatures as 30 ºC, 40 ºC, 50 ºC , 60 ºC, 70 ºC and 80 ºC for a period of 30 hours and assayed.

2.1.2. Optimum pH of purified Laccase

Optimum pH of the partially purified laccase was determined using 0.1 M citrate-phosphate buffer (pH 2.5 – 3.5), sodium acetate buffer (pH 3.5-5.5) and Tris-HCl buffer (pH 7.0-9.0). Ten µg of partially purified protein was incubated at different pH from 2.5 to 9.0, and assayed using ABTS as substrate.

2.1.3. Effect of carbon sources on laccase production

Bacillus subtilis was inoculated in to the basal medium at different types of glucose, sucrose, lactose, maltose and fructose 2 % amended with media. Those flasks were incubated in a shaker at 120rpm for 48 hours. The extra cellular laccase production and biomass were calculated after the incubation period.

2.1.4. Effect of nitrogen sources on laccase production

Bacillus subtilis was inoculated in to the optimized carbon source basal salt production medium at different type of nitrogen sources namely Peptone, yeast extract, ammonium nitrate, ammonium chloride and ammonium sulphate 0.5 % amended with media. The maximum production of laccase and biomass were estimated.

1.2. Inhibition and substrate specificity studies of purified Laccase

Effects of potential inhibitors on laccase activity were determined with 20 µM (ABTS) as the substrate in 50mM phosphate buffer (pH 7.0) and the presence of an inhibitor. The effects of L-cysteine, NaN_3, dithiothreitol (DTT), tropolone, *p*-coumaric acid, kojic acid, acetylacetone, ethylenediaminetetraacetic acid (EDTA), and thioglycolic acid on its activity were determined after 10 min of incubation of the enzyme with the various inhibitors at 30 ºC. Substrate oxidation by the laccase was examined spectrophotometrically at the specific wavelength of each substrate in 50mM of phosphate buffer, pH 7.0 at 30 ºC and the enzyme concentration used was 3.94×10−5 µmol.

1.3. Enzymeatic Degradation of 2.4-DiChlorophenol by Laccase

The degradation of 2.4-Dichlorophenol was performed in 20% acetone in 50 mM malonate buffer (pH 4.5) containing 15 µg ml−1 of each compound, 1 mM MnSO4, 30 mM glucose, 0.5 mM glutathione, 120 mUml−1 glucose oxidase and 850 mUml−1 of the enzyme. The reaction proceeded at 25 ºC in the dark using agitation (90 rpm); five replicate samples were used for each compound. For metabolite analysis, the reaction mixture was acidified with HCl to pH ≈ 3 and the samples were extracted with three portions of ethylacetate. The extracts were concentrated using a rotary evaporator and injected into a gas chromatograph (GC). The intermediates were identified using gas chromatography coupled with mass spectrometry (GC-MS) with an ion trap detector (GCQ, Finnigan, USA). The GC instrument was equipped with split/splitless injector and an HP-5 column was used for separation (30 m, 0.25 mm inner diameter, 0.25 µm film thickness). The temperature program started at 60 ºC and was held for 1 min in splitless mode. Then the splitter was opened and the oven was heated to 150 ◦C at a rate of 25 ºC min^{-1}. The second temperature ramp was up to 260 ºC at a rate of 10 ºC min^{-1}, with this temperature maintained for 20 min. The solvent delay time was set to 5 min. The transfer line temperature was set to 280 ºC. Mass spectra were recorded at 1 scan s−1 under electron impact at 70 eV, mass range 50-350 amu. The excitation potential for the MS/MS product ion mode applied was 0.5 V, and 0.9 V in the case of more stable ions. Methane was used as a medium for chemical ionization (CI).

III.RESULT

3.1. Laccase producing bacteria isolated from agriculture land soil of three different localities of Tamil Nadu by Serial dilution method

The bacteria were isolated from three different localities of Tamil Nadu soil namely Salem, Erode and Kumarapalayam. Screening of all isolated Bacteria for ligninolytic activities was performed using petridish contain malt extract–agar medium, supplemented with 1 mM Guaiacol. The strain salem soil isolated bacteria alone was exhibited a fast and large oxidation of Guaiacol on agar plates, as demonstrated by the dark reddish brown color appeared in the plates than other isolated bacteria it was taken for further study.

