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Research Article

OPTIMIZATION OF PROCESS PARAMETERS FOR LACCASE PRODUCTION BY SOLID STATE FERMENTATION FROM *TRAMETES ELEGANS H6*

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ABSTRACT

Laccases (E.C. 1.10.3.2) are multinuclear enzymes containing copper and belong to the class of oxidoreductase enzyme. The main function of laccase enzymes is to catalyse the oxidation of a variety of phenolic and inorganic compounds, with the consequent reduction of oxygen to water-H_2O. The aim of the present study was to obtain the most potent laccase producer in which after screening a total of 34 fungal strains were obtained from different samples of decayed wood, tree bark and soil from varying biotopes of Valsad and Vapi region of Gujarat, India. These isolates were subjected to primary screening for evaluating their phenol oxidase activity. A total of 16 strains exhibited brown color zone on malt extract agar plate containing orthodianisidine (0.01% w/v). In the secondary screening, Fungal isolate H-6 showed maximum laccase activity with ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) and identified as working strain in solid state fermentation . Isolate H6 was identified as a *Trametes elegans* using ITS sequencing. In the second phase of the study optimization of the process parameters such as incubation time, moisturing agent, innoculam size, pH, Temperature, carbon source and nitrogen source were carried out for the hyper production of laccase using *Trametes elegans H6*.

Keywords: Solid state fermentation, Laccase, White rot fungi, Optimization.

1. INTRODUCTION

Enzymes are biocatalysts, which play an important role in industrial scale processes due to their versatility and being highly selective in nature. Oxidoreductase is an enzyme that catalyzes the transfer of electrons from one molecule-the reductant, also called the electron donor, to another-the oxidant, also called the electron acceptor. Enzymes belonging to oxidoreductase class are of great importance because these enzymes can be used in many important industrial processes.

Laccases (EC 1.10.3.2) are multi-copper oxidases found in plants, fungi, and bacteria. Laccases oxidize a variety of phenolic substrates, performing one-electron oxidations. Most of the structural properties of laccase can be understood from the available literature which is based on study of the purified enzyme. Three dimensional structures revealed that laccases contain four catalytic Cu atoms-the T1 Cu and the tri-nuclear Cu cluster (T2 Cu, T3α Cu and T3β Cu) at the T2/T3 site. The T1 Cu is situated in domain 3 close to the protein surface, and T2 and T3 (α and β) Cu atoms are present at the interface of domains 1 and 3 [1].

White rot fungus, responsible for causing white rot on wood or trees, belongs to the basidiomycetes. Many researchers have reported that these fungi are most potent producer of extracellular laccase. Although, these fungi effectively break down lignin, they are unable to utilize it as an energy source, and it is assumed that they degrade it for its accessibility to the cellulose in the cell wall [2].

Many researchers have reported new microorganisms that can produce higher amount of laccase but the cost of production is quite high. This high cost of enzyme production can be reduced through the use of substrates which are inexpensive. Agricultural wastes such as ligno cellulosic substances are one of the most suitable substances which can be used for enzyme production. As these substances are formed in large amounts year after year, hence finding alternatives for reutilization of these wastes is the main aim that has been strongly taken into account by countries across the world. One of the appropriate approaches for this purpose is solid-state fermentation (SSF), which is defined as the growth of microorganisms on solid materials in the absence or near absence of free water.SSF mimics the natural habitat of

most part of microorganisms – mainly fungi and mold. In this condition, they are able to synthesize considerable amounts of enzymes and other metabolites [3].

Lignin is one of the prominent wastes produced by agricultural and paper pulp sectors. In nature, lignin is the major component of plant cell-wall, tightly bound with hemicelluloses, resulting in matrix formation surrounding cellulose fibres. Laccases are hydrolytic enzymes involved in lignin degradation, making it easier for other biodegradation activities. One of the prominent limitations of laccase is low substrate specificity, which can be applied tactfully towards degradation of structurally similar chemical compounds, such as polyaromatic hydrocarbons (PAH), textile dyes and other xenobiotic compounds. This feature makes laccases most appropriate for pulp delignification and pesticide degradation.

