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Journal of Advanced Scientific Research

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ISSN
0976-9595
Research Article

PRODUCTION AND APPLICATION OF LACCASE IN BIOTRANSFORMATION OF LIGNOCELLULOSIC MATERIAL AND DECOLOURIZATION OF DYES

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Received: 25-10-2021; Revised & Accepted: 13-03-2022; Published: 30-04-2022

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ABSTRACT

Cladosporium cladosporiodes is a novel strain isolated and evaluated for the production of laccase in submerged fermentation. Objective of present work was to optimize the laccase production and assessment of delignification in wheat bran by laccase of *Cladosporium cladosporiodes* in submerged fermentation. The significant laccase production was observed in Byred's medium when grown on wheat bran at static condition. The optimum conditions i.e. pH 5.6, temperature 40°C, wheat bran 4g, $CuSO_4$ concentration 0.1mM were required for laccase production in submerged fermentation and there was no any role of tested six aromatic inducers on laccase production. The extracellular laccase enzyme of *Cladosporium cladosporiodes* produced from lignocellulosic material fermentation involved in delignification of wheat bran, biotransformation of lignin model compounds and decolourization eight different azo, metal complex and triphenyl methane dyes.

Keywords: *Cladosporium cladosporiodes,* Decolourization, Laccase, Wheat bran.

1. INTRODUCTION

Particularly submerged fermentation is a suitable process for the production of enzymes by filamentous fungi. In recent years, the trend has been increased towards the utilization of organic waste residues from forestry, agricultural and alimentary industries as raw materials to produce value-added products such as single-cell proteins, mushrooms, enzymes, ethanol and biologically active secondary metabolites by submerged fermentation technique [1-4].

Lignocellulosic materials are renewable biomass and are most abundant in nature; therefore making use of lignocellulosic materials in various industries are of great importance. Lignocellulosic materials comprises three major group of polymers such as lignin, hemicellulose and cellulose. One of the objectives of biotechnology is the utilization of agricultural and food industry wastes for the production of protein, animal feed, energy, chemicals and combating the pollution of environment [5, 6]. For this reason, many researchers have focused their attention on potential application of lignin degrading microorganisms and their ligninolytic enzymes have received worldwide attention in pretreatment of lignocellulosic substrates for the production of liquid and gaseous fuels [7-10]. Recently, intensive research has revealed the potential of such organisms and enzymes in decolourization of industrial effluents, degradation of environmental pollutants, biopulping and biobleaching in paper industries [2, 3, 11], production of improved cattle feed and biosorption of heavy metals from effluents [12, 13].

Extracellular degradation systems of ligninolytic fungi are basically nonspecific and have been studied as the possible agents of biodegradation, a fact that allows the degradation of mixture of refractory substances [12]. Laccase, Manganese peroxidase and Lignin peroxidase are the major enzymes of white-rot fungi associated with the lignin degrading system [14]. Laccase enzymes are produced in large quantity and these enzyme based decolourization was potentially advantageous to bioremediation technologists and requires less fastidious induction conditions than either MnP or LiP and applications of laccase enzyme in dye decolourization has been shown by many others [15-17].

The aim of the present study is to assess the production of extracellular laccase from *Cladosporium cladosporiodes*

in submerged fermentation by using lignocellulosic materials and applications of laccase in decolourization of different synthetic dyes, bio-transformation.

2. MATERIAL AND METHODS

2.1. Chemicals

The chemicals and dyes used were of highest purity and analytical grade. The following composition (g L^{-1}) of mineral salts medium (MM1 medium) used for the submersed fermentation experiments: KNO_3 5.0, $MgSO_4.7H_2O$ 1.0, K_2HPO_4 2.5, $MnSO_4.H_2O$ 0.001, $CuSO_4.5H_2O$ 0.003, $FeCl_3$ 0.02, Na_2SO_4 1.0. $ZnSO_4.7H_2O$ 0.01 and $Na_2MoO_3.2H_2O$ 0.0015. The mineral salts medium was boiled and pH was adjusted to 5.6 after filtration [18]. For maintenance and propagation of the fungal culturepotato-dextrose agar medium was used.