3.2. Effects of temperature on the stability of purified laccase

The optimum temperature for its activity was 40 ˚C. Thermostability analysis indicated that when the laccase was incubated for 1 h above 40 ºC, it gradually became inactivated. It was, however, stable for up to 1 h at 4 ºC and its respective half-life times at 25 and 40 ºC were estimated at 150 and 20 hours. (Table-1).

3.3. Effect of different pH **for the production of Laccase from *Bacillus subtilis***

Liquid state fermentation was carried out using low nitrogen basal mineral salts solution as different pH ranging from 5.0 to 9.0 Maximum production of enzyme (200 U/ml) and protein 123 mg/ml were observed at pH 8.0 (Graph-1a and Graph 1b).

3.4. Effect of different carbon source for Laccase production from *Bacillus subtilis* in liquid state fermentation

Four different carbon sources such as dextrose, sucrose, maltose and fructose were tested at 2 % for Laccase production in *Bacillus subtilis*. Among the carbon sources, fructose supported a maximum Laccase activity of 213U/ml with a highest extracellular protein of 124 mg/ml at 36 hours (Graph-2a and Graph-2b).

3.5. Effect of different nitrogen source of Laccase production from *Bacillus subtilis* in liquid state fermentation

Five different nitrogen sources such as Yeast extract, peptone, ammonium sulphate, ammonium nitrate and ammonium chloride were tested for extracellular protein and Laccase production in *Bacillus subtilis* among them peptone supported a maximum extracellular protein content of 132 mg/ml at 36 h and Laccase production of 224U/ml at 36 hours (Graph-3a and Graph-3b).

3.6. Effects of inhibitors on the stability of purified laccase

The sensitivity of the strain *Bacillus subtilis* laccase to several putative laccase inhibitors is shown in (Table-4). Most of its activity (>95%) was inhibited by 1mM l-cysteine, 0.1mM DTT, 1mM *p*-coumaric acid, 1m Mkoujic acid, and 1mM thioglycolic acid. Of the metal chelators tested, 1mM *p*-coumaric acid and 1mM thioglycolic acid had strong inhibitory effects, whereas NaN_3, tropolone, acetylacetone, and EDTA produced little or no inhibition on the enzyme reaction.

3.7. Enzymatic degradation of xenobiotic compound (2, 4-Dichlorophenol degradation)

To study degradation of selected xenobiotic 2, 4- Dichlorophenol degradation tested their solubility in a mixture of acetone-water with various ratios and also measured laccase activity with respect to inhibition by acetone. Following these results selected for further experiments a mixture with 20% acetone that was sufficient to dissolve 15 μgml^{-1} of each xenobiotic compound and caused less than 20% enzyme inhibition. The activity of the enzyme during experiments in the water–solvent mixture was stable. The degradation results of xenobiotic

tested only 2, 4- Dichlorophenol degradation exhibited a concentration decrease after 168 hours to 10.1 μgml^{-1} when compared to the control treatment without the enzyme. The enzyme was able to decompose all the 2, 4-Dichlorophenol at different rates. The current status were able to detect major distinct peaks only for 2, 4-Dichlorophenol degradation. Their mass spectral characteristics are given in Graph 4 and they were identified 2, 4- Dichlorophenol degradation respectively.