It is appropriate to realize that Vapi GIDC, being one of the largest industrial area of Gujarat, produces huge amount of industrial waste. Besides this, Valsad district of South Gujarat region is blessed with huge agricultural land, producing considerable agricultural waste. Use of enzyme formulations containing laccase shall be a good alternative towards both the types of waste and their treatments and would be a primary step towards abating environmental pollution. This necessitates screening wide variety of environmental samples for laccase producing microorganisms and their characterization.

With the same objective, the present study was aimed at screening of various environmental samples for laccase producing microorganism, with particular focus on fungal strains and their characterization. The study also focused on optimization of media parameters for maximizing laccase production, using solid substrate fermentation (SSF). The SSF process employed agricultural wastes at large to count upon the economics of the process.

2. MATERIAL AND METHODS

2.1. Materials

ABTS-2,2-Azino-bis (3-ethylbenzthiozoline-6-sulphonic acid), Malt Extract Agar (Himedia, Mumbai, India.), Ortho-dianisidine (Lobachemie, Mumbai, India), Thiamine Hydrochloride, Ammonium Nitrate Cupric Sulphate, Cobalt Chloride, Manganese Sulphate, Ammonium ferric citrate, Magnessiun Sulphate Calcium Chloride, Zinc Sulphate, Potassium Dihydrogen Phosphate, Tween 80 ,Glucose, Fructose, Sucrose, Maltose, Mannitol ,Mannose(Rankem Chemicals, Mumbai, India) Wheat straw (WS) Wheat bran (WB) Rice bran (RB) Rice straw (RS) Sugarcane bagasse (SB) (Collected locally and used as lignocellulosic substrates).

2.2. Screening of the Fungal Strain for the Production of Laccase

Samples of soil and decay wood were collected from Vapi and Valsad region for isolation of white rot fungi.

2.3. Primary Screening for Potent Strain by Plate Assay

Plate assay was performed to detect the laccase producing capacity in malt extract agar plate containing orthodianisidine (0.01% w/v) as a chromogenic substrate. Dark brown color formation indicated the production of laccase. The fungal isolates showing brown color zone were stored and maintained in Malt agar plate at 4°C.

2.4. Secondary Screening for Laccase Production on Solid Substrates

Fungal culture which exhibited positive results in primary screening were subjected to further evaluation for secondary screening under solid state fermentation using lignocellulatic substance.

2.5. Identification of the selected fungal isolate H6.

The cultures were identified on the basis of microscopic characteristics up to family at our laboratory. The molecular identification of the new fungal isolate H-6 was done by using ITS1 and ITS2 gene sequencing at Safron life sciences (Surat, India). Stock culture of the fungal isolates were maintained by sub culturing them at a regular time interval on 2% Malt Extract (ME) agar plate at 30°C and stored at 4°C.

2.6. Enzyme Production in Solid State Fermentation

For inoculum preparation, agar disc of 8 mm diameter taken from the stock culture was transferred on 2% Malt Extract (ME) agar plate with a sterilized cup borer and incubated at 30°C for 8 to 10 days. Freshly grown fungal mycelium culture was used to inoculate in 250 ml erlenmeyer flask containing 5 gm of rice straw as lignocellulatic substance and 20 ml asther medium as a mosituring agent. Contents in the flask were allowed to grow at 30°C for 10 days.

2.7. Extraction of Enzymes

Enzyme was extracted using 20 ml of sodium acetate buffer for maintaining pH 5. The contents of the flask

were mixed with buffer and crushed with the help of a glass rod and constantly shaken for 30 minutes. The whole content was then filtered through a four layered cheese cloth. The filtrate obtained was centrifuged at 5000 rpm for 20 minutes. The clear brown colored supernatant was collected and used as crude enzyme.

2.8. Enzyme Assays

Laccase activity (E.C. 1.10.3.2) was determined by measuring the oxidation of ABTS. Increase in absorbance was measured spectrophotometrically for 3 minutes at 420 nm. Supernatant obtained after centrifugation containing the enzyme extract was mixed with 100µl of 50 mM ABTS and 800 µl of 20 mM Sodium acetate buffer (pH 5.0) and 100 µl of appropriately diluted enzyme extract. One unit of enzyme activity was defined as the amount of enzyme that oxidized 1 µM of substrate per minute at room temperature [4].

