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RESEARCH ARTICLE

ISOLATION AND CULTURE OPTIMIZATION OF EXTRACELLULAR LACCASE ENZYMES OF *PLEUROTUS PULMONARIUS* IN SUBMERGED FERMENTATION

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ABSTRACT

P. pulnonarius produced laccase enzymes extracellularly both in stationary as well as in shaking condition. To obtain the optimum laccase production different cultural parameters were studied. Different carbon sources, nitrogen sources, and pH of the media showed differential influencing effect on laccase production. The laccase production was optimum in PSP (20% potato extract, 2% sucrose, 0.5% peptone) medium in pH 5.0. The value was 72.9 U/ml when measured using guaiacol as substrate. The enzyme showed more affinity towards ABTS than *o*-dianisidine or guaiacol. Three laccase isoenzymes were found in activity staining of the dialysed extracellular culture filtrate. Laccase isolated from *P. pulmonaris* can decolorised synthetic dyes (phenol red and malachite green) which were generally used in textile industries.

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INTRODUCTION

Pleurotus spp. popularly known as oyster mushroom commercially cultivated throughout the world positioned second after the button mushroom *Agaricus bisporus* (Sanchez 2010). The genus *Pleurotus*, comprises a large number of species within which a few are commercially cultivated. This particular genus is very much accepted around the globe not only for the production of protein rich fruiting bodies but also for a number of pharmaceuticals and also some enzymes of industrial importance (Das *et al*., 2015; Mukherjee & Das 2009). The white rot fungus *Pleurotus* produces a large number of lignocellulose degrading enzymes within which laccase is most important (Das *et al*., 1997, Das *et al*., 2011, Das *et al*., 2014). Laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) is a well known lignin degrading enzyme found mainly in fungi though produced in plants, bacteria and insects also (Thurston 1994; Youn *et al*., 1995; Mukherjee and Das, 2009). In addition to lignin degradation different workers suggested diverse biological role of laccase in different organisms like formation of primordia (De vries *et al*., 1986), rapid cell growth (Das *et al*, 1997), Pathogenesis (Galhaup *et al*., 2002) etc. The enzyme can be utilized in various biotechnological industries like paper and pulp, textile, food, pharmaceuticals etc. and also utilized in bioremediation of different pollutants etc (Couto and Herrera, 2006). A laccase- immunoglobulin conjugate has been developed which might be efficiently utilized in immunoblots and immunoassay experiments (Ray *et al*., 2010; 2012).

In this work we have, isolated laccase from the commercially cultivated species *P. pulmonarius* (MTCC 1805), standardized the conditions for optimum laccase production, purified the enzyme partially and studied the substrate specificity and dye decolorization which may be helpful for future applications of this biotechnologically important enzyme.

MATERIALS AND METHODS

Mushroom strain

Pleurotus pulmonarius Cooke (MTCC 1805) was collected from Microbial Type Culture Collections, Institute of Microbial Technology, Chandigarh, India and was maintained on potato-dextrose agar (pH 7.0) containing 20% potato extract, 2% dextrose and 2% agar as reported earlier (Das *et al*., 2014).

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Inoculum source and Liquid submerged fermentation (SMF)

An inoculum was taken from the periphery of 7 days grown cultures on PDA. The production of laccase was studied in liquid medium in stationary /shaking condition containing potato extract (10%/ 20%/ 30%) with different percentage of carbon source (Dextrose/Fructose /Sucrose/Lactose/Maltose) with or without 0.5% nitrogen source (Peptone/Yeast extract/Malt extract) at different pH-(7.0/6.0/5.0) at 25±2 ºC (Das *et al*. 2014). The fungus was cultured for 21 days in stationary/shaking (100 cycles/min) condition and aliquots of culture filtrate was collected in 1 day interval from starting day. After each collection the volume of the culture medium was adjusted to its original volume with fresh medium. After removal of mycelium by filtration extracellular culture filtrates were assayed for enzyme/protein activity.