IV.DISCUSSION

Chlorophenols are important industrial raw materials for producing dye, preservatives, pesticides and chemicals. However, the industrial waste water containing the refractory organics such as chlorophenols is seriously harmful for the environment and human health. A number of physical and chemical processes have been proposed as having potential application in removal of chlorophenols [15]. However, these processes were not environmental friendly. Laccase-mediated removal of chlorophenols is one biochemical process which are low-cost, environmentally friendly and high efficient. Biochemically, laccase [EC.1.10.3.2] is a blue copper oxidase that catalyzes the one-electron oxidation of organic substrates coupled to the four-electron reduction of molecular oxygen to water [16, 17]. Most recently, some laccases from white-rot fungi were found able to catalyze a number of phenolic products, such as chlorophenols (CPs), via various pathways. Laccase from Panus conchatus removed 72.5% trichlorophenol (TCP), a representative of an important class of chlorophenols [18]. Laccase from *Bacillus subtilis* was the major ligninolytic enzyme produced in liquid culture. Submerged fermentation has been considered as an efficient method for enzyme production in biotechnological process due its potential advantages and high yield. In this study, selected submerged fermentation using low nitrogen salt solution. In present study observed that *Bacillus subtilis* strain produced high levels of laccase and very low levels of MnP during the 36 hours incubation. The high level of laccase production could be attributed due to the presence of ferulic acid in wheat bran which was shown to be a better platform for *G. lucidum* growth and production of xylanase enzyme [19](Malarvizhi et al., 2003). Moreover, this is due to the fact that the wheat bran provides the fungus, an environment similar to its natural habitat which is conducive for the high secretion of ligno-cellulolytic enzymes. Copper ions were added to the medium during enrichment culture. It is well known that copper ions are toxic even at low concentrations to lots of bacteria. However some bacterial laccases, such as CueO and PcoA play a role in copper tolerance [20]. The regulation of copper homeostasis of *E. coli* has been analyzed, although the mechanism is still unclear [21]. The main mechanism of CueO in copper resistance has been postulated to be the oxidation of the Cu+ to Cu2+ [23]. This process is effective for copper resistance because the Cu+ is more harmful than Cu2+ [23]. The present study is the first report that demonstrates copper resistance of spore protein. *B. subtilis* can survive in copper-containing medium. However, the strain *B. subtilis* is unable to form melanin-like pigment in the medium containing copper ions though CotA of *B. subtilis* was associated with the formation of a brownish pigment [24, 25]. The strain, *B. subtilis*, also showed a strong endurance to high concentrations of NaCl; it can survive in 10% NaCl. This advantage makes it potentially useful to deal with wastewater containing saline solution and to reduce the time of pretreatment [26]. The pH optimum of the enzyme was broad and slightly higher than that of other laccase purified so far; also, the

temperature optimum was relatively high, although the stability at elevated temperatures was low. Although laccases are mostly secreted in multiple forms [27] SDS-PAGE indicated the presence of only one acidic isoform of *B. subtilis* laccase, whose molecular mass (67 kDa), the molecular masses estimated by gel filtration chromatography and 62 kDa as determined by SDS-PAGE. Biodegradation of chlorophenols by bacterial species has been studied for long time as it is a rather effective approach [28]. Although bacteria individuals can detoxify these materials, if the pollutants are produced on a large scale, then bacteria may be not the ideal organisms to biodegrade of them. It is well-known that laccase-mediated biodegradation systems are succeeding to deal with it [29]. Most laccases from fungi have been proved to possess the ability to degrade most of chlorophenol mixture. Laccase from *Trametes pubescens* was evaluated had the ability to degrade a chlorophenol mixture. In the previously report showed by [30] that 82% of TCP (15 mg/L) is degraded after 4 h reaction under the optimum condition. Laccase-producing fungi have also been reported to be useful tools for xenobiotic removal in liquid effluents as well as in soil bioremediation [31]. The degradation of xenobiotic by peroxidation enzyme was first described in *Phanerochaete chrysosporium* as a lipid peroxidation-dependent process [32]. It was later demonstrated that xenobiotic degradation by some MnPs also occurs directly: MnP from *Nematoloma frowardii* degrades anthracene, phenanthrene, pyrene, fluoranthene and benzo[a]pyrene, leading to partial mineralization [33]. The major degradation products of the studied 2, 4-D degradation, but current status were able to detect major distinct peaks only for 2, 4-D degradation. Their mass spectral characteristics were identified 2, 4-D degradation respectively. The oxidation of 2, 4-D as a temporal intermediate [34]. The 2, 4-D degradation experiments showed that MnP isolated from *I. lacteus* was able to efficiently degrade three and four ring 2, 4-D compounds with IP higher than 7.8 eV which are not degraded by the enzyme from *S. coronilla*. The MnP from *I. lacteus* had a tolerance to acetone comparable to that of MnP from *Bjerkandera adusta* and *Phanerochaete chrysosporium* [35].