2.9. Optimization of Process Parameters for Production of Laccase by *Trametes elegans H6*

The production of laccase enzyme were investigated as per the one-factor-at-a-time (OFAT) method of designing experiments to observe the possible optimum levels of the parameters by changing one value alone at a time. To determine the best process parameters, stepwise modifications of the production medium were done that affected fungus biomass and laccase production for H6 isolate. Enzyme activity was assayed using the standard assay method as described earlier. Optimum conditions were like wisely selected for further studies at each step.

2.9.1. Influence of Incubation Time (days)

The fungus isolate H6 was cultivated in rice straw for 18 days. After the specific days interval, the culture mycelium was separated from specific flask and enzyme activity was determined in filtrate. Thus,optimum incubation time was selected for further studies.

2.9.2. Influence of Moisturing Agent

Moisturing agents with different composition were used to evaluate their effect on laccase production in solid state fermentation. The composition of different moistening agents is as follows:

Following mediums were employed / devised / used for optimum growth and production of laccase:

Medium 1: Asther medium [5]. The medium was supplemented with Tween-80 (0.01 % v/v) and buffered with 20 mM phthalate buffer pH 5.5.

Medium 2: YMPG medium (Yeast extract, Malt extract, Peptone, Glucose [6]

Medium 3: Dhawan medium [7]. pH of the medium was adjusted to 5.4 with HCl 0.5 N.

Medium 4: Moistening agent 5 (MA 5) [8]. All components were dissolved in citrate buffer.

Medium 5: Olga medium.

Medium 6: Coll medium [9].

Composition of the mediums are listed in Table 1

2.9.3. Influence of Lingo Cellulosic Substrates

Isolate *Trametes elegans H6* was grown in 250 ml Erlenmeyer flasks containing 5 gm each of the lignocellulosic substrates - Wheat bran, Wheat straw, Rice bran, sugarcane bagassae and Rice straw, moistened with Asther medium and incubated at 30°C for 14 days.

2.9.4. Influence of Inoculum Size

The effect of inoculum size on laccase production was examined by taking different number of agar discs from 4 to 12 number of 8mm diameter from stock culture plate and inoculating these agar discs into the production medium containing Rice straw.

2.9.5. Effect of Medium pH

In order to evaluate the effect of initial culture pH on laccase production, the initial pH of Asther medium was adjusted between 2.5-7.0 using 1M HC1 and 1M NaOH, and then used as a moistening agent for rice straw in SSF process.

2.9.6. Effect of Temperature

In order to study the effect of incubation temperature on laccase production,solid state fermentation flasks were incubated in a temperature range of 20-50°C.

2.9.7. Effect of Co-substrates

To study the effect of different co-substrates on laccase production, various co-substrates like glucose, fructose, sucrose, maltose, mannitol, mannose were supplemented in Asther medium at a concentration of 10 gm/1it.

2.9.8. Effect of Nitrogen Source

To study the effect of nitrogen source on laccase production, both organic and inorganic nitrogen sources like ammonium sulphate, yeast extract, casein, peptone, urea, malt extract and gelatin were supplemented in Asther medium at a concentration of 0.2 gm/1it.

Table 1: Composition of the six mediums

Components	Medium Concentration in gm/lit					
	1	2	3	4	5	6
NH_4NO_3	0.5					
$CuSO_4.5H_2O$	0.007					
$CoC1_2.6H_2O$	0.007					
$MnSO_4.7H_2O$	0.035				0.05	
$MgSO_4.7H_2O$	0.05	1	0.035	0.5	0.5	1.0
Ca(NO3)2.7H2O			0.5			
$CaCl_2.2H_2O$	0.0132			0.1		
$ZnS0_4.7H_2O$	0.0462				0.001	
$FeS0_4.7H_2O$					0.0005	0.01
KCl				0.5		
$(NH_4)_2SO_4$				0.9		
KH_2PO_4	0.2	1	0.5	2.0	0.6	0.5
K_2HPO_4					0.4	
Ammonium Ferric Citrate	0.085					
L-aspargine	1.0	1.0				1.0
Thiamine-HCl	0.0025	1.0				
Yeast extract	0.5	2.0				0.5
Malt extract		10.0	20.0			
Glucose	10.0	10.0		2.0	10.0	10.0
Peptone		2.0			3.0	
Tween-80	0.01 % v/v					
pH	5.5	5.0	5.4	5.0	5.0	5.0

3. RESULTS & DISCUSSION

3.1. Primary Screening of the Fungal Strain for the Production of Laccase

A total of 88 samples of fruiting bodies and decay wood and bark were collected from various biosphere of Valsad and Vapi region.