Laccase Activity assay

Laccase activity was spectrophotometrically performed as described by Das *et al*. (1997) with guaiacol, *o*-dianisidine or ABTS (Bose *et al*, 2007) as the substrate. Except the substrate specificity all other assays were done using guaiacol as substrate. The reaction mixture generally contained 0.2 ml Culture filtrate, 0.1 ml 1M acetate buffer pH 7.0, 0.3ml 3mM 0-dianisidine/0.5ml 20mm guaiacol (in 20% acetone) or 0.1 ml 1mM ABTS. The volume was adjusted to 1ml. The absorbances were taken in 420nm for ABTS ($\varepsilon = 3.6 \times 10^4$ $M^{-1} cm^{-1}$), 460nm for *o*-dianisidine ($\varepsilon = 11300$ $M^{-1}cm^{-1}$), 470 nm for Guaiacol ($\varepsilon = 6740$ $M^{-1}cm^{-1}$) and. Enzyme activity was expressed in International Unit.

Protein estimation

Protein concentration was determined using bovine serum albumin as standard according to the method of Lowry (1951).

Activity staining

Activity staining of enzyme after native polyacrylamide gel electrophoresis (PAGE) of the concentrated culture filtrate was done with solutions of guaiacol, in acetate buffer, pH 5.0 as reported earlier (Das *et al*., 2011).

Partial purification of Laccase

The laccase activity present in the culture filtrate was partially purified after 0-40% and 40-80% ammonium sulphate precipitation, followed by gel permission chromatography.

Polyacrylamide Gel Electrophoresis (PAGE)

Non-denaturing PAGE was also done using tubes (11.5 cm x 0.5 cm) according to the method of Das *et al* (2011). The gel was run at a constant current of 2 mA per tube for 3-4 hours. After the tracking dye had reached the lower edge, the gel was removed from tube and subsequent staining was done using the standard method.

Dye decolorization studies

Phenol red, malachite green, congo red and eosin yellow were tested for dye decolorization studies. The reaction mixture contained equal volume of an aqueous solution of dye and 20U crude laccase in 0.2 M acetate buffer (pH 5.0). The residual dye conc. was measured according to Das *et al.* (2014).

Statistical Analysis

Two way ANOVA (Analysis of Variance) was done using GraphPad Prism ver 5.01(2007) software.

RESULTS

Effect of shaking and stationary conditions on laccase production

Pleurotus pulmonarius produced laccase in extracellular medium. The laccase production was studied in PD media (Potato extract 10%, Dextrose -1%, pH-7.0) in both shaking and stationary conditions. The optimum laccase production (12.2U) was found in 5 days in shaking condition whereas 10.64 U laccase was produced in 16th day in stationary condition. It was found that the laccase obtained from stationary condition was more stable than its counterpart in shaking condition (data not shown) so further experiments were done in stationary condition.

Effect of different carbon sources on laccase production

Laccase production was studied in five different (1%) carbon sources such as dextrose, maltose, lactose, sucrose, and fructose. It was found that dextrose was the best substrate followed by sucrose, maltose, fructose and then lactose. The optimum laccase production was found on 14th day in case of sucrose, lactose and for other carbon sources like dextrose, maltose and fructose optimum result was found at 16 day.

Effect of different percentage of Potato extract (PE) on laccase production

The optimum laccase production was shown in 20% potato extract containing media (1% Carbon source, pH-7.0) than 10% potato extract containing media(1% Carbon source, pH-7.0) (Fig.1). So, 20% potato extract was used for further study.

Effect of different conc. of carbon sources on laccase production

The optimum laccase production was shown in 2% carbon source followed by 3% and 1% carbon source (Fig.2). It was found that 2% sucrose is the best substrate followed by 2% dextrose, 2% fructose, 2% maltose and then 2% lactose. So, 2% Carbon source was used in further study.