2.2. Role of different lignocellulosic materials, concentration on laccase production

Studies were conducted in Erlenmeyer flask (250 ml) containing mineral salts medium (100 ml) with different lignocellulosic substances such as corn cobs, oat saw dust, coconut coir rice bran, wheat straw, bagasse and sterilized at 121°C for 15 min and then inoculated with 1cm^2 of fungal mycelium. The effect of selected lignocellulosic material concentration (1-5%) on laccase production was studied at static and shaking conditions.

2.3. Effect of temperature, pH, aromatic inducers, $CuSO_4$ on laccase production

The effect of pH ranging from 3-8 and temperature ranging from 20-60°C on laccase production in flask containing Byred's medium with selected lignocellulosic material were studied. In all the experiments, an un-inoculated culture flask was used as a control. The effect of different concentrations $CuSO_4$ (0.05-0.80 mM) and aromatic inducers on laccase production were also studied (20 mg per 50 mL of MM1 medium).

2.4. Dye decolourization, biotransformation of lignocellulosic materials by partially purified laccase

The eight-day-old culture supernatant was collected by centrifugation at 10,000 × g for 10 min at 4°C. Slowly solid ammonium sulfate powder was added to the culture supernatant up to 40% saturation and then resulting precipitate was removed by centrifugation (15,000 × g for 20 min at 4°C). Again solid ammonium sulfate was added to the 40% saturation fraction up to 80% and then precipitate of 40-80% saturation was collected by centrifugation (15,000 × g for 20 min at 4°C). This precipitate was re-dissolved in 10 ml of citrate buffer (25 mM, pH 3.5) and dialyzed overnight with two changes of fresh citrate buffer (25 mM, pH 3.5). This supernatant was used as a partially purified laccase after removing of unwanted precipitated material by centrifugation (3000×g for 10 min at 4°C). By using partially purified laccase, the biotransformation of aromatic compounds and the decolourization of structurally different dyes were determined over 20 min and 15 min time periods, respectively. The 2 ml of assay reaction mixture contains 10 units of enzyme solution and 0.2mg of respective aromatic compounds or dyes in 25 mM citrate buffer (pH 3.5) and was analyzed from 200 to 600 nm at intervals of 5 min.

2.5. Enzyme assay

Spectrophotometrically the laccase enzyme activity was determined by measuring the increase in absorbance at 420 nm of 0.5 mM 2, 2'-azino-bis (3-ethylbenz-thiazoline-6-sulphonate) ($\varepsilon 420 = 36{,}000\ M^{-1}\ cm^{-1}$) in sodium acetate buffer (50 mM, pH 3.5) [19]. Per minute the amount of enzyme required to oxidize 1 μmol of substrate into products was defined as one unit of enzyme activity.

2.6. Extraction of metabolites

After standardization of various physical parameters, the flasks were kept for 30 days and un-inoculated flask was used as control. The submerged fermentation medium was filtered through the cheese cloth and this filtrate was centrifuged at 8,000 x g for 20 minutes. The pH of supernatant was adjusted to 2-4 with dilute HCl (4N) and transferred to separating funnel containing two volumes of ethyl acetate, mixed well and separated ethyl acetate fraction was evaporated to small volume and subjected to various chromatographic techniques for the metabolite analysis.

2.7. Statistical analysis

The values are mean of three independent observations and statistics are calculated using the parametric statistic programmer, version 1.01 (Chagrin Falls, OH, USA, Lundon Software, Inc.).