V.CONCLUSION

The optimum temperature for its activity was 40 ˚C. It was, however, stable for up to 1 h at 4 ˚C and its respective half-life times at 25 and 40 ˚C were estimated at 150 and 20 h. Maximum production of enzyme (200 U/ml) and protein 123 mg/ml were observed at pH 8.0. Among the carbon sources, fructose supported a maximum Laccase activity of 213 U/ml with a highest extracellular protein of 124 mg/ml at 36 h. From different nitrogen sources peptone supported a maximum extracellular protein content of 132 mg/ml at 36 h and Laccase production of 224 U/ml at 36 h. Most of its activity (>95%) was inhibited by 1mM l-cysteine, 0.1mM DTT, 1mM *p*-coumaric acid, 1m Mkoujic acid, and 1mM thioglycolic acid. Of the metal chelators tested, 1mM *p*-coumaric acid and 1mM thioglycolic acid had strong inhibitory effects, whereas NaN_3, tropolone, acetylacetone, and EDTA produced little or no inhibition on the enzyme reaction. The major degradation products of the 2, 4-Dichlorphrnol degradation by lacase, current status were able to detect major distinct peaks only for 2, 4-Dichlorphrnol degradation

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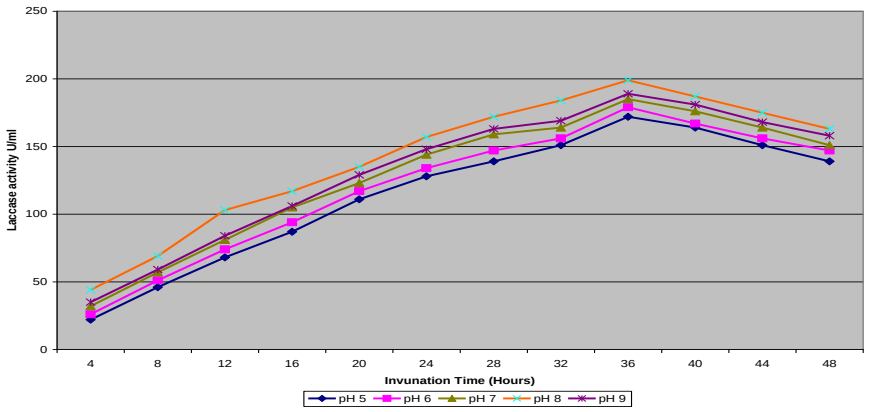
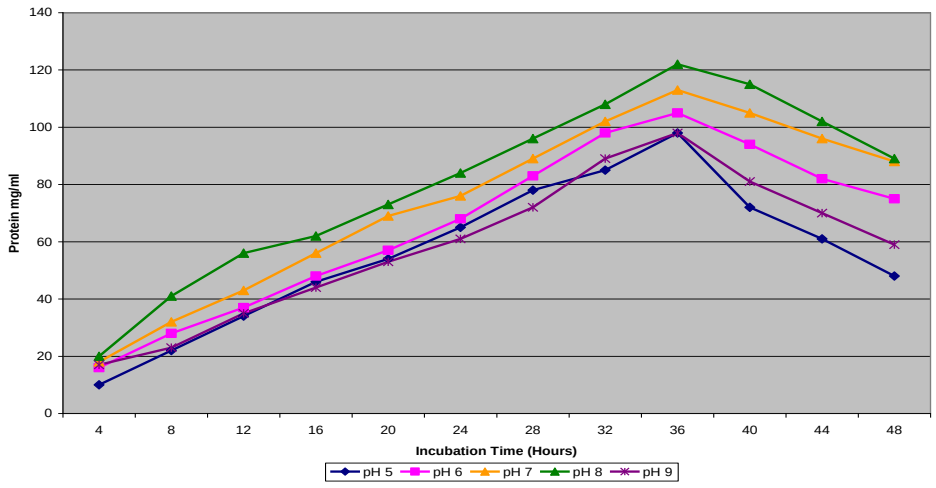
Graph-1a. Effect of different pH for the production of Laccase from Bacillus subtilis