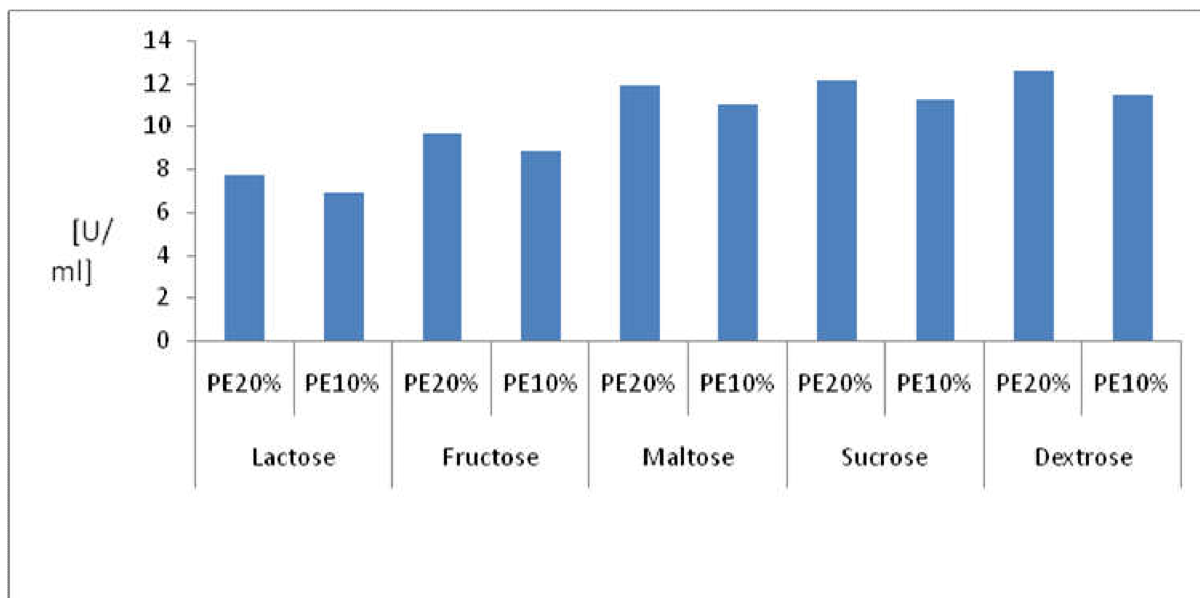
Effect of yeast extract on laccase production

When yeast extract was added in the media with 2% carbon source, 20% potato extract at pH 7, the highest laccase activity was observed in PLY (potato-lactose-yeast) media

Table 1. Effect of different carbon sources on laccase production[a] in stationary condition

Carbon source	Laccase activity U/ ml								protein (mg)		Specific activity (U/mg protein)	
	day								14th	16th	14th	16th
	10th	11th	12th	13th	14th	15th	16th	17th				
Lactose	4.16	4.44	4.93	5.84	6.82	3.28	1.74	1.6	10.5		.65	
Fructose	1.24	2.56	3.50	5.98	6.72	8.12	8.76	7.25		14		.62
Maltose	1.17	2.50	3.38	6.52	8.58	9.46	10.84	9.48		12		.9
Sucrose	9.9	10.24	10.34	10.82	11.14	10.48	8.65	6.70	13.2		.84	
Dextrose	1.48	2.12	2.78	7.62	8.42	9.96	11.46	9.35		12.25		.93

[a]Carbon source-1%, Potato extract 10%, pH-7.0



[a]Carbon source -1%, pH-7.0

Fig. 1. Effect of different percentage of Potato extract (PE) on laccase production[a]