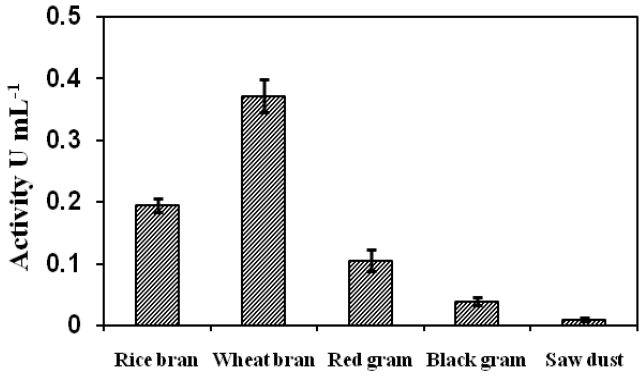
3. RESULTS AND DISCUSSION

Solid lignocellulosic materials used acted as either inert or non-inert materials for the cultivation of fungi. Non-inert materials are called support substrate, which function as an attachment place and also supplies some

nutrients to the fungi, while inert materials only act as an attachment place for the fungi [20]. Lignocellulose based agricultural wastes contain lignin, hemicelluloses and cellulose, which are rich in sugar, these wastes can be utilized as support substrate. The submerged fermentation approach can contribute to the production of industrially valuable products such as laccase enzyme, which was used in the pretreatment of bio-transformation of lignin model aromatic compounds, lignocellulosic substrates and decolourization of different synthetic dyes.

3.1. Effect of different lignocellulosic materials

The suitable carbon source screening is an essential step for the production of laccase by the *Cladosporium cladosporiodes*. In the present study, various lignocellulosic substrates with different structural compositions were used for achieving higher laccase production as well as to diminish the production cost of fermentation process.



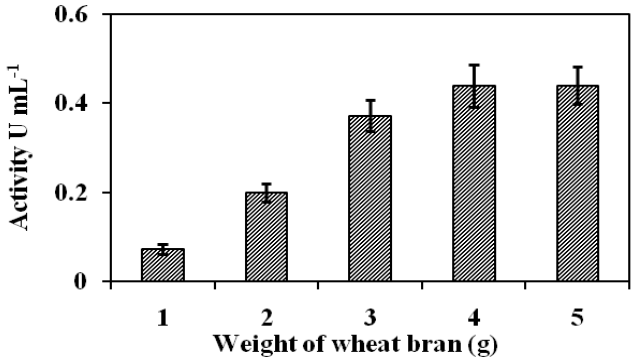
Results are the average of triplicate flasks

Fig. 1: Role of different lignocellulosic materials on laccase production by *Cladosporium cladosporioides*

Fig. 1 depicts the effect of various lignocellulosic materials (3% in MM1 medium) on laccase production by *Cladosporium cladosporiodes*. Among these lignocellulosic substrates tested for laccase production, wheat bran showed maximum laccase activity of 372 U L^{-1} followed by rice bran 194 U L^{-1}, red gram 105 U L^{-1}, black gram 39 U L^{-1} and very low activities was detected from the saw dust 9 U L^{-1}. Erden *et al.* (2009) reported that the maximum laccase activity 47.09 U L^{-1} from *T. versicolor* cultivated in powdered hazelnut cobs [21]. *T. trogii* was obtained maximal laccase activity PASS containing media 350 U L^{-1} and *T. versicolor* showed maximum laccase activity 1216 U L^{-1}in media containing PB [6]. Gonzalez *et al.* (2013) showed the highest specific laccase activity 177 U L^{-1} under submerged conditions supplemented with copper (5 mM) with coffee husk as substrate [22]. These results indicates that different lignocellusic materials have different role in laccase secretion and also by *Cladosporium cladosporiodes*.