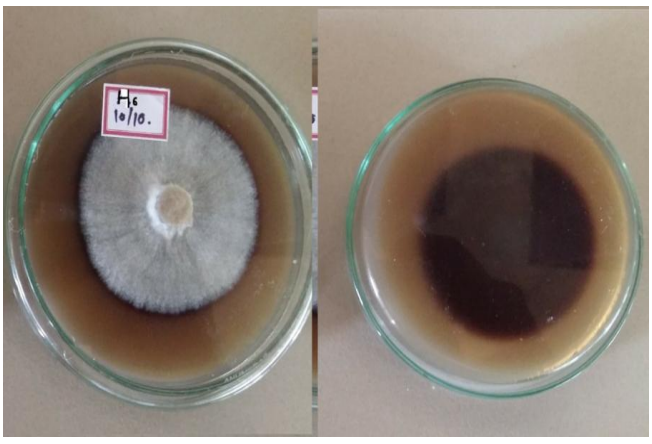

Fig. 1: Plate assay for Laccase on MEA plate containing ortho-dianisidine as chromogenic substrate

Total 34 fungal isolates were obtained from these samples. Out of 34 fungal isolates 16 fungal isolate gave positive results in the primary screening by exhibiting brown color zone on malt extract plate containing 0.01% ortho-dianisidine (Fig.1).

3.2. Secondary Screening for Laccase Production on Solid Substrates:

16 isolates that displayed positive results during primary screening were subjected to secondary screening by solid state fermentation. Out of the 16 isolates maximum activity was shown by isolate H6 (2306.66 U/gm) whereas isolate H10 shown lowest activity under solid state fermentation (Table 2). Isolate H6 was selected for the further study for the optimization of laccase production as it displayed excellent result in the production of enzyme.

3.3. Identification of the selected fungal isolate H6.

The *isolate H*6 was non-spore forming fungus under laboratory cultivation conditions and forms pure white cottony growth on MEA after 96-120 h of incubation

which is typical characteristics of basidomycetes fungi. Identification of isolate H6 was further corroborated by studies on its ITS I and ITS 2 gene sequences carried out by Safron Life Sciences, Gujarat, India. Blast similarity search analysis based on ITS 1 and ITS 2 gene sequences

Table 2: Secondary Screening of laccase production by various white rot fungus isolates

Strain No.	Isolate Code	Primary Screening Chromogenic Substrates (0.01%) ortho-dianisidine	Enzyme Production (U/gm of substrate)
1	H-1	+	383.33
2	H-2	+	233.33
3	H-3	++	760.00
4	H-4	+	132.001
5	H-5	+	250.0
6	H-6	++++	2606.636
7	H-7	++	486.115
8	H-8	+	383.33
9	H-9	++	577.761
10	H-10	+	111.1
11	H-11	+++	1111
12	H-12	+	344.43
13	H-13	++	577.761
14	H-14	++	396.669
15	H-15	+++	1099.96
16	H-16	++	737.75

+ - Poor producer, ++ - Moderate producer, +++ - Good producer and ++++ - Excellent producer of color on agar plate.

3.4. Optimisation of the process parameters for laccase production by laccase production by *Trametes elegans H6*

3.4.1. Influence of Incubation Time (Days)

Ligninolytic enzyme production was studied for varied incubation periods. Isolate *Trametes elegans H6* was grown on lignocellulic substrate with Asther medium as a moistening agent for the enzyme production. After 2-3 days of incubation initial fungal growth was observed and complete colonization of fungus on substrate was observed on 6thday of incubation. Laccase activities were found to increase with an increase in incubation period (Fig. 3). Maximum activity of laccase (3111.878U/gm) was obtained on 14thday of incubation. Moreover, gradual decline in laccase production was observed after the 14th day of incubation. This decrease can be attributed to the depletion of macro and micro nutrients in the fermentation medium which stressed the fungal physiology resulting in the inactivation of secretory machinery of the enzymes.