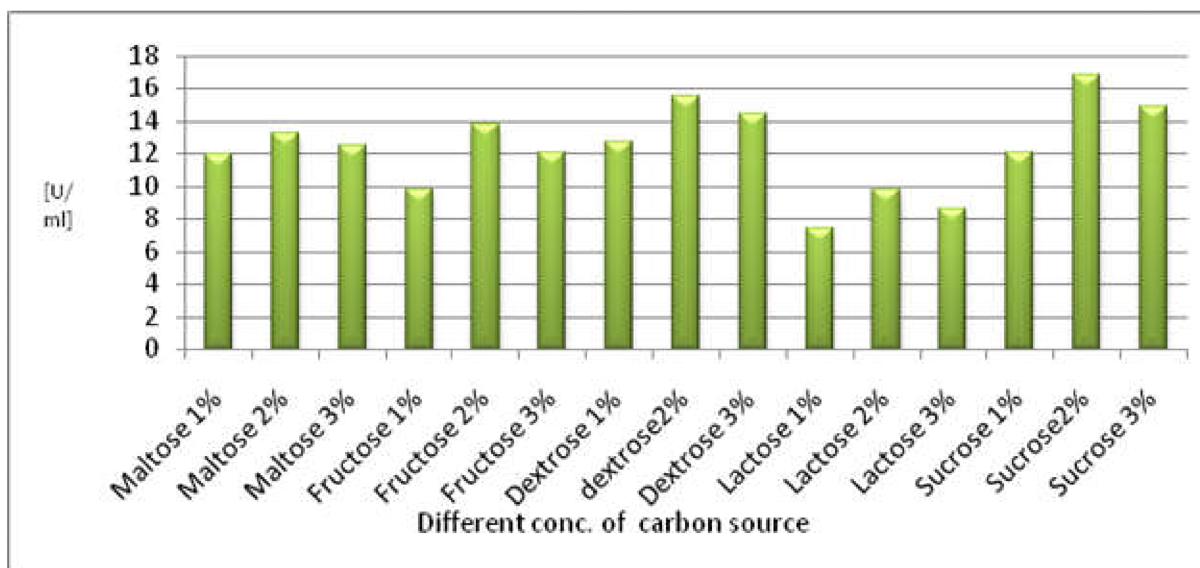
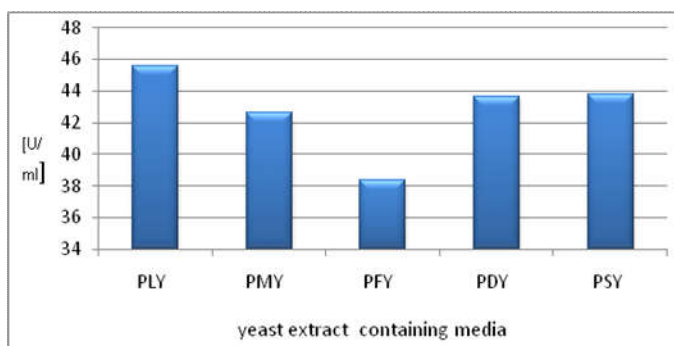


Fig. 2. Effect of different conc. of carbon sources on optimum laccase production, (Maltose, Fructose and Dextrose were considered for 16th day whereas Lactose and Sucrose were considered for 14th day , Potato extract 20%, pH-7)

followed by PSY (potato-sucrose-yeast) media, PDY (potato-dextrose-yeast) media, PMY (potato-maltose-yeast) media and lowest was recorded from PFY (potato-fructose-yeast) media (Fig.3).

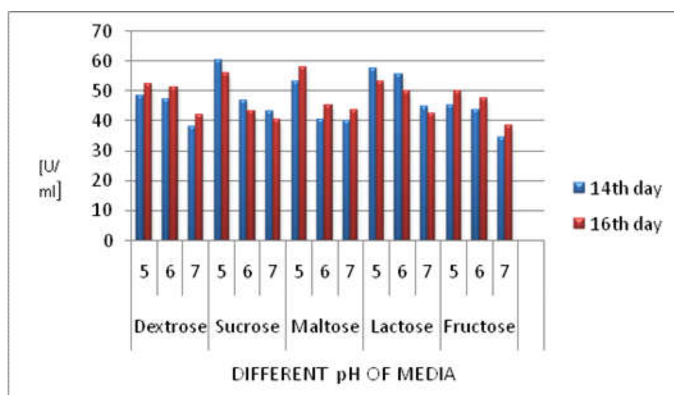
Effect of pH of media on laccase production

The optimum laccase production was found at pH 5 in PSY media. In higher pH the laccase production decreased in all the tested media (Fig.4.).