3.2. Effect of wheat bran concentration on laccase production

Fig. 2 shows the optimum concentration (1, 2, 3, 4 and 5% w/v) of wheat bran required for the production of laccase in submerged fermentation. The maximum laccase production was occurred at 4% of wheat bran and increase in wheat bran concentration (1-4%) increases the production of laccase 73 U L^{-1}, 200 U L^{-1}, 372 U L^{-1}, 439 U L^{-1} respectively and above 4% it was constant (5% - 440 U L^{-1}). Laccase activity was detected on 3rd day and reached 439 U L^{-1}after 8 days at 4% wheat bran concentration and this concentration (4%) was selected for further experiments. Kachlishvili *et al.* (2014) reported the 89 U L^{-1} laccase productions by *Cerrena unicolor* at 4% wheat bran [23] and Chawachart *et al.* (2004) detected less extracellular laccase activity by *Coriolus versicolor* strain RC3 with wheat bran [24]. In 2010, Kurt and Buyukalaca investigated yield performance of maximum laccase of *P. ostreatus* and *P. Sajor-caju* cultivated on wheat bran [25]. The maximum laccase activity of *Cladosporium cladosporiodes* showed at 4% of wheat bran concentration and requires more carbon source for its heavy biomass to secrete laccase.



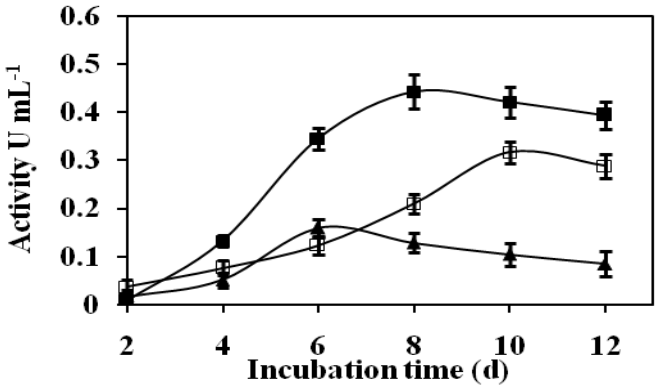
Results are the average of triplicate flasks

Fig. 2: Role of various concentrations of wheat bran on laccase production by *Cladosporium cladosporioides*

3.3. Role of Medium, shaking and static condition on laccase production

Three different media such as distilled water, buffer and MM1 medium were used for the production of laccase

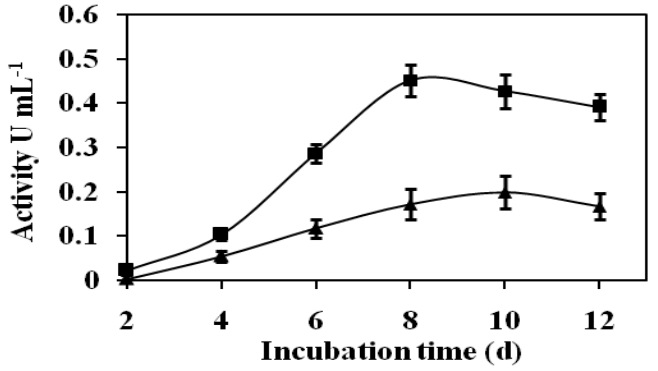
at 4% wheat bran concentration. The maximum production of laccase was observed in MM1 medium 443 U L^{-1} and slightly decreases the laccase production in sodium citrate buffer 317 U L^{-1}, distilled water (159 U L^{-1}) (fig. 3).



Results are the average of triplicate flasks

Fig. 3: Role of media on laccase production by *Cladosporium cladosporioides* (Water ▲ Buffer □; Byrde's medium ■)

From these results, it was concluded that MM1 medium required for the maximum laccase product by *Cladosporium cladosporiodes*. In the MM1 medium, effect of static and shaking conditions on the production of laccase was tested (fig. 4).



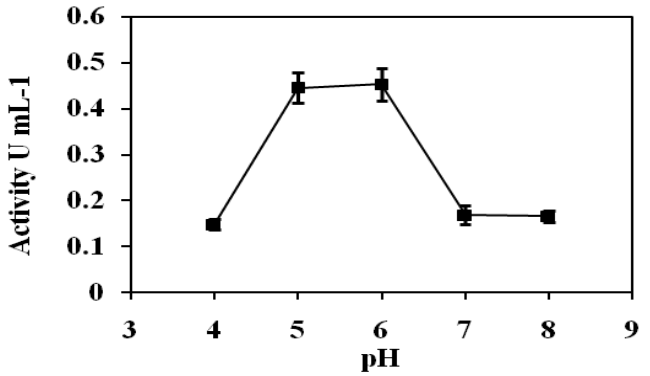
Results are the average of triplicate flasks