Graph-1b. Effect of different pH for the production of Laccase protein from Bacillus subtilis



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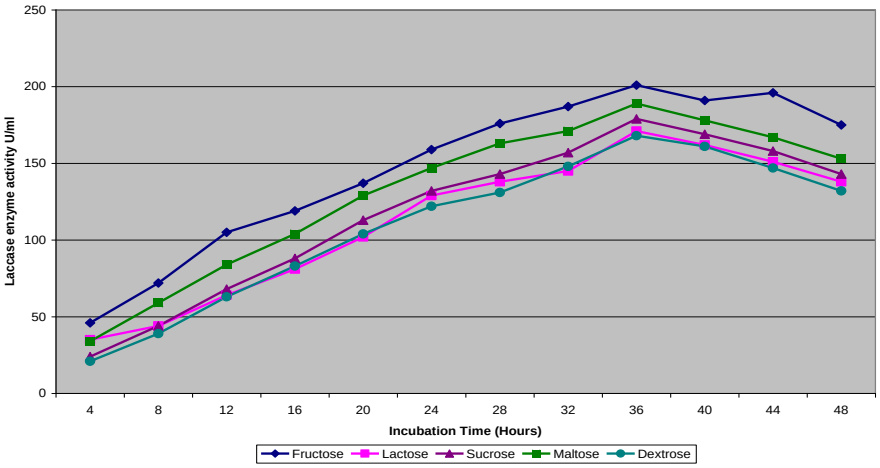
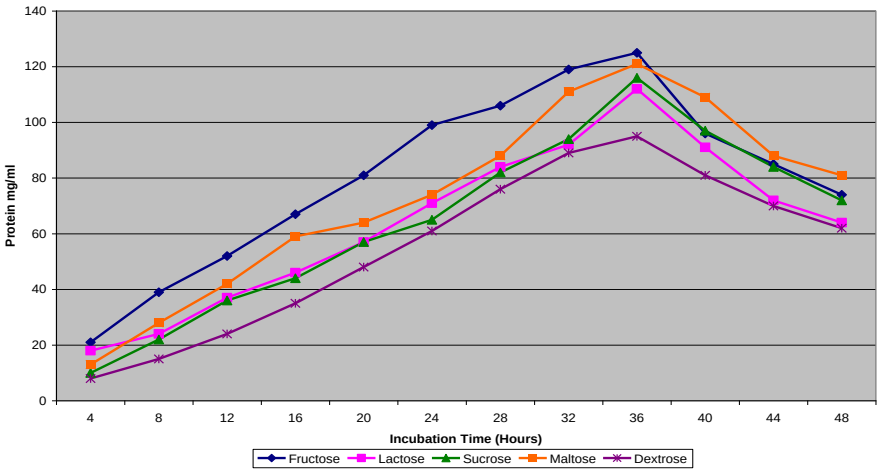
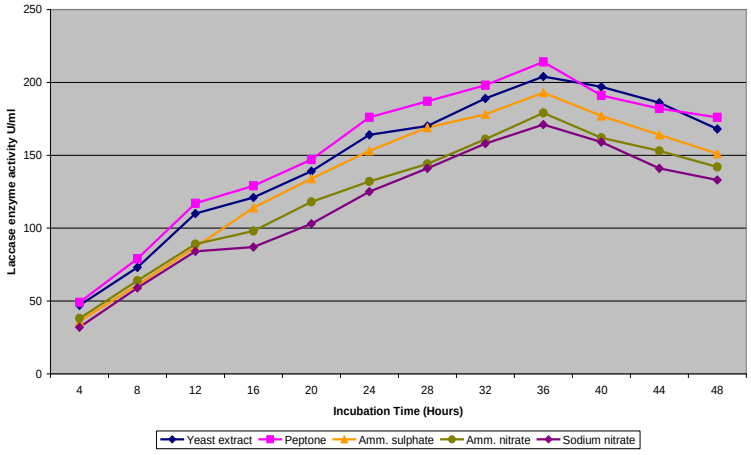
Graph-2a. Effect of different carbon source for Laccase production from Bacillus subtilis in liquid state fermentation