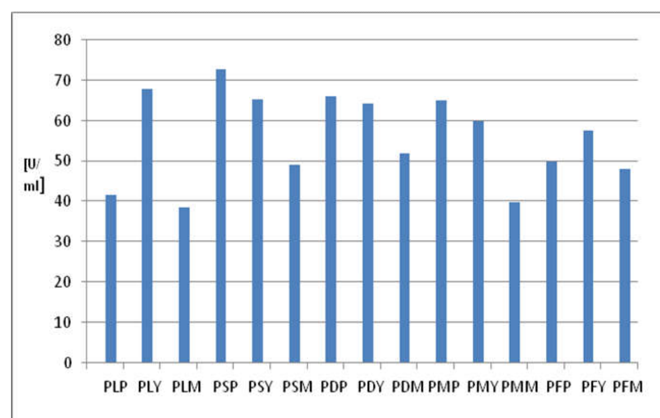
[a]Carbon source 2%, Nitrogen source 0.5 % , Potato extract 20%, pH-7.0

Fig. 3. Effect of Yeast extract (Nitrogen source) on Laccase production[a]



[a] N-source-yeast 0.5%), potato extract-20%, C- source :2.0%

Fig.4. Effect of pH of media on laccase production[a]



[a]Carbon source 2%. Nitrogen source 0.5 %, pH-7.0, Potato extract 20%

Fig. 5. Effect of different nitrogen sources on laccase production[a]

Effect of different nitrogen sources on laccase production

Different nitrogen sources e.g. malt extract, yeast extract, peptone were given along with different carbon sources as media for *P. pulmonarius*. The highest laccase activity was found in PSP (potato sucrose peptone) media followed by PLY (potato lactose yeast) media then PDP (potato dextrose peptone) media and lowest was recorded from PLM (potato lactose malt) media (Fig.5). It was clear from the Fig. 5 that malt extract was least influencing nitrogen source.

Optimum laccase production

So, the optimum laccase production was found in PSP medium (72.9 U/ml) followed by PLY (67.8 U/ml) medium at pH 5.0 (Fig.5).

Effect of $CuSO_4$ and phosphate on laccase production

When phosphate was added in potato sucrose peptone (PSP) media, laccase production was slightly increased which is not significant but when $CuSO_4$ was added then laccase production was significantly decreased (Fig.6). If $CuSO_4$ and phosphate both were added in PSP media then laccase production was also significantly decreased.