Fig. 4: Effect of shaking and static on laccase production by *Cladosporium cladosporioides* (Static ■; Shaking ▲)

The maximum secretion of laccase was obtained under the static condition (452 U L^{-1}) and very less amount of laccase was produced in shaking (172 U L^{-1}) condition. At the static condition *Cladosporium cladosporiodes* secrets the laccase in the liquid medium by growing on the surface and in shaking condition laccase secretion decreases because of lack of mycelial growth or enzyme may denatured by interaction with wheat bran particles. Hess *et al.* (2002) showed that considerably decreased laccase production by *Trametes multicolour* and when fungus was grown in the stirred tank reactor, mycelia were damaged [26] and Mohorciˇcˇ *et al.* (2004) showed that cultivation of white-rot fungus *Bjerkanderaadusta* in a stirred tank reactor with very low enzyme activities [27].

3.4. Effect of pH and temperature on laccase production

The optimum pH for the production of laccase was determined over a pH range of 4-8 by changing pH of MM1 medium. The production of laccase was increased with increase in pH from 4.0 - 6.0 (fig. 5) and maximum laccase production was obtained at pH 6.0 (452 U L^{-1}). Further, increase in the medium pH above 6.0 (pH 7.0-168 U L^{-1}) will decreases the laccase secretion and the variation in pH affects the growth of the fungus *Cladosporium cladosporiodes*. On either side of this, optimum pH range decreases the growth of the fungus and simultaneously decreases the secretion of laccase. Fungi produced excess of laccase at pH 5.0 and most of the studies showed that pH between 4.5 and 6.0 is suitable for enzyme production [28].

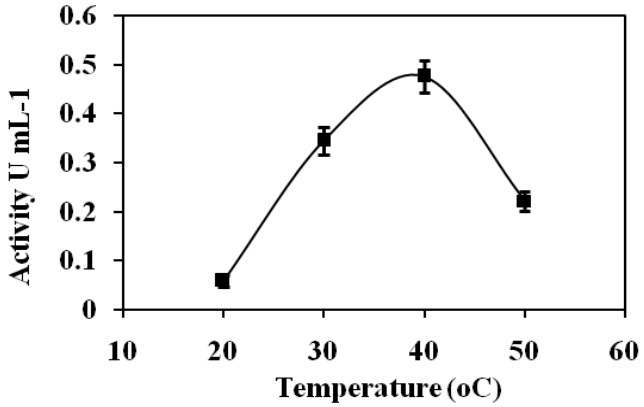


Results are the averages of triplicate flasks

Fig. 5: Effect of pH on laccase production by *Cladosporium cladosporioides*

Temperature is one of the most important physical parameters for the production laccase. Traditionally mesophilic range is used, whereas degradation is too slow within the psychrophilic range and maintaining high temperature is generally thought uneconomical [29]. In order to determine the optimum temperature for the production of maximum laccase over a

temperature range 20-50°C was studied. Fig. 6 showed that increase in temperature from 20°C-40°C increases the production of laccase and maximum laccase (477 U L^{-1}) secretion was observed at 40°C.



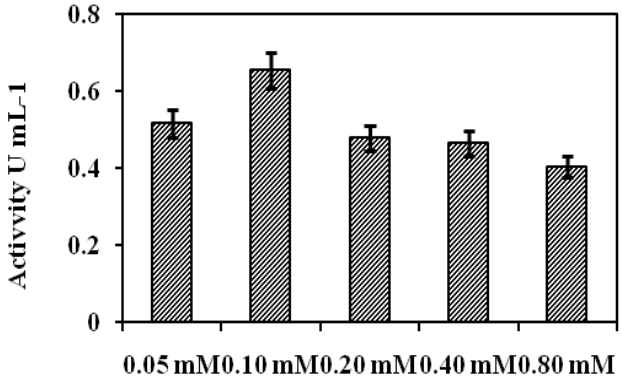
Results are the averages of triplicate flasks