3.4.2. Influence of Moisturing Agent

From the experimental data obtained it was observed that amongst different media used, maximum enzyme production-3125 U/gm was observed in Asther medium at pH 5.5 (Fig. 4), whereas comparable laccase activity was recorded in YPMG-1755U/gm and Dhavan media-1601 U/gm. Rest of the media exhibited comparatively less laccase activity. Hence, on the basis of these results, Asther medium was selected for further studies for the growth and maximum production of laccase from isolate *Trametes elegans H6*. Similar results of laccase production have been reported by Chhaya and Gupte [11].

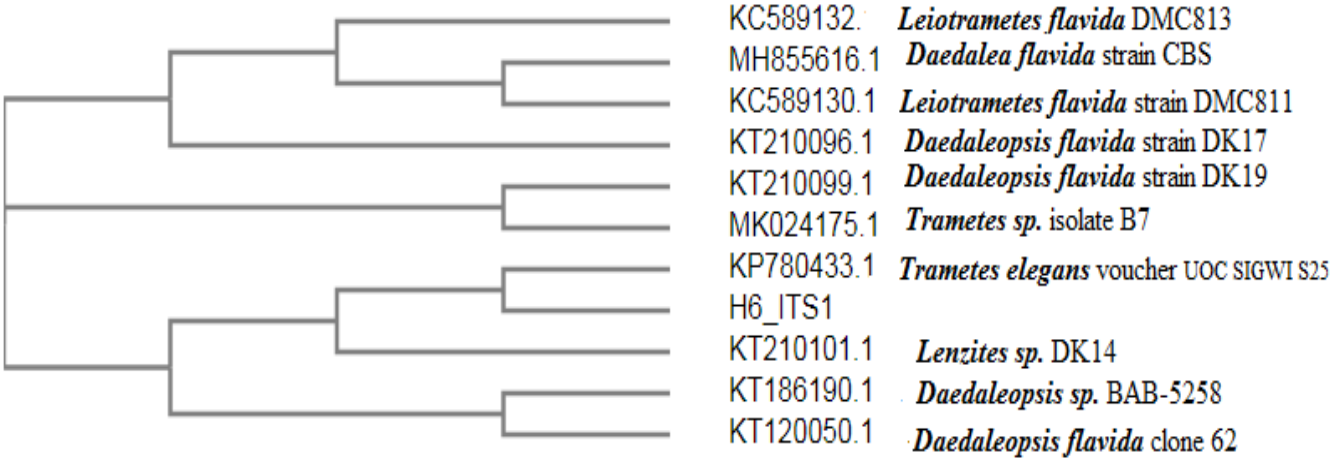

Fig. 2: Evolutionary tree of isolate H6 constructed by Neighbor Joining method

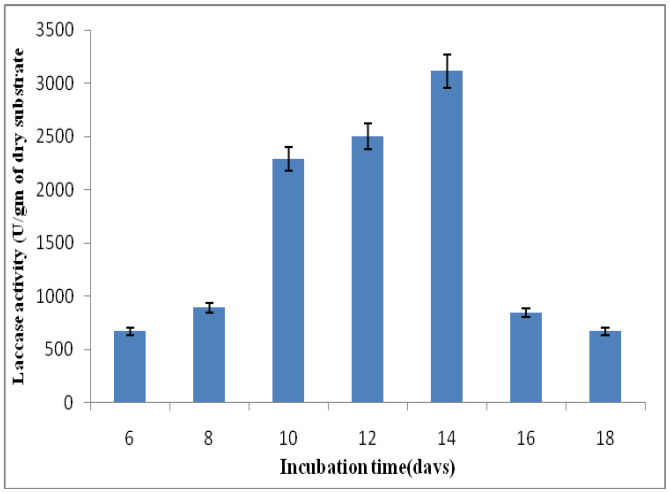

Fig. 3: Effect of incubation time on laccase production

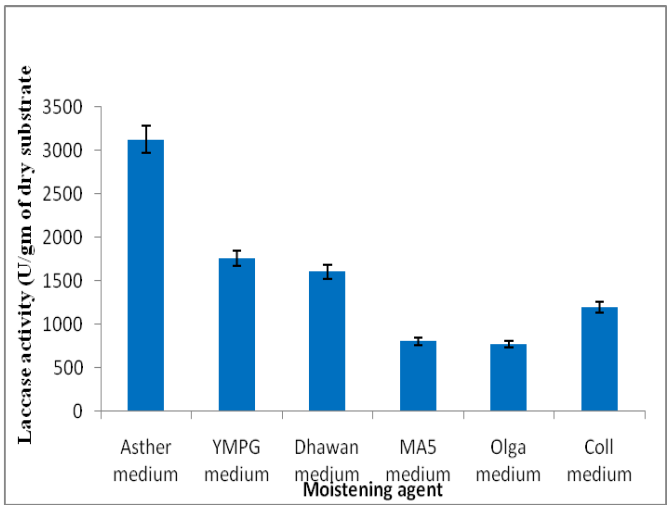

Fig. 4: Effect of moistening agent on laccase production

3.4.3. Influence of Ligno Cellulosic Substrates

Different types of lignocellulosic substrates comparativelybear an impact on enzyme production as some lignocellulosic substrate significantly stimulates higher ligninolytic enzyme production, hence selection of best lignocellulosic substrate is very important. Various lignocellulosic substrates like wheat straw, wheat