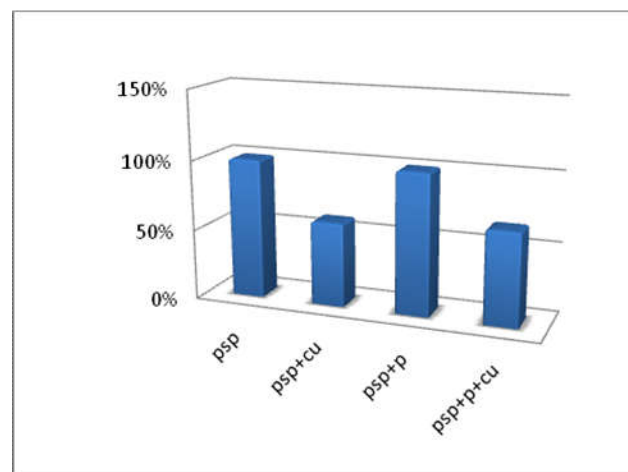


Fig.6. Effect of $CuSO_4$ and phosphate on laccase production

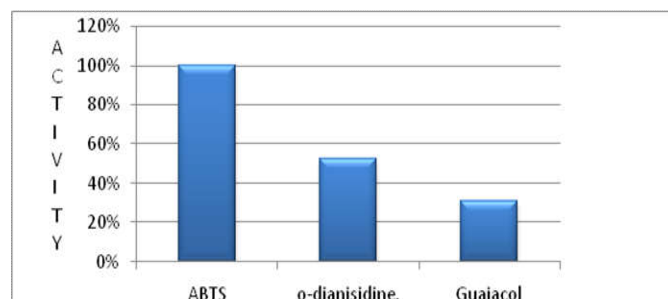


Fig. 7. Substrate specificity

Substrate specificity

In the present experimental conditions three substrates viz. Guaiacol, ABTS and o-dianisidine were tested for laccase activities. It was found that *P. pulmonaris* culture filtrate showed maximum laccase activities against ABTS followed by *o*-dianisidine than guaiacol (Fig.7).

Zymogram

In zymogram more than one laccase isozymes were found in presence of guaiacol. Fig. 8 showed activity staining of enzyme by native polyacrylamide gel electrophoresis (PAGE) with guaiacol. in acetate buffer pH 5.

Table 2. Partial purification of Laccase of *P. Pulmonarius*

Purification steps		Total enzyme activity	Total protein (mg)	Sp. Activity (U/mg protein)	Purification fold	Yield (%)
Culture Filtrate	800ml	11272U	12000mg	0.939U/ mg protein	1	100
Ammonium sulphate	0-40%	2492U	300 mg	8.3 U/mg protein	8.8	22.10
Precipitation	40-80%	4780U	1260mg	3.79U/ mg protein	4.0	42.40
Biogel P-200	0-40%	120 U	6.8 mg	17.64U	18.78	1.06
	0-80%	210 U	17.6mg	11.93	12.70	1.86

Partial purification of Laccase of *P. Pulmonarius*

Laccase was purified partially by ammonium sulphate precipitation followed by Biogel P-200 (Table-2). Two laccase isozymes were separated in differential ammonium sulphate precipitation (Fig.8).

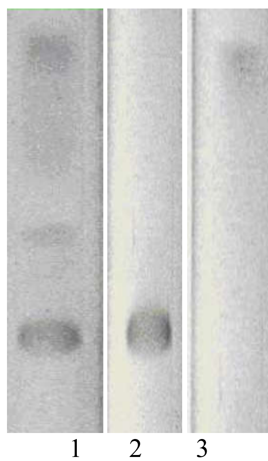


Fig. 8. Laccase Isozymatic pattern (1.Crude culture filtrate, 2. 40-80% Ammonium sulphate cut, 3. 0-40% Ammonium sulphate cut)

Dye decolorization

In presence of 20 U crude laccase 19.16% colour of phenol red was decolorized in 7.30 hours whereas 13.34% malachite green was decolorized in 8 days. Congo red and Eosin yellow were not decolorized in present experimental conditions.

DISCUSSION

On the basis of substrate degradation, fungi are divided as brown rot and white rot. Brown rot can degrade cellulose and hemicellulose but not lignin whereas white rot can degrade cellulose, hemicellulose as well as lignin also. *Pleurotus* is a large group of white rot fungi having more than 25 species. All the spices are edible. *P.pulmonarius* (MTCC 1805) is a white rot edible mushroom which can efficiently grow in the agro-climatic conditions of India. *P. pulmonarius* produces different enzymes required for degradation of substrates (viz. cellulose, hemicelluloses and lignin). As lignin encrusted cellulose or hemicelluloses so the fungus first attacks the lignin layer. There are number of lignin degrading enzyme like lignin peroxidase, manganese peroxidase, laccase etc (Dos Santos Bazanella *et al*., 2013) within which *P*. pulmonarius produces enormous amount of laccase (De Souza and Peralta, 2004) in submerged as well as in solid state fermentation (Tlecuitl-Beristain, 2008) both intracellularly as well as extracellularly. In the present investigation, it was found that *P*. pulmonarius produced laccase in both stationary condition as well as shaking conditions. The laccase produced in shaking condition is much more in amount and less time consuming than stationary condition but the laccase produced in stationary condition is much more stable than its counterpart in shaking condition (data not shown). De Souza *et al*. 2004 showed that the composition of culture media and culture conditions have massive effect on extracellular enzyme production in filamentous fungi like *P. pulmonarius*. Laccase production was studied using different carbon sources (1%) containing 10% potato extract (Table 1). It was found that the optimum laccase production took place within 14 to16 days Similar results observed by some other workers (De Souza etal,2004). Dextrose followed by sucrose showed the optimum results. It was found that potato extract is very much essential in laccase production in *P. florida* (Das *et al*., 1999). When the conc. of potato extract varies laccase activity showed its optimum at 20% (Fig.1).