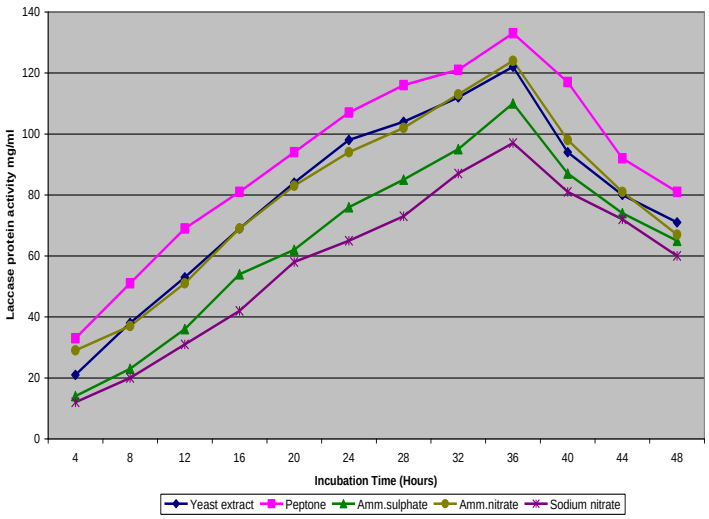
Graph-2b. Effect of different carbon source for Laccase protein production from Bacillus subtilis in liquid state fermentation



Graph-3a. Effect of different nitrogen source of Laccase production from Bacillus subtilis in liquid state fermentation

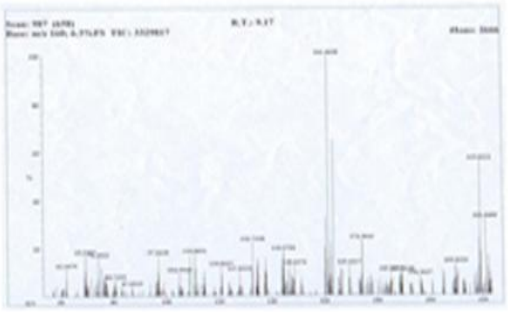


Graph-3b. Effect of different nitrogen sources for the production of Laccase (Protein) from Bacillus subtilis



Graph 4. The enzyme was able to decompose all the 2, 4- Dichlorophenol degradation at different rates. Their mass spectral characteristics are identified 2, 4- Dichlorophenol degradation respectively.

Control



Treated

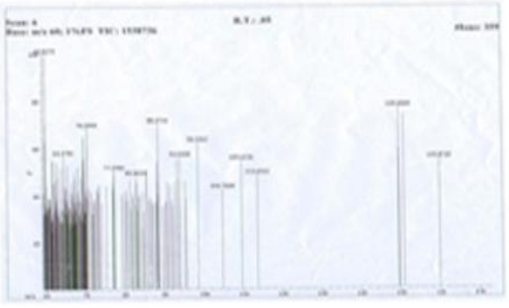


Table-1. Effect of Different Temperature on laccase Stability

Different Temperature	U/ml
30 ˚C	114
40 ˚C	122
50 ˚C	104
60 ˚C	78
70 ˚C	66
80 ˚C	52

Table-2. Effect of putative laccase inhibitors on oxidation of syringaldazine by purified laccase

Compound	Concentration (mM)	Inhibition (%)
L-Cysteine	1.0	96
NaN_3	10.0	53
DTT	0.1	100
Tropolone	6.0	58
p-Coumaric acid	1.0	94
Kojic acid	1.0	93
Acetylacetone	10.0	89
EDTA	25.0	1
Thioglycolic acid	1.0	94