bran, rice bran, rice straw, sugarcane bagasse, were screened for their capability of ligninolytic enzyme production using the Asther's medium as a moistening agent. All different agro-residues tested promoted a good growth of fungi. However, Rice straw was found to be the best lingocellulosic substrate as maximum laccase activity 3556 U/gm obtained from the incubating flask having rice straw as a substrate, while lowest laccase activity was obtained from flask having Sugar cane baggase as a substrate(Fig. 5).

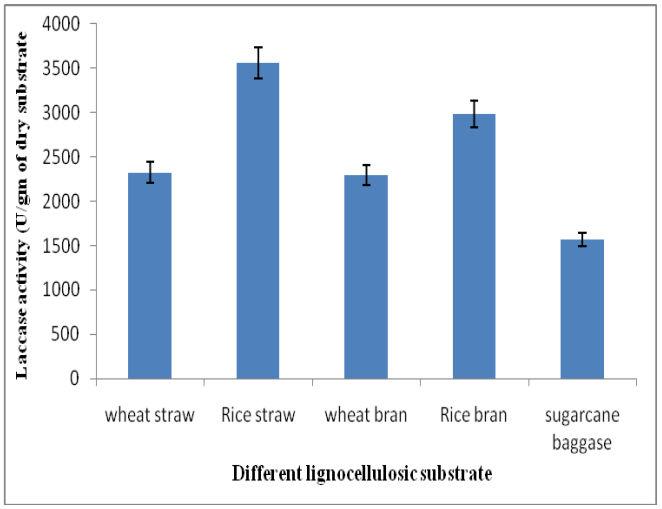

Fig. 5: Effect of different lignocellulosic substrates on laccase production

3.4.4. Influence of Innoculam Size

Inoculum plays an important role in any fermentation process. Better inter particle translocation is obtained on even distribution of inoculum. Increase in production of laccase was observed with an increase in agar plugs from 5 to 10. Maximum production of laccase 3677 U/gm was obtained in the flask having ten agar plugs(Fig. 6). When more than 10 agar plugs were inoculated in the flask, a decrease in laccase activity was observed which may be due to the fast depletion of nutrients amongst the organisms. However Elsayed et al. [12] reported six agar plugs of *Pleumtiis ostreatus* ARC280 as an optimum inoculum size for laccase production.