Carbon and nitrogen sources played major role in laccase production (Gochev and Krastanov 2007). As excessive amount of carbon source may have inhibitory effect (Eggert *et al*., 1996) so, to determine the optimum conc. of carbon sources present experiment are designed in presence of 1 to 3% carbon sources (Fig. 2). The optimum carbon conc. is found as 2%. When the sugar concentration increased more than 2% there was an inhibitory effect on laccase production. According to Eggert *et al*. (1996) carbon sources such as glucose, sucrose etc. allows constitutive production of the enzyme but repress its induction. Here 2% sucrose conc. Shows optimum effect on laccase production (16.84U/ml) in 14th day (Fig.2). Nitrogen source is another key factor in laccase production (Das *et al*., 1997). In present experiment it was found that yeast extract induces the laccase production in every cases (Fig. 3). Highest activity was found in potato lactose yeast (PLY) media (45.60 U/ml) though laccase production was very least in potato lactose media (Table-1), possibly synergistic effect of lactose and yeast extract. To see the effect of other nitrogen sources peptone and malt are also used in addition to yeast extract. Potato sucrose peptone (PSP) medium showed the highest activity (48.65u/ml) which is more than Potato sucrose yeast (PSY) (43.65u/ml) medium) and Potato lactose yeast (45.24u/ml) (PLY) in pH 7 (Fig-5). The pH is another foremost factor for laccase production which varies not only from species to species but also within same species but in different nutritional conditions. Most of the cultural studies of the laccase restricted within pH 5 to 7 (Das *et al*., 1997 & Prasad *et al*, 2005 etc.). The optimum laccase production was found in pH 5.0 (Fig. 4). As laccase is a copper containing enzyme different workers

suggested the inducing effect of copper containing compound (viz $CuSO_4$) on laccase production (Prasad *et al*., 2005). However in the present experimental condition $CuSO_4$ (0.01%) showed inhibitory effect (Fig-6)). Similar result was observed by Das *et al*. (1999; 2013) which might be due to the excess amount of $CuSO_4$ in the media. Some workers showed the inducing effect of phosphate on laccase production. In the present experimental condition phosphate has slight inducing effect (Fig. 6) but it is not statistically significant.

So, from the cultural studies it can be concluded that *P. pulmonarius* produced highest amount of laccase in PSP medium containing potato extract (20%), sucrose (2%), peptone (0.5%), in pH 5 (Fig.5). Laccase has a large number of substrates (Das etal,1997). In the present experimental condition three substrates are chosen within which ABTS showed more activity than *o*-dianisidine & guaiacol (Fig-7). Many workers showed laccase as an enzyme used in bioremediation for decolorization of many industrial dyes (Mukherjee & Das, 2009; Das *et al*., 2014). In the present experimental condition phenol red and malachite green were decolorized to some extent though no decolorization was found in Congo red & Eosin yellow. However, Zilly *et al*. (2002) showed that *P. pulmonarius* laccase completely decolorized Congo red within 8 to 10 days. The copper containing enzyme laccase showed different isoforms depending on cultural and environmental condition. In present experimental condition three laccase isoform was found. Similar result was obtained by different workers (Das *et al*., 2011; De Souza *et al*.,2004). For characterization of any enzyme purification is very much necessary. After ammonium sulphate precipitation, one isozyme was found in 0-40% fraction and another is found in 0-80% fraction (Fig. 8). The third enzyme has been lost during present purification process possibly due to its lower concentration.

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