Fig. 6: Effect of temperature on laccase production by *Cladosporium cladosporioides*

Increase in temperature up to 50°C, the secretion of laccase decreases (222 U L^{-1}). Further, all studies for the production of laccase were carried out at the optimum temperature of 40°C.Farnet *et al.* (2000) reported that the optimum temperature range is between 25°C-30°C for the production of laccase [30]. The present study results suggested that the optimum pH 6.0 and temperature of 40°C were required for maximum laccase production using wheat bran (4%) as a substrate in submerged fermentation by *Cladosporium cladosporiodes*. Hence, it can be fulfilled that pH and temperature exerts a similar effect on laccase secretion and growth irrespective of the mode of fermentation.

3.5. Effect of $CuSO_4$and aromatic compounds on secretion of laccase

Copper is a micronutrient, induces both laccase transcription and production, also has a key role as metal activator [31]. Hence, the effect of copper was a complex mechanism on laccase secretion by *Cladosporium cladosporioides*. Wheat bran based medium supplemented with various concentrations of $CuSO_4$ (0.05 mM-0.80 mM) was studied for the production of laccase at 40°C without shaking. The production of laccase in the control experiment in which the addition of $CuSO_4$ to the growth medium was omitted was 120 U L^{-1} and marked increase in laccase activity observed even in supplementation low concentrations of Cu (fig. 7).



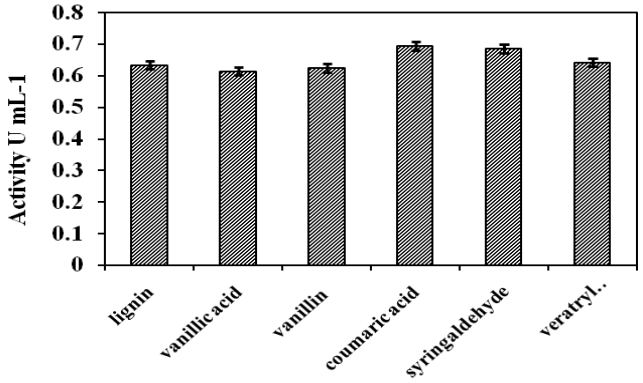
Results are the average of triplicate flasks

Fig. 7: Effect of various concentrations of $CuSO_4$ on laccase production by *Cladosporium cladosporioides*

The optimal copper concentration 0.10 mM required for laccase secretion by *Cladosporium cladosporiodes* and obtained maximum laccase activity was 654 U L^{-1}. Many reports showed that copper induced the secretion of fungal laccase by different *Trametes* species and *Coriolus versicolo*r, but at higher copper concentration, the fungal biomass decreases with laccase secretion. This is due to toxic effect of copper at higher concentration on the fungal culture [32, 33].

The next step was to enhance laccase production in submerged fermentation using putative aromatic inducers. No significant results obtained when different lignin model compounds used as an inducer. The different inducers such as Lignin, Vanillic acid, Vanillin, Coumaric acid, Syringaldehyde and Veratryl alcohol were added (20 mg per 50 mL of medium) before inoculation. Effect of different aromatic compounds on laccase production by *Cladosporium cladosporiodes* containing the basal level of Cu^{2+} (0.10 mM) was shown in fig. 8.

All six aromatic compounds Lignin, Vanillic acid, Vanillin, Coumaric acid, Syringaldehyde and Veratryl alcohol showed the presence fungal growth with laccase activity of 633 U L^{-1}, 613 U L^{-1}, 624 U L^{-1}, 693 U L^{-1} , 685 U L^{-1} and 641 U L^{-1}, respectively. The aromatic compounds were used to boost laccase secretion in other organisms [35, 36], but here, there was no significant induction of laccase by any of the lignin model compounds tested. The induction of laccase by lignin was reported in *Heterobasidion annosum* [34] and gallic acid, catechol, veratric acid and ferulic acid were induced production of laccase in *Trametes pubescens* and *Phlebia radiata* [35, 36].