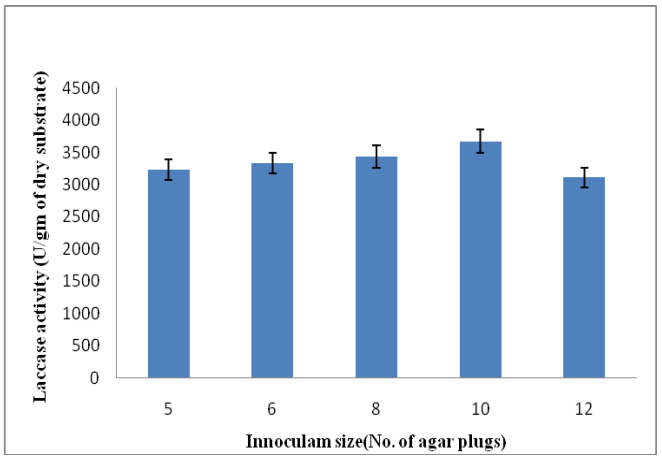

Fig. 6: Effect of inoculum size on laccase production

3.4.5. *Influence of pH*

pH of the medium is one of the most influencial parameters because growth of the microorganism is dependentupon pH. pH changes may affect the metabolic activity as well as on the action of enzymes. It was found that best was pH 5 for the laccase production by isolate *Trametes elegans H6* (Fig. 7).

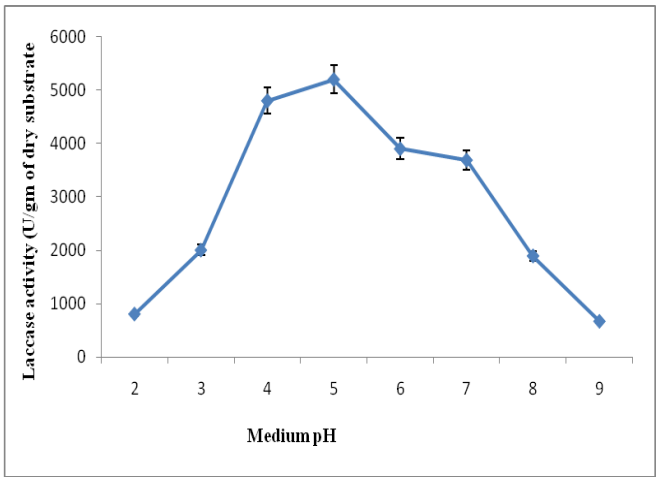

Fig. 7: Effect of medium pH on laccase production

After pH 5, a decrease in the laccase production is observed. Highly acidic pH is also unfavorable for laccase production by isolate H6. The reason for the less production in pH between 2 to 4 is due to lesser growth of the isolate H6. Similar results have been reported by Rehan A. Abdet al. [13] using *T. harzianum*. However sukhbir kaur et al. [14] reported 4.5 as an optimum pH for lacasse production using fungal strain SL4.

3.4.6. *Influence of Temprature*

Changes in incubation temperature showed significant effect on the production of extracellular laccase by isolate *Trametes elegans H6*. The fungus was grown at different temperatures ranging from 20-50°C and maximum laccase activity-5014 U/gm was observed at 30°C as compared to other temperatures tested for laccase production (Fig. 8). Further increase in incubation temperature, resulted in a gradual decrease in enzyme production as recorded upto 40°C. Similar result was also reported by V. Sivakami et al. [15] as optimum temperature for laccase for *Pleurotus ostreatus* LIG 19. However, Savitha et al. [16] reported 45°C as an optimum temperature for lacasse production using *Tricoderma spp.*

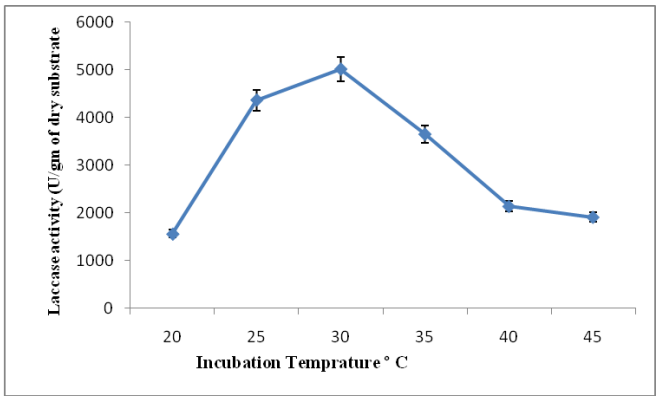

Fig. 8: Effect of incubation Temprature on laccase production

3.4.7. Effect of co-substrate

Maximum production of laccase-5210.96 U/gm was obtained when mannitol was used as the co-substrate (Figure-9), followed by glucose-5018.0 U/gm and Maltose-4988.74 U/gm. The additional supplement of co-substrate, thus has a profound effect on laccase production. However Ding et al. [17] reported glucose as the most effective co-substrate for higher laccase production by *Ganoderma lucidum*.

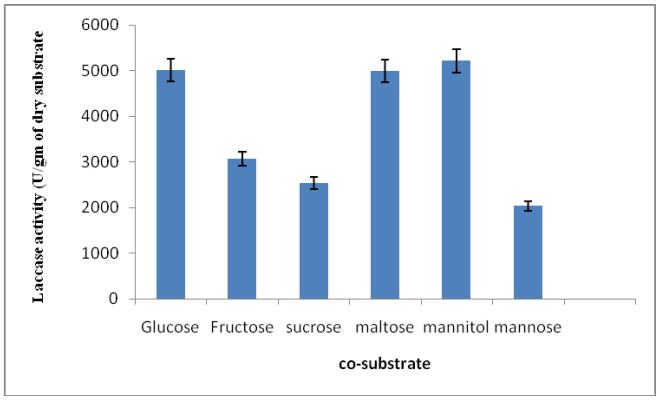

Fig. 9: Effect of co-substrate on laccase production

3.4.8. *Influence of Nitrogen Source*

In the present investigation the effect of different nitrogen sources was studied by replacement of various nitrogen sources like Ammonium sulphate, Urea, Pepton, Yeast extract, Caesein, Malt extract, Gelatin at a concentration of 0.02 gm/l. Maximum laccase production obtained with Urea-6222.04 U/gm (Fig. 10).

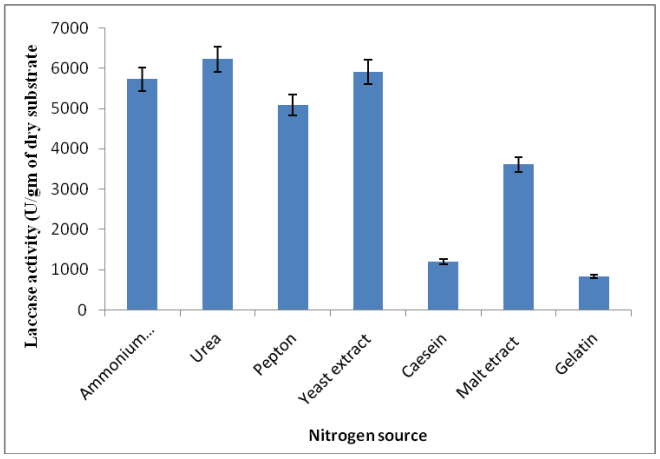

Fig. 10: Effect of Nitrogen source on laccase production

From the results obtained it can be deduced that replacement of yeast extract by other nitrogen sources increased laccase production. Similar results have also been reported by Asgher et al. (2010) [18].However Revanker and Lelc. [19] reported that yeast extract is the best nitrogen source for the laccase production.

4. CONCLUSION

In the present study, isolate H6 was found to be the highest laccase producer amongst various isolates tested. It was identified as *Trametes elegance H6* as per molecular identification. Present investigations also confirm that optimization of cultural condition and process parameters of the fermentation process leads to higher laccase enzyme production. Incubation time of 14 days of incubation was best for optimum production. Best lignocellulosic substrate for laccase production was found to be Rice straw which is inexpensive and easily available. Moisture content is an important factor in SSF process; Asther medium was found to be optimum for maximum laccase production Innoculam size of 10 agar discs of 8 mm was found to be optimum for laccase production. Maximum laccase production was obtained at pH 5 and temperature 30°C. Mannitol was considered as best carbon source and urea was found as a best nitrogen source. The overall optimization of all the cultivation conditions using "one-factor-at-a-time" methodology, increased the laccase production by 2.96 fold compared to un-optimized conditions.

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