Results are the average of triplicate flasks

Fig. 8: Effect of lignin and different lignin model compounds on laccase production by *Cladosporium cladosporioides*

3.6. Determination of lignin content and analysis of biotransformed products from wheat bran by a fungus

We have estimated the amount of lignin and percent of weight loss in the wheat bran sample of control (uninoculated) as well as in test sample (inoculated) and compared before and after treatment by the fungus. The amount of lignin estimated in the control sample was 257±1.5 mg g^{-1} and in the fungal treated sample was 187±1.4 mg g^{-1} of wheat bran. The percentage of weight loss in control sample was 5-7% and in the fungal treated sample was 35-39%. In both samples, 5-7% loss of weight is common because of the soluble carbohydrates passed through the filter and enters into the filtrate solution. These results showed that the biotransformation of wheat bran lignin in submersed fermentation by *Cladosporium cladosporiodes*.

Two volumes of ethyl acetate were used to extract metabolites from fermented medium, which was filtered through the cheese cloth. Extracted sample was then passed through GC-MS spectrometer and analyzed for the presence of different reduced and oxidized products of lignin from wheat bran. Here the low molecular weight compounds released from lignin degradation were analyzed by suitable technique (GC-MS) [37, 38]. The Total Ion Chromatography (TIC) corresponding to the compounds extracted with ethyl acetate from the acidified supernatant obtained from un-inoculated (control) and inoculated with *Cladosporium cladosporiodes* peaks identity were depicted in table 1. The compound released from lignin degradation may be identified as Ethanedioic acid, Benzene acetic acid, Propanedioic acid, Acetoguiacone, 3,4,5,-trimethoxy benzaldehyde, Bis-(2-methoxy ethyl) phthalate on the basis of peak of retention time at 10.5, 12.7, 13.1, 15.6, 18.6, 20.8. The observed results in the present study were similar to the results obtained by Raj *et al.* (2007) and Belanger *et al.* (2015) which showed the presence of lignin signatures (vanillyls, p-hydroxyls, syringyls, cinnamyls) by analysis of the interrelated terrigenous organic matter ofdominant plant species and soil horizon [39, 40]. From these results we confirmed that *Cladosporium cladosporiodes was* involved in the biotransformation of lignocellulosic material (wheat bran).

Table 1: The compounds identified in ethyl acetate extract from control and fungal degraded wheat bran products

Peak Retention Time	Control Figure	Test Figure	Retention time matched compounds
10.5	-	+	Ethanedioic acid
12.7	-	+	Benzene acetic acid
13.1	-	+	Propane dioicacid
15.6	-	+	Acetoguiacone
18.6	-	+	3,4,5,-trimethoxy benzaldehyde
20.8	-	+	Bis-(2-methoxy ethyl) phthalate

- Absent, + Present

3.7. Decolourization of dyes by laccase produced in submerged fermentation

The partially purified extracellular laccase of *Cladosporium cladosporiodes* was used for the decolourization of different synthetic dyes (50 mg L^{-1}). It was observed that acid black 210 and acid blue 193 were completely decolourized within 20 h, whereas Acid Orange 12, Crystal Violet, Fast Red E, Reactive Blue BL/LPR, Reactive Blue B (S), Acid Red 151 were partially decolourized in 20 h. Mainly effectiveness of decolourization depends on complexity and structure of each dye [41]. Relatively small structural differences can markedly affect decolourization.

4. CONCLUSION

The optimization of culture parameters influence the production of thermophilic laccase in submerge fermentation by *Cladosporium cladosporiodes* and involved

in bio-transformation lignin model compounds and decolourization of 8 different synthetic dyes. These results proved that properties of the secreted laccasewere potential for industrial and biotechnology applications.

Conflict of interest
None declared

Source of funding
None declared

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